

Supporting Information Appendix

Auranofin exerts broad spectrum bactericidal activities by targeting thiol-redox homeostasis

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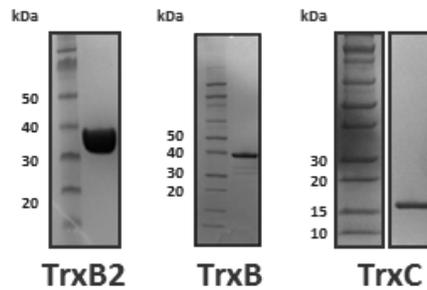
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Supplementary Tables and Figures

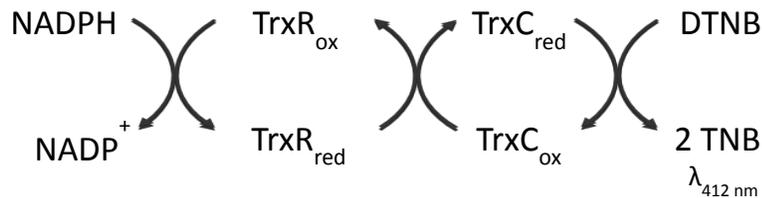
Table S1:

Medium	Auranofin MIC against H37Ra
Sauton's minimal medium	0.5 mg/L
Sauton's minimal medium + albumin (5g/100mL)*	10 mg/L

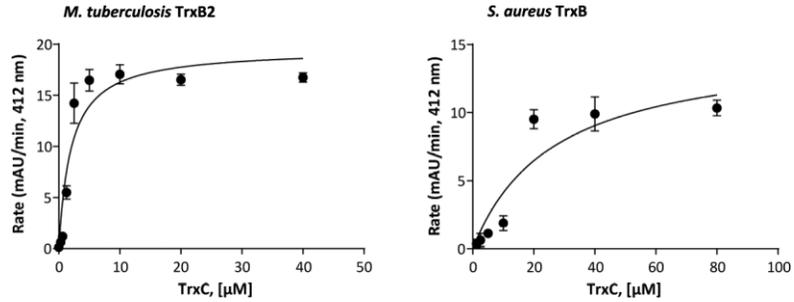
*The albumin concentration corresponds to the albumin concentration found in OADC.



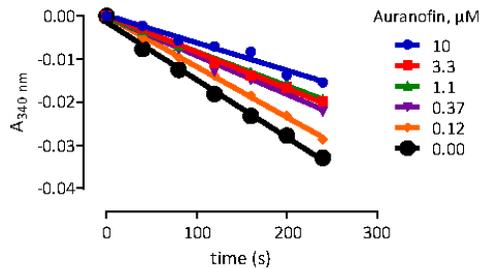
S1: Purification of TrxR and Trx. *trxB2* and *trxC* were cloned from *M. tuberculosis* strain H37Ra and *trxB* was cloned from *S. aureus* NCTC8325. All three were cloned into the pET28a/b expression vectors and purified via Ni-NTA chromatography as described in Supplementary Materials and Methods.



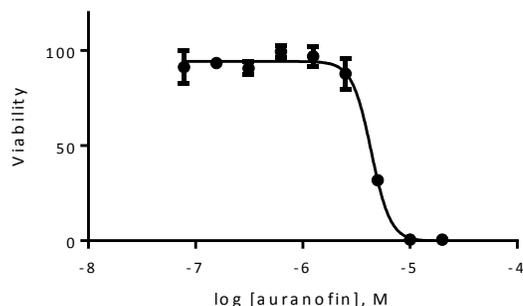
S2: Schematic of the thioredoxin reductase assay for TrxB2 and TrxB. Oxidized TrxC is regenerated by DTNB. TNB has a yellow color and can be monitored at 412 nm. No activity is seen in controls lacking TrxC.



S3: TrxB2 and TrxB K_m determination for TrxC. Various concentrations of *M. tuberculosis* TrxC were incubated with either TrxB2 or TrxB and NADPH (100 μM) and DTNB (200 μM) at pH 7.5 and 37°C.



