Supplementary Information

Phagosomal Copper–promoted Oxidative Attack on Intracellular *Mycobacterium tuberculosis*

M. Daben J. Libardo[1], Cesar de la Fuente-Nuñez[2-4], Kushi Anand[5], Gopinath Krishnamoorthy[6], Peggy Kaiser[6], Stephanie C. Pringle[7], Christopher Dietz[1], Scott Pierce[1], Michael B. Smith[1], Amy Barczak[7,8,9], Stefan H.E. Kaufmann[6], Amit Singh[5,], and Alfredo M. Angeles-Boza[1,10,]**

[1]Department of Chemistry, University of Connecticut, Storrs, CT 06269, USA

[2]Synthetic Biology Group, MIT Synthetic Biology Center, Department of Biological Engineering, and Department of Electrical Engineering and Computer Science, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

[3]Research Laboratory of Electronics, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

[4]The Broad Institute of MIT and Harvard, Cambridge, MA 02139, USA

[5]Department of Microbiology and Cell Biology, Center for Infectious Disease Research, Indian Institute of Science, Bangalore 560012, India

[6]Department of Immunology, Max Planck Institute for Infection Biology, Berlin 10117, Germany

[7]The Ragon Institute of Harvard, MIT, and Massachusetts General Hospital, Cambridge, MA 02139, USA

[8]Division of Infectious Disease, Massachusetts General Hospital, Boston, MA 02114, USA

[9]Department of Medicine, Harvard Medical School, Boston, MA 02115, USA

[10]Institute of Materials Science, University of Connecticut, Storrs, CT 06269, USA

*Correspondence: alfredo.angeles-boza@uconn.edu and asingh@iisc.ac.in

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METHOD DETAILS

Mycobacterial Srains. Frozen glycerol stock solutions of *M. smegmatis* mc^{2}155, *M. bovis* BCG (SSI 1331), *M. tuberculosis* H37Rv, and the clinical isolates BND320, JAL2287, and MYC431 were used to routinely grow mycobacteria in Middlebrook 7H9 + 10% OADC supplement (in liquid culture) or in Middlebrook 7H11 + 10% OADC supplement (in solid media). All manipulations done with *M. tuberculosis* were performed in a Biosafety Level 3 (BSL3) laboratory.

Macrophage Cell Culture. RAW264.7 cells were grown in 10% FBS in DMEM with 200 U/mL each of Penicillin and Streptomycin. THP-1 cells were cultured in DMEM (for *ex vivo* efficacy studies) or RPMI-1640 (for viability studies) with 10% FBS, 200 U/mL each of Penicillin and Streptomycin, and 0.05 mM 2-mercaptoethanol (only for RPMI medium). THP-1 cells were differentiated prior to infection by addition of phorbol 12-myristate 13-acetate (PMA) to a final concentration of 40 ng/mL and incubation for 72 hours. Cells were grown in a 37°C incubator with 5% CO_2.

Mice. BALB/c mice were purchased from Jackson Laboratories and maintained in HEPA-filtered cages in a BSL3 facility throughout the experiment. Where indicated, mice were given water with 118 mg/L $CuSO_4$ from the time of infection.

Synthesis and Purification of Peptidomimetics. DAB-6 and DAB-10 were synthesized using Fmoc/tBu chemistry and purified using RP-HPLC based on previously reported methods.[1] 5(6)-carboxytetramethylrhodamine or 5(6)-carboxytetramethylfluorescein were used for dye conjugation to the ε-amino group of key lysine residues. Briefly, Fmoc-Lys(Mtt)-OH was used in the peptide synthesis to mark the fluorophore attachment site. Once the full peptide sequence has been synthesized, Mtt deprotection was achieved by 1% TFA in DCM for 20 mins followed by exhaustive washing with 1% TFA. Fluorophores were attached via HBTU coupling. The peptide was cleaved from the resin and purified as reported.[1]

Antimicrobial Susceptibility Testing, Checkerboard Assay and Time Kill Kinetics. Activity testing was done using the broth microdilution method as suggested by Hancock.[2] Mycobacteria were grown

