Supplementary information (SI)

1 Supplementary figures

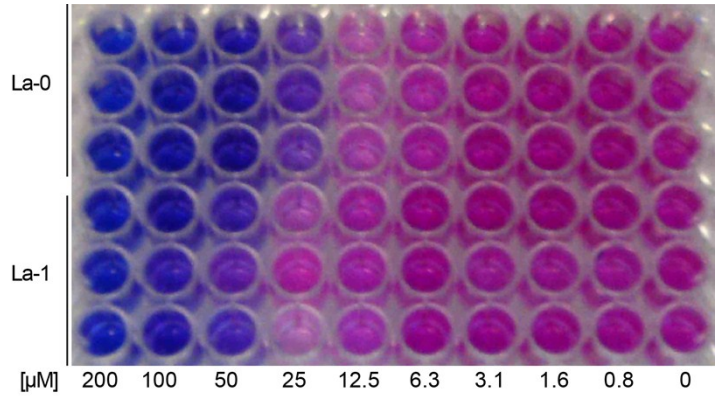


Fig. S1 MIC determination of La-0 and La-1 in *M. tuberculosis* H37Rv. Purple: dead cells. Pink: alive cells.

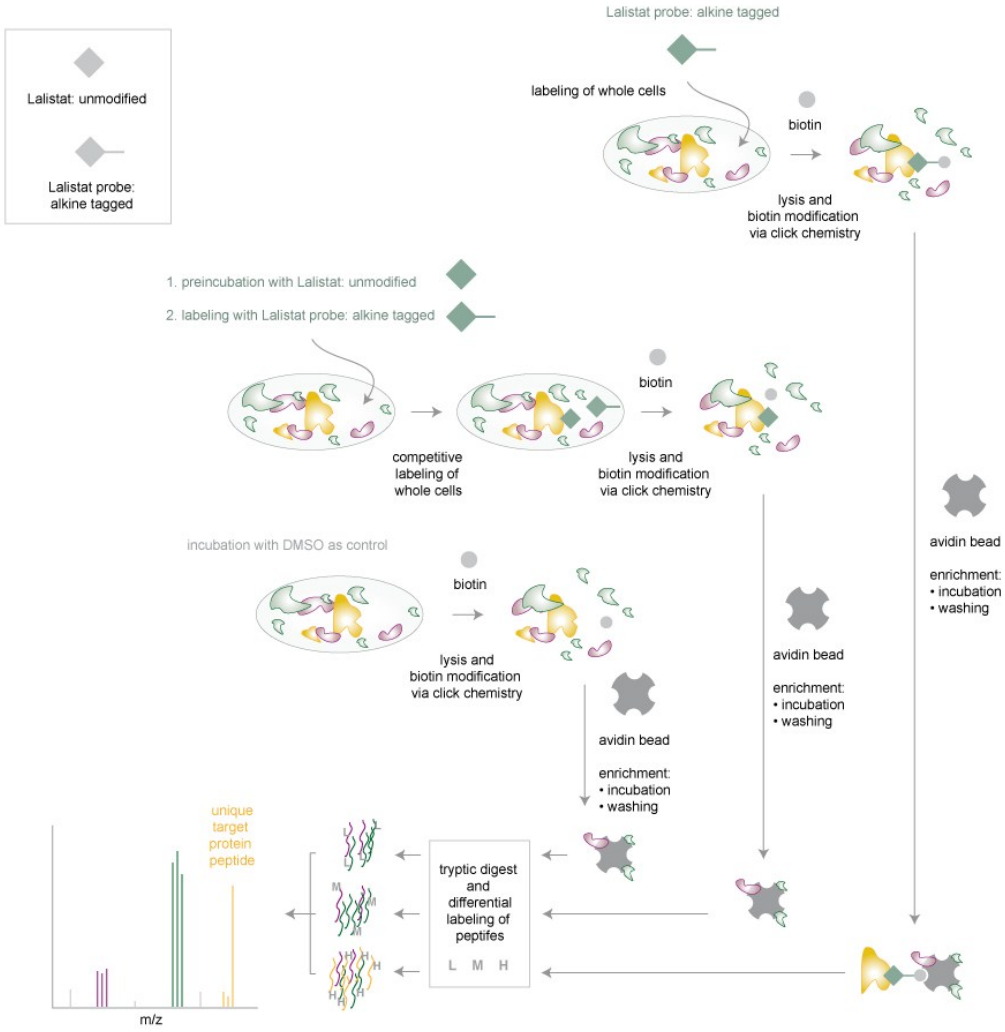


Fig. S2 Activity based protein profiling (ABPP) workflow for quantitative, gel-free proteomics including DMSO control and competition control.