S4: Auranofin inhibition of mycothione reductase. Mycothione reductase (Mtr) was cloned from *M. tuberculosis* strain H37Ra and heterologously expressed in *E. coli* BL21(DE3)pLysS (Promega). Mtr was preincubated with NADPH and varying concentrations auranofin. Reactions were initiated by the addition of mycothione and activity was monitored by measuring a decrease in absorbance at 340 nm (oxidation of NADPH).



S5: Auranofin toxicity on HepG2 cells. Auranofin cytotoxicity was assayed against HepG2 cells for 48 hr at varying concentrations. Viability was measured using CellTiter-Glo. The CC_{50} for auranofin was 4.5 μ M.

Supplementary Materials and Methods

Chemicals. Auranofin was purchased from Chem-Impex International (Wood Dale, IL) and Sigma-Aldrich (St. Louis, MO). Mycothiol was purchased from JEMA Biosciences (San Diego, CA). All other compounds were purchased from Sigma-Aldrich.

Bacterial medium and strains. For carbon starvation, cells were washed three times in PBS supplemented with tyloxapol (0.05% v/v) and finally cultured in PBS/tyloxapol for 96 hr. Where indicated, 10% (v/v) ADS (50 g albumin, 20 g dextrose, 8.5 g NaCl in 1 l H₂O) replaced OADC. Solid medium was Middlebrook 7H10 (BD) supplemented with 10% (v/v) OADC. Sauton's medium contained 0.5 g KH₂PO₄, 0.5 g MgSO₄, 4.0 g asparagine, 60 ml glycerol, 0.05 g ferric ammonium citrate, 2.0 g citric acid, 0.1 ml 1% (w/v) ZnSO₄ per liter. *E. coli* knockout strains were acquired from the *E. coli* Genetic Stock Center at Yale University and are from the Keio Collection of single gene knockouts (1).

Drug susceptibility testing. Auranofin minimum inhibitory concentrations (MICs) were determined by broth microdilution methodology according to Clinical Laboratory Standards

Institute guidelines. Controls in all assays included strain-appropriate antibiotics, bacteria alone, or media alone. For non-replicating persistence assays, CFUs for respective compounds were determined against cultures that had been carbon-starved for 96 hr in PBS/tyloxapol (0.05% v/v). Assays were performed in 1 ml of culture ($OD_{600\text{ nm}} = 0.1$). After 5 days of treatment all cultures were washed once in PBS and then plated as serial dilutions on 7H10 plates. Colonies were enumerated after incubation for 3 weeks at 37°C.

Deletion of thioredoxin reductase (*Rv3913*, *trxB2*) in *M. tuberculosis*.

The *Rv2855* and *Rv3913* genes were replaced by a $\gamma\delta$ (*sacB-hyg*) $\gamma\delta$ cassette as described in Jain and colleagues . The following primers were used to PCR-amplify the left and right flanking regions of the *Rv3913* gene:

trxB2_LL TTTTTTTTCCATAAATTGGAGCATTTCGGCGGCCTTAC,

trxB2_LR TTTTTTTTCCATTTCTTGGCATGACTATTAACCTAGCGGGATGTCT
CACTGAGGTCTCTCCGGAGCCGATAACGATCAC,

*trxB2_RL*TTTTTTTTTCCATAGATTGGCCTGAGTATCGTGAGACTAACGAGTGTCTGGTCTCGT
AGGCAGCAACCGGAGAAGCTGA,

trxB2_RR TTTTTTTTCCATCTTTTGGACTCAAGGGCGACGTTACGG

Briefly, the PCR products were cloned into pYUB1471. The plasmids were linearized with *pacl*, ligated to *pacl*-cut shuttle phasmid phAE159, and the resulting phasmids were packaged in vitro. High-titer phage lysates were used to transduce cultures of *M. tuberculosis* H37Rv and CDC1551. The transductions were plated on 7H10 plates supplemented with hygromycin (75 mg/l) and the plates were incubated at 37°C for 4-12 weeks. The transductants were checked for the deletion of their corresponding genes by PCR using the following primers: *trxB2F*

CAAGCAGGTCTGCTGGAC, trxB2R AAATCCCGGGTAGTTCTC, uptag
GATGTCTCACTGAGGTCTCT.

