Supporting Information

Structure-Based Virtual Screening and Biological Evaluation of *Mycobacterium tuberculosis* APS Reductase Inhibitors

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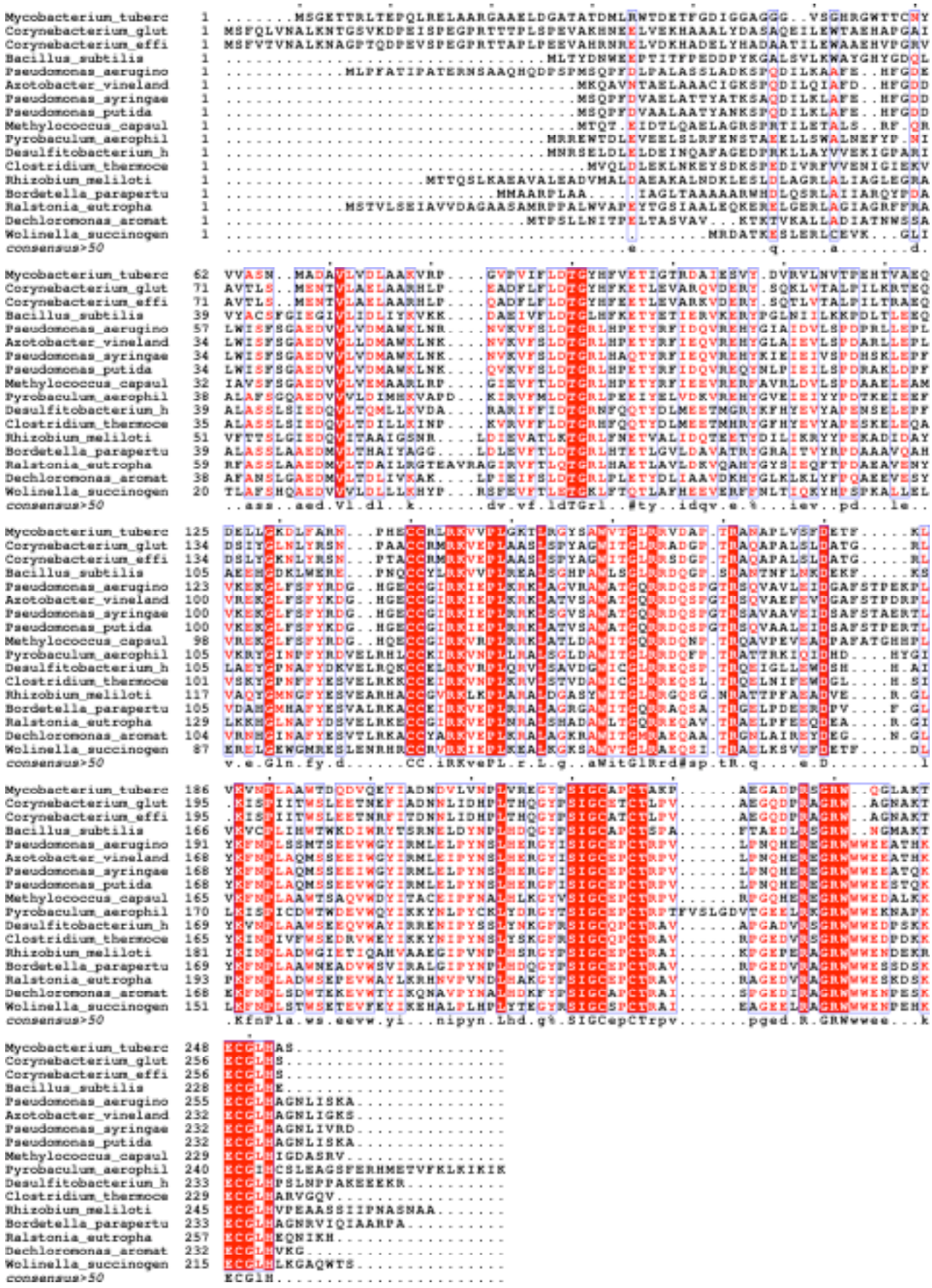
Contents of Supporting Information:

Supplementary Figure 1. Structure based sequence alignment of 17 APS reductases from prokaryotes. The ClustalW Multiple Sequence Alignment program was used. Strictly conserved residues are outlined in red, red letters indicate conserved residues and conserved regions are boxed in blue. Alignment pictures were rendered with the server ESPript 2.2 (http://espript.ibcp.fr)

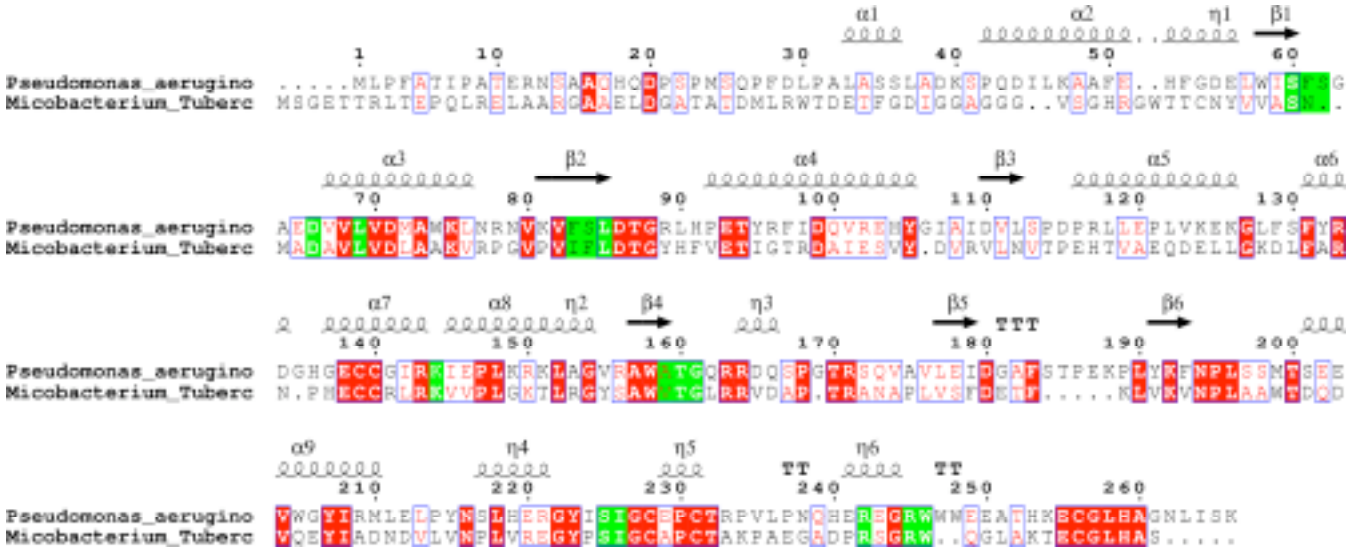
Supplementary Figure 2. Structure based sequence alignment of *Pseudomonas Aeruginosa* and *Micobacterium tuberculosis* APS reductases. The ClustalW Multiple Sequence Alignment program was used. Strictly conserved residues are outlined in red, red letters indicate conserved residues and conserved regions are boxed in blue. Residues flanking the active site are outlined in green.

Experimental Section.

Supplementary Figure 1.



Supplementary Figure 2.



Experimental Section.