2 Materials and methods

2.1 Synthesis

2.1.1 Synthesis of 4-(Piperidin-1-yl)-1,2,5-thiodiazol-3-yl piperidine-1-carboxylate (La-0)

La-0 was prepared according to Rosenbaum et al.[1]

2.1.2 Synthesis of 4-(Piperidin-1-yl)-1,2,5-thiadiazol-3-yl 4-ethynylpiperidine-1-carboxylate (La-1)

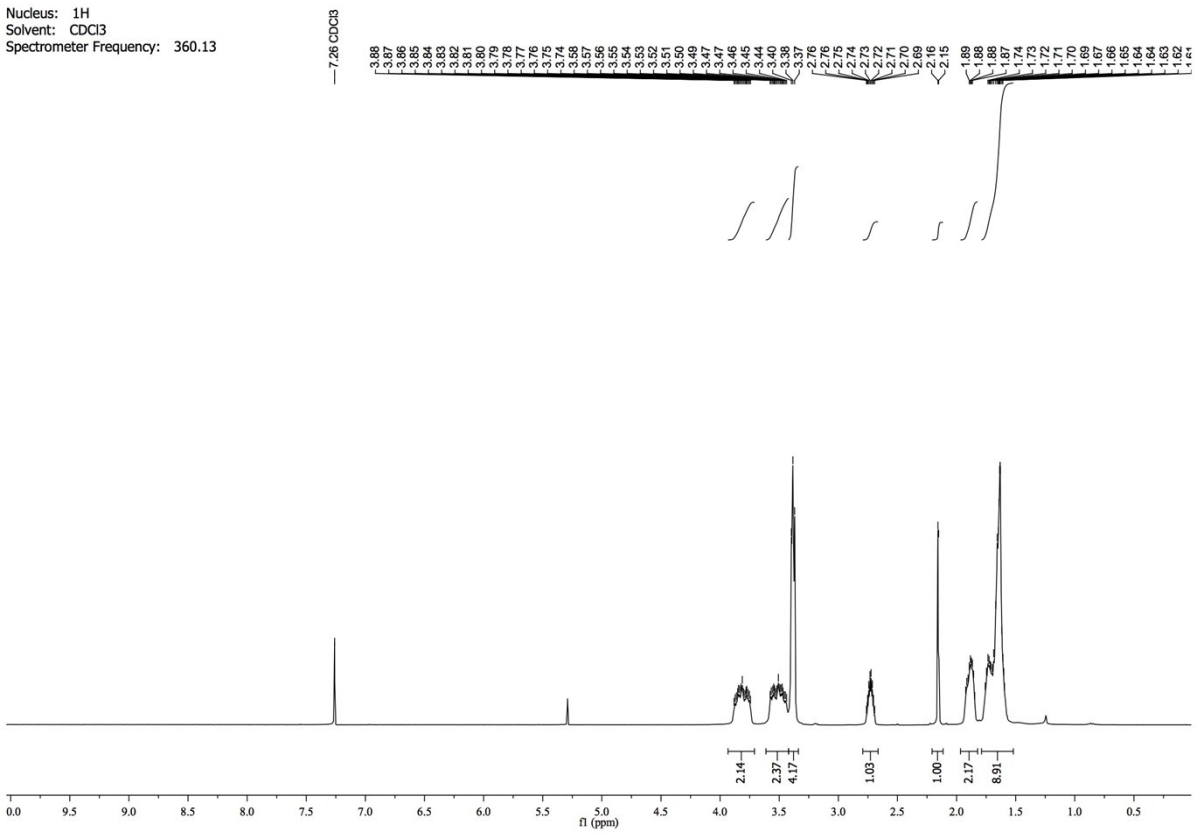
4-(Piperidin-1-yl)-1,2,5-thiadiazol-3-ol (76.7 mg, 0.41 mmol) was prepared according to Rosenbaum et al. [1] and dissolved in THF (10 mL). NaH (16.4 mg, 60% in mineral oil, 0.41 mmol) was added and the resulting suspension was stirred until full dissolution indicating complete deprotonation. A phosgene solution (234 µL, 20% in toluene, 1.75 M, 0.41 mmol) was added and the resulting solution was stirred at rt for 10 min. 4-Ethynylpiperidine-TFA salt (144 mg, 0.70 mmol), prepared as described by Braisted et al.[2], and DIPEA (120 µL, 0.70 mmol) were combined in THF (2 mL) and stirred for 5 min. This solution was added to the *in situ* formed chloroformate solution and stirred at rt for 20 min. The reaction was stopped by addition of 1 M HCl (10 mL) and diluted with EtOAc (25 mL). After separation of phases the organic layer was washed three times with saturated NaHCO3 solution, dried over $MgSO_4$ and concentrated *in vacuo*. The product was purified by RP-HPLC as the HCl salt to yield as a clear oil.