Thioredoxin and thioredoxin reductase expression and purification. *Mycobacterium tuberculosis* thioredoxin reductase (Rv3913) was cloned into the pET28a expression vector (EMD Millipore, Billerica, MA) (with an LEVLFQGP N-terminal amino acid sequence, corresponding to the PreScission Protease cleavage site) and electroporated into *E. coli* BL21(DE3)pLysS cells. Cultures were grown at 37°C in LB medium supplemented with 100 mg/l kanamycin. Protein expression was induced with 500 mM isopropyl-b-D-thiogalactopyranoside (IPTG) and cells were grown overnight at 16°C. Cultures were then harvested by centrifugation (4000 g, 4°C, 10 min). The bacterial pellet was resuspended in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM dithiotreitol (DTT), 10 mM imidazole, 0.1% Triton X-100 v/v, pH 7.5) and lysed by passing through a M-110P microfluidizer at 4°C (Microfluidics, Westwood, MA) and then centrifuged at 8000 g, 4°C, 45 min. The cleared lysate was incubated with washed Ni-NTA agarose beads (500 µl per 10 ml of lysate) for one hour while shaking at 4°C. After applying the beads to a polypropylene column, non-specifically bound proteins were removed by washing (50 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, 20 mM imidazole, 0.1% Triton X-100 v/v, pH 7.5). The protein was eluted in 50 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, 200 mM imidazole, 0.1% Triton X-100 v/v, pH 7.5. Fractions with the highest protein content were pooled and the His-tag and was removed by incubation with PreScission Protease (GE Healthcare, Pittsburgh, PA). The enzyme was further purified by Ni-NTA chromatography and concentrated using an Amicon Ultra 15, 30 kDa (EMD Millipore). *M. tuberculosis trxC* (Rv3914) was cloned into pET28a and expressed and purified in a similar fashion.

The gene encoding *trxB* was PCR amplified from genomic DNA of *Staphylococcus aureus* NCTC 8325 with forward primer 5'-GCGGCAGCCCATATGACTGAAATAGATTTTGGAT-3' and reverse primer 5'-GGTGGTGCTCGAGTT AAGCTTGATCGTTTAAAT-3'. The PCR product

was subsequently cloned into the expression vector pET28b (EMD Millipore, Billerica, MA). *E. coli* BL21(DE3)pLysS competent cells were electroporated with the plasmid pET28b-trxB and grown on LB agar plates supplemented with 50 mg/l kanamycin for transformant selection. Transformants were cultured in LB medium (supplemented with 50 mg/l kanamycin) at 37°C in a shaker until OD_{600nm} reached 0.6, and protein expression was induced with 200 µM isopropyl-β-D-thiogalactopyranoside (IPTG) at 26°C overnight. Cultures were then harvested by centrifugation (8000 g, 4°C, 10 min) and the cell pellet was resuspended in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM dithiotreitol (DTT), 10 mM imidazole, 0.1% Triton X-100 v/v, pH 7.5), lysed by passing through a M-110P microfluidizer at 4°C, and then centrifuged at 10000xg, 4°C, 20 min. The cleared cell lysate was incubated with Ni-NTA agarose beads (500 µl per 10 ml of lysate) for one hour with mild shaking at 4°C. The beads were applied to a polypropylene column, and washed with washing buffer (50 mM Tris - HCl, 150 mM NaCl, 1 mM DTT, 20 mM imidazole, 0.1% Triton X-100 v/v, pH 7.5) to remove nonspecifically bound proteins. The protein was then eluted with elution buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, 200 mM imidazole, 0.1% Triton X-100 v/v, pH 7.5). Fractions with purified protein were pooled and the His-tag was removed by incubation with thrombin (GE Healthcare, Pittsburgh, PA). The enzyme was further purified by Ni-NTA chromatography and concentrated using an Amicon Ultra 15, 10 kDa.