until exponential phase in Middlebrook 7H9 broth with 10% OADC (Oleic acid, Albumin, Dextrose, Catalase) supplement and 0.05% Tween 80. For experiments without catalase, media contained 4.7 g/L of 7H9 base, 5 g/L BSA, 0.85 g/L NaCl, 2 g/L glucose, 0.4% glycerol, and 0.05% Tween-80. Peptidomimetic stock solutions were diluted in growth medium and 50 μL of a 2-fold serial dilutions were placed on separate wells of a sterile 96-well round-bottom polypropylene plate (polystyrene plates were used for *M. tuberculosis* and *M. bovis*). To each well, 50 μL of bacterial suspension (OD_{600} of 0.2-0.4 diluted 1:1000) was added to yield a final inoculum of $5x10^5$ CFU/mL per well. Plates were incubated in seal plastic bags at 37°C for 24 hrs for *M. smegmatis*, and 7 days for *M. bovis* and *M. tuberculosis*. Following incubation, 10 μL of Alamar Blue was added and the color was allowed to develop for an additional 4 hrs for *M. smegmatis* and 24 hrs for *M. bovis* and *M. tuberculosis*. Fluorescence of the plate was read using a microplate reader and was converted into percent growth inhibition. The Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration that resulted in 90% growth inhibition. Data shown was obtained from three independent trials done in duplicates. To test synergy of DAB-10 and rifampicin, a similar method was adopted, except that DAB-10 was added to the rows of the 96-well plate while the anti-TB drugs were added to the columns. Heat maps represent data obtained from three independent trials done in duplicates. The time kill kinetics experiments were done by mixing *M. smegmatis* mc^2155 or *M. tuberculosis* H37Rv with the indicated concentration of DAB-10 with or without 100 μM tetrathiomolybdate (TTM) or 50 μM $CuCl_2$ in a total volume of 1 mL. At each time point, a 20 μL aliquot was withdrawn and diluted 100- and 1000-fold. Then 100 μL of the dilutions were plated in 7H11 plates and incubated for three days (Msm) or three weeks (Mtb) in sealed bags at 37°C. Colonies were enumerated manually and results show Mean ± SEM from three trials done in duplicates.

Assay for Activity vs Non-replicating Mtb. *M. tuberculosis* H37Rv was grown to exponential phase (OD_{600} ~1.0) in 7H9 + 10% OADC it was diluted 1:5 in Dubos medium 24 hrs prior to transferring the culture to air-tight glass bottles equipped with magnetic stir bar. The cultures were incubated at 37°C with mixing for 3-4 weeks. The bottles were then transferred to an anaerobic glovebox where it was opened. 1

mL of the non-replicating cell suspension were added to peptides at the indicated concentration or 100 μM of metronidazole in separate wells of a 24-well plate. The plate was placed in a sealed bag inside the anaerobic glovebox and was left for 7 days, afterwhich, dilutions were plated in 7H11/OADC plates and incubated at 37°C aerobically. Colonies were enumerated manually and data represent results from two independent experiments done in duplicates.

Measurement of Intramycobacterial Reduction Potentials *in vitro* and *ex vivo*. Calibration curves were first generated by treating *M. smegmatis* mc^{2}155 or *M.* tuberculosis H37Rv expressing MRx1-roGFP2 with varying ratios of DTT_{ox} and DTT_{red} corresponding to known potential values based on the Nernst Equation with a fixed total concentration of 10 mM. Bacilli were suspended in degassed PBS and the DTT mix was added. After a 5 min treatment, cells were pelleted and immediately fixed with 4% paraformaldehyde for 15 mins. Cells were washed and resuspended in 10 μL of degassed PBS for flow cytometry measurements (Msm) or plate reader measurement (Mtb). For Msm treated with DAB-10, bacilli were incubated with DAB-10 at its MIC and 2X MIC in Middlebrook 7H9 at 37°C for 1 hour. Afterwards, the cells were washed and fixed with 4% paraformaldehyde for 15 mins and analysed using a BD FACSCalibur flow cytometer. The fluorescence of 100,000 cells were measured and the data analyzed using FlowJo v.10. The fluorescence intensity values at 405 nm and 488 nm was obtained and the ratio converted to E_{MSH} values using the previously generated Calibration Curves. A similar approach was used for DAB-10-treated Mtb, except a BMG labtech microplate reader was used to measure fluorescence at 390 and 492 nm, using orbital averaging. Data shown represent Mean ± SEM obtained from three independent trials done in duplicates. For *ex vivo* measurements, PMA-differentiated THP-1 cells were infected with *M. tuberculosis* H37Rv expressing Mrx1-roGFP2 (MOI 1:10) for 4 hrs, followed by a 12 hour recovery. Then, peptides were added at 40 μM or INH at 5 μM in fresh DMEM with or without 100 μM of TTM and incubated at 37°C, 5% CO_2 incubator for 12 hours. The fluorescence was read using a BMG labtech microplate reader using orbital averaging. Fluorescence ratio was converted to

E_{MSH} values using the calibration curve obtained *in vitro*. Data represent results from two independent experiments performed in duplicates.

TUNEL Assay. *M. smegmatis* mc^2155 was treated with DAB-10 at the indicated concentration in the presence or absence of 100 μM tetrathiomolybdate (TTM) for 1 hour at 37°C . Following treatment, cells were prepared for TUNEL labeling according to a previously reported procedure.[1] The cells were then analyzed using a BD FACSCalibur flow cytometer to which fluorescence of 100,000 cells were measured and data were analyzed using FlowJo v.10. The events were gated first for propidium iodide (PI) fluorescence to differentiate the cells from debris and then gated for green fluorescence to select for TUNEL positive cells. Total TUNEL (+) cells were divided with the total number of PI (+) cells to determine % TUNEL (+) cells. Bars represent data obtained from two independent trials done in triplicates.