RP-HPLC (analytical setup, method: gradient 2% B → 98% B over 10 min): t_R = 8.73 min.

^{1}H-NMR (360 MHz, $CDCl_3$): δ [ppm] = 3.89-3.73 (m, 2 H), 3.59-3.43 (m, 2 H), 3.42-3.35 (m, 4 H), 2.73 (tq, J = 3.7, 7.0 Hz, 1 H), 2.15 (d, J = 2.4 Hz, 1 H), 1.95-1.83 (m, 2 H), 1.78-1.68 (m, 2 H), 1.67-1.57 (m, 6 H).

^{13}C-NMR (91 MHz, $CDCl_3$): δ [ppm] = 153.8, 150.9, 146.6, 85.5, 70.5, 49.1, 43.2, 42.8, 31.4, 20.9, 26.4, 25.5, 24.3.

HRMS (ESI): calc. for $C_{15}H_{25}N_2O_2$ $[M+H]^+$: 321.1380; found: 321.1372.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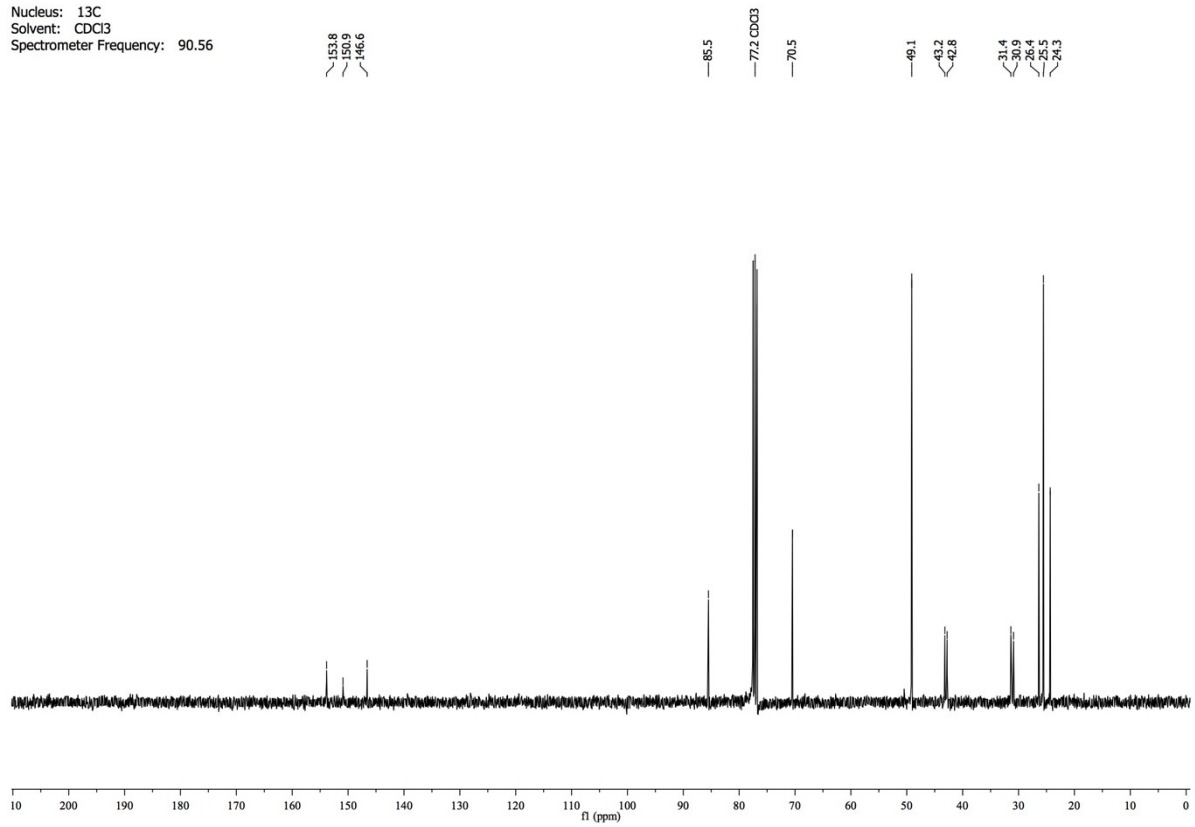
2.17
8.91
f1 (ppm)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85.5
77.2 CDCl3
70.5
49.1
43.2
42.8
31.4
30.9
26.4
25.5
24.3
f1 (ppm)

2.2. Minimal inhibitory concentration (MIC) determination

Compound-mediated growth inhibition was carried out using 96-well plates. A culture of stationary phase growing bacteria was diluted to a final OD_{600} = 0.001 in fresh media. 100 µL of diluted bacteria was added to 100 µL of two fold inhibitory concentration of compound to be tested and diluted two fold in each well. 96-well plates were incubated at 37 °C under a water impermeable membrane. To determine the growth of mycobacteria 100 µL of a 0.02 % resazurin solution was added 7 days for *M. tuberculosis*. A color change from purple to pink within 2 to 4 days indicated viable cells while purple colored wells suggested no bacterial growth.