Virtual Screening Calculations. The AutoDock 4.0 (AD4)[1,2] software package, as implemented through the graphical user interface called AutoDockTools (ADT),[3] was used to dock small molecules to APS reductase. The enzyme file was prepared using published coordinates (PDB 2GOY).[4] The terminal residues were modified to charged quaternary amine and carboxylate forms. The [4Fe-4S] cluster was retained with the protein structure. Charges of this group were manually assigned. In our case, the cluster is believed to have two ferric (+3) and two ferrous (+2) irons.[5] Since the eight sulfur atoms (four belonging to the cluster and four belonging to the four cysteines) have a net charge of -1, the total net charge of the system should be of -2. Noodleman and co-workers calculated the ESP charges for models of the cluster in this oxidation state.[6] These charges were added to APS reductase iron-sulfur cluster atoms and to the four sulfur of the coordinating cysteines (Table 1).

Table 1. Calculated Charges for $[Fe_4S_4(SCH_3)_4]^{2-}$

Atom	ESP Charges
Fe_{ox}	+0.642 (_2)
Fe_{red}	+0.635 (_2)
S^*_{ox}	-0.584 (_2)
S^*_{red}	-0.580 (_2)
S_{ox}	-0.574 (_2)
S_{red}	-0.571 (_2)

All other atom values were generated automatically by ADT. The docking area was assigned visually around the enzyme active site. A grid of 80 Å x 80 Å x 80 Å with 0.375 Å spacing was calculated around the docking area for 13 ligand atom types using AutoGrid4. These atom types were sufficient to describe all atoms in the NCI database. For VS, compound structures of the NCI Diversity Set and the ones derived from the similarity search were prepared using the ZINC database server (http://zinc.docking.org/upload.shtml)[7] to take into account the different protomeric and tautomeric states of each compound. All the ligands were then converted in the AutoDock format file (.pdbqt). For each ligand, 100 separate docking calculations were performed. Each docking calculation consisted of 10 million

energy evaluations using the Lamarckian genetic algorithm local search (GALS) method. The GALS method evaluates a population of possible docking solutions and propagates the most successful individuals from each generation into the subsequent generation of possible solutions. A low-frequency local search according to the method of Solis and Wets is applied to docking trials to ensure that the final solution represents a local minimum. All dockings described in this paper were performed with a population size of 150, and 300 rounds of Solis and Wets local search were applied with a probability of 0.06. A mutation rate of 0.02 and a crossover rate of 0.8 were used to generate new docking trials for subsequent generations, and the best individual from each generation was propagated to the next generation. The docking results from each of the eight calculations were clustered on the basis of root-meansquare deviation (rmsd) between the Cartesian coordinates of the atoms and were ranked on the basis of free energy of binding. The top-ranked compounds were visually inspected for good chemical geometry. Pictures of the modelled ligand/enzyme complexes were rendered with PMV.[3]

Preparation of NCI Compounds. Compounds determined by AD4 to have low binding energies to APS reductase were requested in groups of 40 and received from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Insititute (http://dtp.nci.nih.gov/branches/dscb/repo_request.html). Chemical compounds were dissolved in DMSO to 10 mM final concentration and stored at room temperature.

Enzyme purification. Purification of APS reductase was carried out as previously described.[4]

APS Reductase Activity Assay. APS reductase activity was assayed using a modification of an assay for monitoring $^{35}SO_4^{2-}$ release from ATP-sulfurylase as follows.[5] Reactions were performed in a final volume of 100 µL. At various time points, a 10 µL aliquot was removed from the reaction and added to 0.5 mL of a 2% (w/v) charcoal solution containing 20 mM Na_2SO_3. The suspension was vortexed, clarified by centrifugation and a 400 µL aliquot of the supernatant solution, containing the radiolabeled sulfite product, was counted in 10 mL of scintillation fluid. ^{35}S-labeled APS was synthesized and purified as previously described[8] with the inclusion of an additional anion exchange purification step (5 ml Fast Flow Q column (GE Healthcare) eluting with a linear gradient of ammonium bicarbonate, pH 8.0, from 0.005 to 0.7 M.