Thiol depletion assay. Bacteria were treated for 15 min (*S. aureus*) or 3 hr (*M. tuberculosis*) with the indicated concentrations of auranofin, ampicillin, or isoniazid. After treatment bacteria were washed twice in PBS and then resuspended in 100 mM potassium phosphate, monobasic, pH 7.4, containing 1 mM ethylenediaminetetraacetic acid, and lysed using a Precellys 24 homogenizer. Thiols were quantified using the Thiol Detection Assay Kit (Cayman Chemical, Ann Arbor, MI).

Murine peritonitis infection model

Briefly, MRSA strain Sanger 252 (a sequenced hospital-associated MRSA strain) was grown to mid-logarithmic phase in Todd-Hewitt broth, washed into phosphate-buffered saline, and $\sim 10^9$ colony-forming units were injected intraperitoneally (i.p.) into 8 week old female CD1 mice (Charles River Laboratories, Wilmington MA). One hour after infection, the mice were divided into three groups and treated i.p. with auranofin (either 0.12 or 0.012 mg/kg) or vehicle (n = 8 per group). Treatment was continued IP once daily throughout the study, mortality was monitored for 7 days, and mice that became moribund were humanely euthanized. This protocol was reviewed and approved by the UC San Diego Institutional Animal Care and Use Committee.

Mycothione reductase expression and activity assays. *Mtb* mycothiol reductase from H37Ra (100% identity to Rv2855c from H37Rv) was cloned into the pET28a expression vector (EMD Millipore) (with an LEVLFQGP N-terminal amino acid sequence) and electroporated into *E. coli* BL21(DE3)pLysS cells. Cultures were grown at 37°C in LB medium supplemented with 100 mg/ml kanamycin. Protein expression was induced with 500 mM isopropyl-b-D-thiogalactopyranoside (IPTG) and cells were grown overnight at 16°C. Cultures were then harvested by centrifugation (4000 g, 4°C, 10 min). The bacterial pellet was resuspended in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM dithiotreitol (DTT), 10 mM imidazole, 0.1% Triton X-100 v/v, pH 7.5) and lysed by passing through a M-110P microfluidizer at 4°C (Microfluidics) and then centrifuged at 8000 g, 4°C, 45 min. The cleared lysate was incubated with washed Ni-NTA agarose beads (500 μ l per 10 ml of lysate) for one hour while shaking at 4°C. After applying the beads to a polypropylene column, non-specifically bound proteins were removed by washing (50 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, 20 mM imidazole, 0.1% Triton X-100 v/v, pH 7.5). The protein was eluted in 50 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, 200 mM imidazole, 0.1% Triton X-100 v/v, pH 7.5. Fractions with the highest protein content were pooled and the His-tag and was removed by incubation with PreScission protease (GE Healthcare).

The enzyme was further purified by Ni-NTA chromatography and concentrated using an Amicon Ultra 15, 30 kDa (EMD Millipore).

Mycothione reductase activity assays were performed in a total volume of 50 μ l in black clear-bottom 384-well plates at 30°C. Standard reaction mixes contain 500 μ M MSSM and 500 μ M NADPH unless otherwise noted, in 50 mM HEPES, pH 8.0, with 2 mM EDTA. Enzyme activity was monitored in kinetic mode on a Spectramax M5 plate reader by measuring loss of absorbance at 340 nm.

For K_m determination, thioredoxin reductase activity assays were performed in a total volume of 50 μ l in black clear-bottom 384-well plates at 37°C. Standard reaction mixtures contain 25 nM thioredoxin reductase, 100 μ M 5,5'-dithiobis-(2nitrobenzoic acid) (DTNB), 100 μ M NADPH in 50 mM HEPES, pH 7.5, with 2 mM EDTA. Assays were performed in triplicate with varying concentrations of TrxC. Absorbance at 412 nm was read on a Spectramax M5 plate reader as an indication of enzyme activity. The initial rate during the first five min of the assay was calculated as the change in change A_{412nm} over time. K_m was calculated using GrapPad Prism.

1. Baba T, *et al.* (2006) Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* 2:2006 0008.