Laser Confocal Microscopy. 30,000 RAW264.7 cells were seeded into each well of a chambered cover glass and allowed to adhere overnight. For peptidomimetic localization; 1, 2, 4, and 8 μM of tetramethylrhodamine-labeled DAB-10 (TMR-DAB-10) or DAB-6 was added to each well and peptide labeling was allowed to proceed for 30 mins at 37°C. For endocytosis inhibitor experiments, cells were pre-treated with either 50 μM Amiloride or 80 μM Dynasore (both inhibitors were from stock solutions in DMSO and final DMSO concentration was <0.5%) for 1 hr. The cells were then washed once with growth medium and replaced with fresh media containing the inhibitors and desired concentration of DAB-10. For co-localization experiments, *M. smegmatis* constitutively expressing GFP[3] or *M. tuberculosis* harboring pMSP12-GFP plasmid[4] were grown in Middlebrook 7H9 + 10% ADC with 50 μg/mL Kanamycin was washed twice with PBS, and resuspended in 10% FBS in DMEM. The bacteria was quantified via OD600 measurements. The RAW264.7 cells were then infected at MOI 1:100 for 1 hr for Msm and MOI 1:10 for 4 hrs for Mtb, washed exhaustively with PBS and fresh growth medium was added. Infected cells were then treated with TMR-DAB-10 for 30 mins and then washed and replenished with fresh media. For copper co-localization studies, cells were simultaneously treated with 1 μM CS-1

and 4 μM TMR-DAB-10 for 30 mins. Whenever activated RAW264.7 cells were used, 100 ng/mL of Lipopolysaccharide (LPS) from *E. coli* was added 24 hours prior to cell staining. Cellular organelles were labeled with MitoTracker Deep Red and/or LysoTracker based on the manufacturer's instructions. Samples were analyzed using a Nikon A1R Laser Confocal Microscope using the appropriate filter sets.

Quantification of DAB-10 Uptake by Macrophages. 50,000 RAW264.7 cells were seeded into 24-well polystyrene plates and allowed to adhere overnight. TMR-DAB-10 or fluorescein-labeled DAB-10 (FL-DAB-10) was added at the indicated concentration and allowed to incubate for 30 mins at 37°C. After incubation, cells were washed exhaustively and lysed with 200 μL RIPA lysis buffer. The fluorescence of the lysate was measured using a Molecular Devices FlexStation 3 microplate reader via a top-read mode. The absolute quantity of fluorescent peptide in the lysate was established by using a calibration curve generated from peptide standards dissolved in the lysis buffer. Data represent Mean ± SEM obtained from three independent trials done in duplicates.

Isothermal Titration Calorimetry. Binding constants were measured on a TA instruments NanoITC calorimeter with the peptidomimetic being titrated into a solution of Cu^{2+} at 25°C. Solutions used were 2 mM DAB-10 (in the ITC syringe) and 400 μM $Cu(NO_3)_2$ (in the ITC cell) both in 20 mM MES buffer at pH 6.40. Blank titrations were run to determine heats of dilution. Data were analyzed using TA Instruments NanoAnalyze software and represent values obtained from three independent titrations.

Mammalian Cell Viability. 10,000 RAW264.7 or THP-1 cells were seeded and differentiated (for the case of THP-1 cells) in 96-well polypropylene plates. 100 μL of media containing 2-fold serial dilutions of the test agent was added to each well and the cells were incubated for 24 hrs at 37°C. Then, the cells were washed with PBS twice and replaced with fresh media containing 5 mg/mL of MTT. The color was allowed to develop for 4 hrs in the incubator afterwhich, the cells were washed with PBS twice. 200 μL of DMSO was then added to each well to lyse the cells and quantify degree of MTT oxidation. The absorbance of each well at 595 nm was recorded and used to calculated % cell viability. Data shows Mean ± SEM obtained from three independent trials done in duplicates.

Activity against Intracellular *Mycobacteria*. 20,000 THP-1 cells were seeded into 24-well plates and PMA-differentiated for 72 hrs. *M. tuberculosis* H37Rv was grown until exponential phase and was used to infect fully differentiated THP-1 cells at an MOI of 1:5. The infection was allowed to proceed for 4 hours followed by a 12 hr recovery time. Macrophages were then washed with PBS thrice. Then fresh medium was replaced into the cell containing either the peptides at the indicated concentration or INH at 5 μM in the presence or absence of 100 μM of either bathobuproinedisulfonate (BCS) or TTM. The cells were incubated for 48 hours, 4 days, or 7 days, media was changed and fresh compound was added every 48 hours. Then, macrophages were lysed and the lysate serially diluted and plated in 7H11 agar plates. The plates were incubated in sealed bags for four weeks and colonies were enumerated manually. Data shows values obtained from two trials done in duplicates.