General Kinetic Methods. Unless otherwise specified, the reaction buffer was 100 mM Bis-Tris pH 7.5, 5 mM DTT, and the temperature was 30 °C. The auxillary protein reductant, thioredoxin was added at

10 μM. With the exception of slow reactions, the enzymatic reactions were monitored to completion (≥ 3 half lives) and rate constants were obtained by nonlinear least-squares fit to a single exponential (Kaleidagraph). To ensure single-turnover reactions, the concentration of APS reductase was kept in excess of the concentration of labeled APS (~1 nM). Two or three concentrations of APS reductase were chosen that were at least 10-fold below the K_M value. Under these conditions, the observed rate constant was linearly dependent on enzyme concentration. Thus, reactions were first order in APS and APS reductase in all cases. Under subsaturing conditions, with [APS] << K_M, the Michaelis-Menton equation (eq 1) simplifies to equation 2.[9] The reaction progress curve was plotted as a function of time and the fractional extent of reaction, and fit by a single-exponential function (eq 3) to yield k_{obs}, which is the product of enzyme concentration and the apparent second-order rate constant (eq 4). Kinetic data were measured in at least two independent experiments and the standard error was typically less than 15%.

$$V_{obs} = [E][S]k_{cat}/(K_m + [S]) \quad (1)$$

$$V_{obs} = (k_{cat}/K_m)[E][S] \quad (2)$$

$$\text{fraction product} = 1 - e^{-kobst} \quad (3)$$

$$k_{obs} = (k_{cat}/K_m)[E] \quad (4)$$

Inhibitor Screening. For initial screening, compounds were tested in kinetic assays at 100 μM final concentration. Compounds that inhibited more than 50% at this concentration were analyzed further as described below.

Analog Dissociation Constants. The standard assays and conditions described above were used to monitor the K_{cat}/K_M for reduction of APS in the presence and absence of inhibitor. Values of K_i were determined from the dependence of the observed rate constant (k_{obs}) on inhibitor concentration. With subsaturing APS, the inhibition constant is equal to the dissociation constant ($K_i = K_d$). Except in cases where solubility was a limiting factor, a range of inhibitor concentrations was employed from at least 5-fold below to 10-fold above the inhibition constant. Nonlinear least-squares fits of the equation for competitive inhibition (eq 5) gave excellent fits in all cases, and the standard error was typically less than 15%.

$$(k_{cat}/K_M)^{obs} = (k_{cat}/K_M)/(1 + [I]/K_i) \quad (5)$$

Catalytic inactivation of APS reductase by 2-nitro-9,10-phenanthrenedione. APS reductase (9 μM) was incubated with compound 23180 (0.9 μM) or DMSO and enzyme activity was measured at 1, 15 and 30 min. No statistical difference was observed in the activity of the enzyme in these experiments indicating that compound 23180 did not catalytically inactivate APS reductase.

Thiol Quantitation. Labeling of APS reductase by the thiol-reactive probe NBDCl was carried out using a modification of a following the published procedure.[10] Briefly, APS reductase (10 μM) was incubated, at room temperature, in a final volume of 1 mL of buffer containing 50 mM BisTris (pH=7.5), 1 mM EDTA, and 1 mM DTT with (a) DMSO or (b) 10 μM compound 23180. NBDCl (50 μM) was added to each of the resulting solutions and incubated for 30 minutes at room temperature. Excess NBDCL was removed from the labeled APS reductase by ultracentrifugation prior to the UV-vis scan. No loss in APS reductase thiol labeling was observed in the presence of inhibitor.

Promiscuous Inhibition. At the suggestion of one reviewer, we tested members of each structural class of inhibitor for promiscuous inhibition. Assays were carried out as described above in the presence of 0.01% Triton, and showed no significant difference in Kd with the assay without detergent. We also performed a gel shift assay of trypsin activity acting on APS reductase in the presence of inhibitors. By this gel assay, none of the inhibitors at concentrations of 50 uM changed the proteolysis pattern of trypsin and qualitatively indicates that the compounds are not inhibiting trypsin.

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