***In vivo* efficacy study of DAB-10.** Mice were exposed to Mtb strain H37Rv via the aerosol route using an AeroMP aerosolizing device (Biaera Technologies) at a dose resulting in implantation of approximately 50 CFU per mouse. Five mice were sacrificed following infection, and lungs were recovered, homogenized, and plated on 7H10 media to quantitate implanted CFU. On day 8, mice were administered DAB-6 by intraperitoneal injection; DAB-6 mice immediately became acutely ill and were sacrificed. On days 8 through 14 post-infection, mice were administered DAB-10 by intraperitoneal injection daily with no adverse effects. At day 15 post-infection, mice were sacrificed and lungs were harvested, homogenized, diluted in 7H9 media, and plated on 7H10 media for quantitation of CFU.

Mouse protocols were reviewed and approved by the Massachusetts General Hospital Institutional Animal Care an Use Committee. Massachusetts General Hospital's Animal Welfare Assurance Number is D16-00361 (A3596-01). All protocols are in accordance with institutional guidelines, AAALAC guidelines, and animal studies guidelines in the ACS Ethical Guidelines.

Image Quantification. Microscopy images were analyzed using FIJI (Image J). For co-localization analysis, single channel images were converted to 32-bit type images and a line was drawn across the mycobacterial cell. Fluorescence intensity per pixel were obtained from the Analysis tool of FIJI. For calculations of Costes P-value of colocalization, single channel images were converted to 32-bit type

images. Regions of interests (ROIs) were drawn along the boundary of each puncta in the green channel (for CS-1 and peptide colocalization). The Costes method of randomization was applied using the green channel as the basis, and representative P-values for multiple puncta was calculated using the Coloc 2 function of FIJI ($n > 10$).

Statistical Analysis. Data were plotted and analyzed for statistical differences using GraphPad Prism 7.0. One-way or Two-way ANOVA was used to determine statistical significance which was set at $P<0.05$.

SUPPLEMENTARY FIGURES AND TABLES

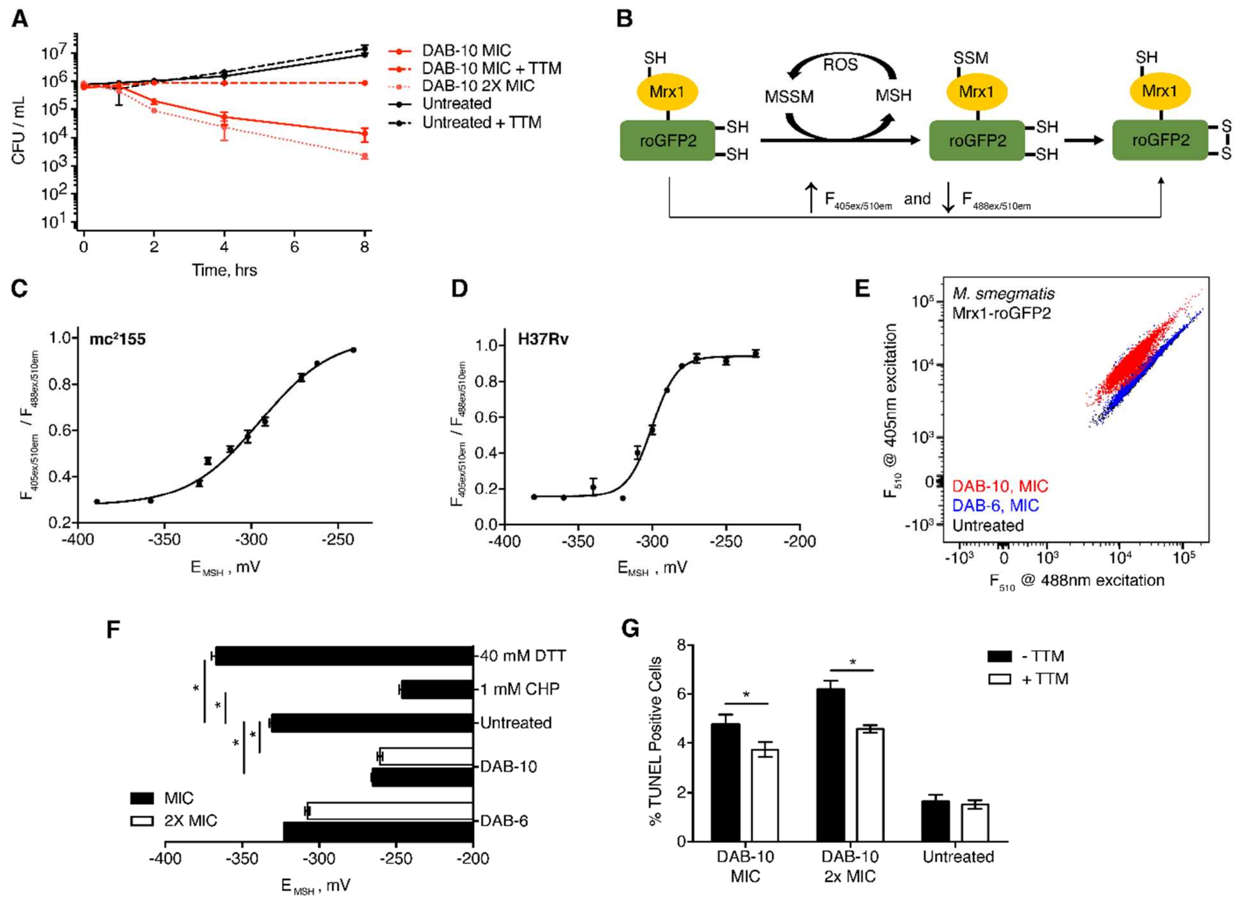


Figure S1. Cu-dependent oxidative activity of DAB-10 against mycobacteria. (A) Time-kill curves of *M. smegmatis* cells treated with DAB-10 in the presence or absence of 100 µM of the Cu chelator, TTM. Data represent mean ± SEM (n = 3 duplicates). (B) Schematic representation of changes occurring in the Mrx1-roGFP2 sensor as a result of oxidation by intracellular ROS. (C and D) Calibration curve for E_{MSH} determinations. Msm (C) and Mtb (D) were treated with varying ratios of DTT_{red} and DTT_{oxd} corresponding to calculated E_{MSH} values (in mV) using the Nernst equation. These E_{MSH} values were plotted against 405/488 fluorescence ratios obtained from flow cytometry (for Msm) or 390/492 fluorescence ratio obtained from the microplate reader (for Mtb). Plot represents data obtained from three independent experiments and shown as mean ± SEM (n = 2 triplicates). (E) Representative scatter plot obtained from flow cytometric determination of DAB-10-treated Msm, demonstrating oxidation of probe

Mrx1-roGFP2 only during DAB-10 treatment. (F) The 405/488 ratio of Mrx1-roGFP2 resulting from DAB-10 treatment of Msm were converted to E_{MSH} values using the Nernst equation. Bars represent mean ± SEM (n = 3 duplicates). *, *P*<0.05. (G) Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP Nicked End Labeling (TUNEL) assay was used to quantify extent of genomic DNA damage arising from oxidative activity of DAB-10. Exponential phase Msm cells were treated with DAB-10 at indicated concentration with or without 100 μM of TTM in 7H9 + 10% OADC. Bares represent mean ± SEM (n = 2 triplicates). *, *P*<0.05.

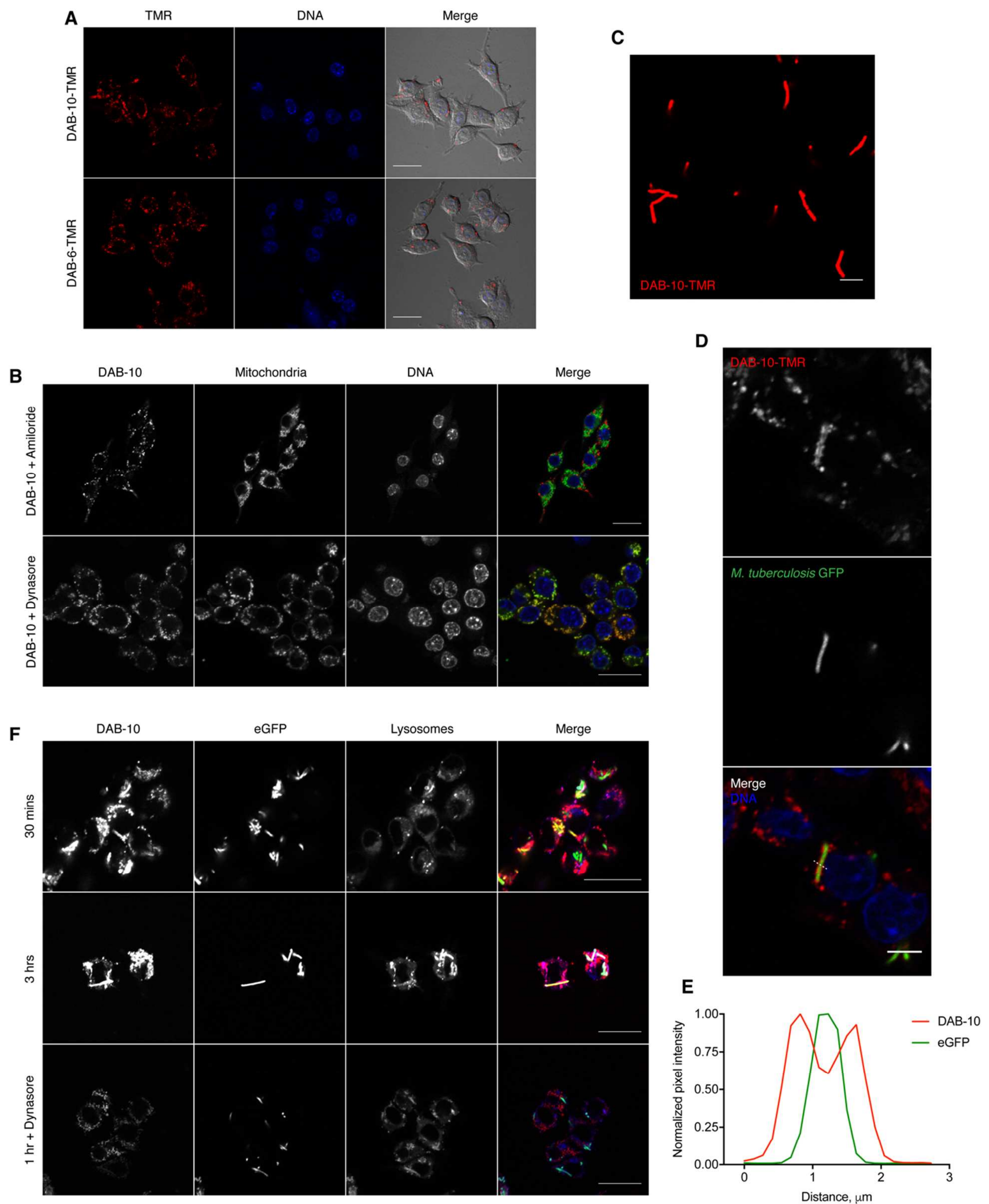
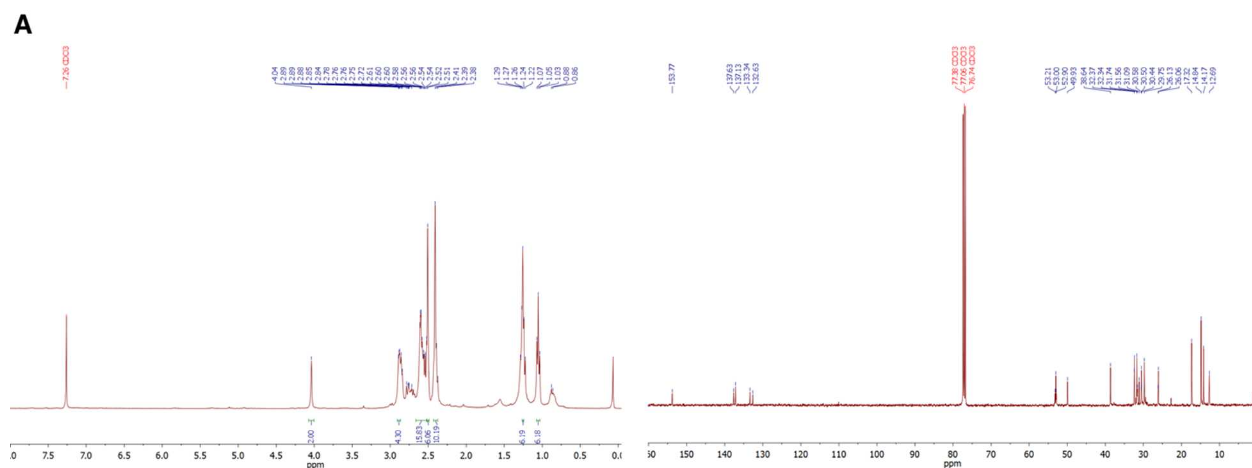


Figure S2. DAB-10 localization in macrophages and mycobacteria. (A) 1 μM of DAB-10 or 4 μM of DAB-6 was incubated with RAW264.7 cells for 1 hr at 37°C and localization was followed using laser

confocal microscopy. Hoescht dye was used to visualize the DNA. Scale bar = 20 µm. (B) RAW264.7 cells were treated with either 50 µM amiloride or 80 µM dynasore prior to incubation with 4 µM DAB-10-TMR. Localization was followed by confocal microscopy. MitoTracker deep red and Hoescht dye was used to label the mitochondria and DNA respectively. Scale bar = 20 µm. (C) A suspension of 10^6 CFU/mL of Msm was treated with 1 µM of DAB-10-TMR for 30 mins and localization was visualized by confocal microscopy. Scale bar = 5 µm. (D) RAW264.7 cells were infected with GFP-expressing Mtb H37Rv for 4 hrs at 37°C prior to addition of 5 µM DAB-10-TMR for 30 mins. White dashed line in the merge channel was used for fluorescence intensity measurement. Scale bar = 5 µm. (E) Plot of length of the white dashed line in (D) versus pixel intensity along the red and green channel obtained using the Analysis tool of FIJI. (F) RAW264.7 cells were infected with eGFP-expressing Msm (MOI 1:100) prior to incubation with 4 µM DAB-10-TMR and LysoTracker for 30 mins in the dark. 80 µM of dynasore was added for the experiment in the bottom panel. Localizations were visualized using confocal microscopy. Scale bar = 20 µm.



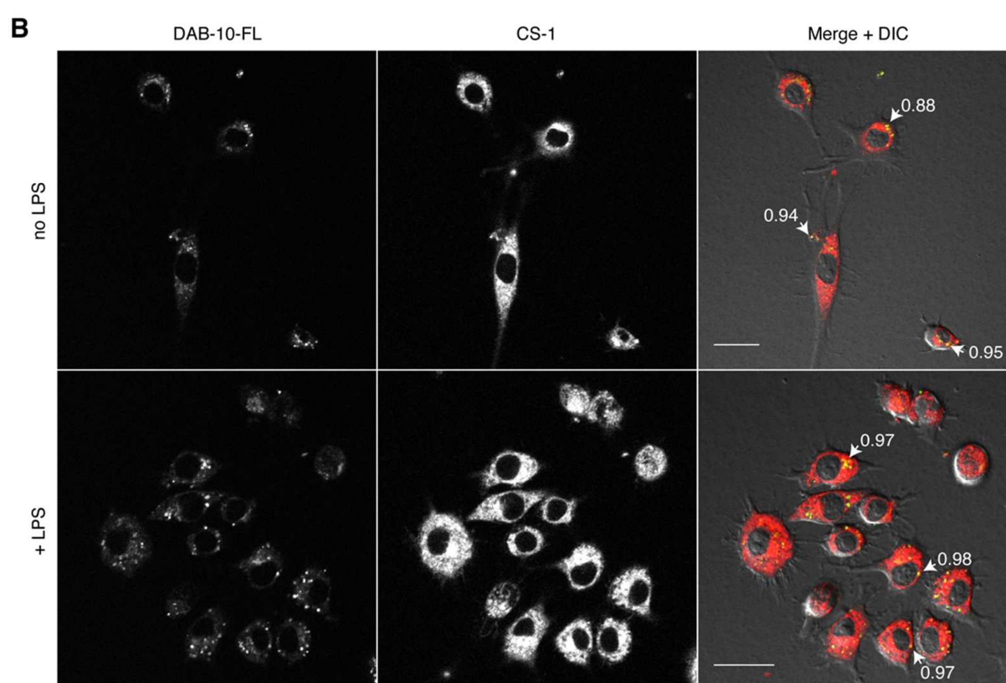


Figure S3. DAB-10 Co-localization with intracellular copper. (A) ^{1}H (left) and ^{13}C (right) NMR spectra (in $CDCl_3$) of synthesized CS-1 copper fluorescent probe. All peaks are accounted for and match the literature values.[5] (B) Naïve and LPS-activated RAW264.7 cells were incubated with fluorescein (FL)-labeled DAB-10 at 8 μM and 1 μM of CS-1 for 1 hr followed by visualization with confocal microscopy. Values in white in the merge channel represent the Costes P-value of colocalization (calculated using the Coloc 2 function of FIJI) for the discrete puncta pointed by the white arrow. Scale bar = 20 μm.

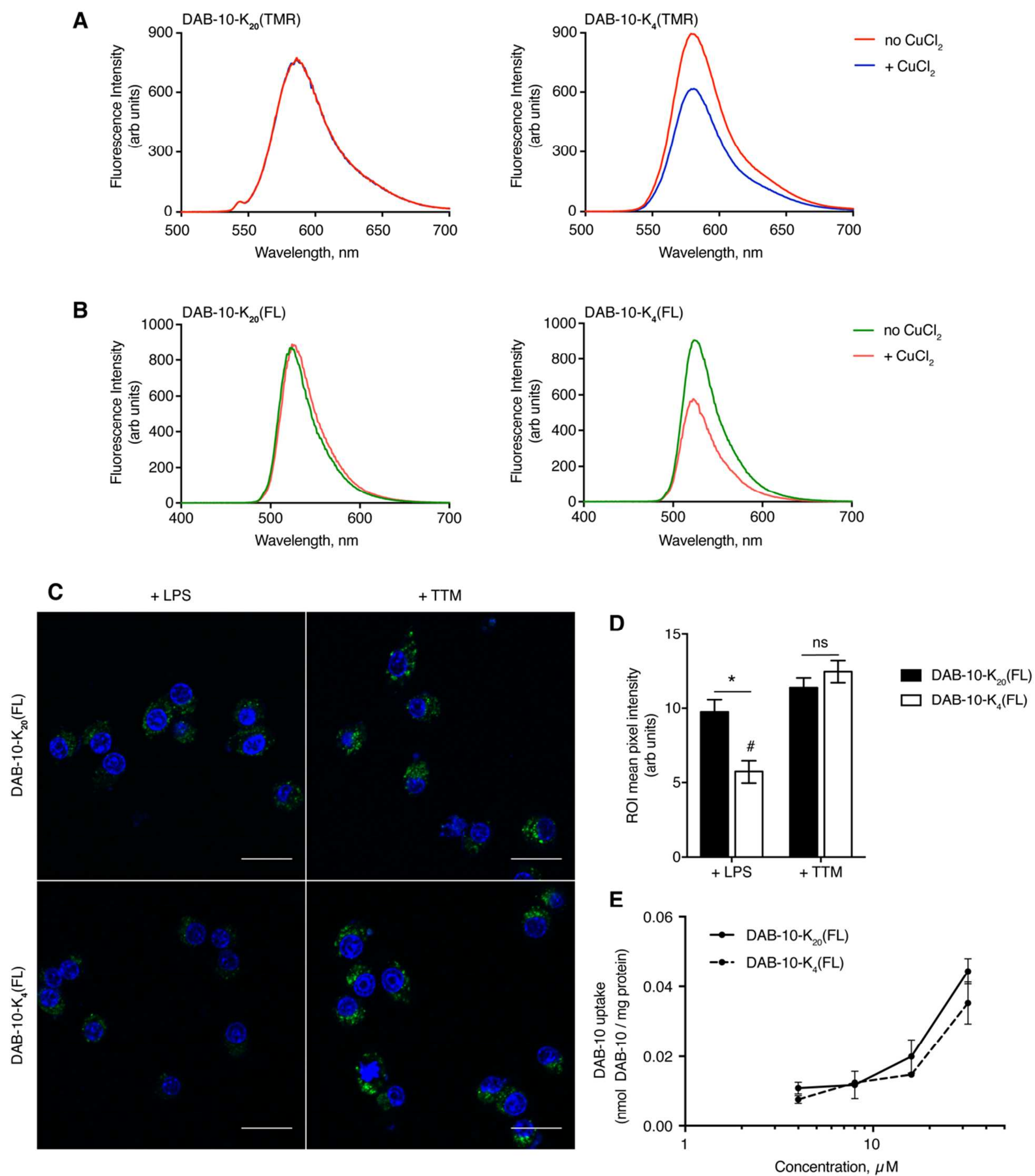


Figure S4. Intracellular Cu binding by DAB-10 probed by fluorescence studies. (A) The emission spectra of 2 μM of DAB-10-K_{20}(TMR) or DAB-10-K_4(TMR) was measured before and after addition of 2 μM $CuSO_4$ using a CaryWin Varian spectrofluorometer. (B) A similar experiment was done as in (A), using fluorescein-labeled DAB-10 instead of TMR. Both (A) and (B) indicates that fluorescence

suppression phenotype is independent of the identity of the fluorophore and is only a function of its position relative to the copper binding site. (C) LPS-activated RAW264.7 cells were incubated with different FL-labeled DAB-10 derivatives at 8 μM (with or without 100 μM of TTM) for 30 mins prior to confocal microscopy. Scale bar = 20 μm. (D) Regions of interest (ROIs) were drawn using the green channel and followed the region covered by the green puncta in the images. Pixel intensity was obtained from the Analysis tool of FIJI. Fluorescence intensity was measure for all the cells shown in the field of view. *, $P<0.05$. (E) Analytical Quantification of DAB-10-FL uptake in RAW264.7 following 1 hour incubation. Data represent mean ± SEM (n = 3 duplicates).

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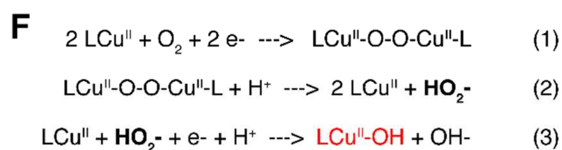

Figure S5. Various efficacy and cytotoxicity studies of DAB-10. (A) RAW264.7 cells were incubated for 24 hrs with the indicated concentration of either DAB-6 or DAB-10. Viability was assessed using the MTT assay. Data is the mean ± SEM obtained from three experiments done in duplicates. (B) PMA-

differentiated THP-1 cells were infected with Mtb H37Rv (MOI 1:5) prior to incubation with 10X the *in vitro* MIC of DAB-6 (80 μM) and DAB-10 (20 μM) for 48 hours with or without 100 μM of the copper chelators. Results represent mean ± SEM from three trials done in duplicates. *, $P<0.05$. (C) PMA-differentiated THP-1 cells were incubated with the copper chelators at the indicated concentrations for 24 hrs. Cellular viability was assessed using the MTT assay. Data is the mean ± SEM obtained from three experiments done in duplicates. (D) *in vivo* efficacy of DAB-10 was assessed in BALB/c mice aerosol infected with Mtb H37Rv and fed with either pure water or water containing 118 mg/L $CuSO_4$. Mice were treated with 5 mg/kg of DAB-10 everyday for 7 days prior to sacrificing the animal, homogenizing the lung, and plating for CFUs. (E) The checkerboard assay was used to assess the interactions between DAB-10 and rifampicin. Mtb H37Rv were treated with the test agents either alone or various proportions for 7 days. Heat maps show percent viability in each well determined via measurement of alamar blue fluorescence. Data shows values obtained from three independent trials done in duplicates. Values inside heat maps are fractional inhibitory concentration (FIC) indices for the corresponding wells. FIC Indices < 0.5 indicate synergistic interactions. Bars to the right of the heat maps show color scale for % bacterial viability. (F) Proposed mechanism of ROS formation by Cowan and co-workers. The deprotonated H_2O_2 which is likely scavenged by catalase is highlighted in bold letter, and the formal ROS produced is shown in red fonts.

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