2.3. FICI determination

Determination synergistic drug effects were calculated using fractional inhibitory concentration index (FICI):

$$FICI = \frac{MIC\ A+B}{MIC\ A} + \frac{MIC\ B+A}{MIC\ B}$$

MIC A+B – MIC of drug A when combined with drug B

MIC B+A – MIC of drug B when combined with drug A

Results were evaluated as follows: synergism: ≤ 0.5, indifference: > 0.5 – 4, antagonism > 4.[3]

For the combination of La-0 and Vancomycin were following values obtained:

MIC La-0 = 50 µM

MIC La-0 + Vanc. (0.4 µM) = 12.5 µM

MIC Vanc. = 6.3 µM

MIC Vanc. + La-0 (12.5 µM) = 0.4 µM

$FICI_{La\text{-}0/Vanc} \approx 0.31$

2.4 *Mycobacterium tuberculosis* Growth Analysis

GFP-expressing *Mycobacterium tuberculosis* H37Rv [4] was generated using the plasmid 32362:pMN437 (Addgene), kindly provided by M. Niederweis (University of Alabama, Birmingham, AL) [5]. $1x10^6$ bacteria were cultured in 7H9 medium supplemented with oleic acid-albumin-dextrose-catalase (OADC) (10%), Tween 80 (0.05%), and glycerol (0.2%) in a total volume of 100 µl in a black 96 well plate with clear bottom (Corning Inc, Corning, NY) sealed with an air-permeable membrane (Porvair Sciences, Dunn Labortechnik, Asbach, Germany). Growth was as measured as RLU at 528 nm after excitation at 485 nm in a fluorescence microplate reader (Synergy 2, Biotek, Winooski, VT) at indicated time points.

2.5 Analysis of *M. tuberculosis* growth in human macrophages

Mononuclear cells were isolated from peripheral blood (PBMC) of healthy volunteers by density gradient centrifugation. Monocytes were separated (purity consistently >95%) by counterflow elutriation. Human monocyte-derived Macrophages (hMDM) were generated in the presence of 10 ng/ml recombinant human M-CSF from highly purified monocytes as described.[6] *M. tuberculosis* growth in human macrophages was analyzed as described.[7] In brief $2x10^5$ hMDMs were cultured in 500µl RPMI 1640 with 10% FCS and 4mM L-glutamine in 48-well flat-bottom microtiter plates (Nunc) at 37°C in a humidified atmosphere containing 5% CO_2. Macrophages were infected with *M. tuberculosis* strain H37Rv with an MOI of 1:1. Four hours post infection, non-phagocytosed bacteria were removed by washing three times with 0.5ml Hanks' balanced salt solution (HBSS; Invitrogen) at 37°C. After washing and after 3 days of cultivation, 0.5 ml media was added to the macrophage culture. At day 7 supernatants were completely removed and macrophage cultures were lysed at 4 hours and 7 days post infection

by adding 10 µl 10% Saponin solution (Sigma) in HBSS at 37°C for 15 min. Lysates were serially diluted in sterile water containing 0.05% Tween 80 (Merck, Darmstadt, Germany) and plated twice on 7H10 agar containing 0.5% glycerol (Serva) and 10% heat-inactivated bovine calf serum (BioWest, France). After 3 weeks at 37°C the CFUs were counted.

2.6 Activity based protein profiling (ABPP)

M. tuberculosis H37Rv was derived from frozen stock ($2.5x10^8$ bacteria/ml). Homogenous bacterial suspension was prepared in 7H9 medium (50 mL) supplemented with oleic acid-albumin-dextrose-catalase (OADC) (10%), Tween 80 (0.05%), and glycerol (0.2%). 25 mL each was incubated in 30 mL square medium bottles (Nalgene) at 37°C without shaking for three days. Preculture was diluted to 450 mL and incubated for four days. Bacteria were washed with PBS and an optical density at 600 nm of 40 was adjusted. For regular ABPP experiments (probe / DMSO control) 1 mL suspension was supplemented with 30 µM lalistat-1 (La-1; lalistat probe) or DMSO as a control and incubated for 30 min, vortexed and incubated another 30 min. For competition experiments (probe / competition control) 1 mL suspension was supplemented with 60 µM lalistat (La-0) or DMSO control and incubated for 30 min, vortexed and incubated another 30 min. Both samples were now additionally supplemented with 30 µM lalistat-1 (La-1; lalistat probe), incubated for 30 min, vortexed and incubated another 30 min. For both regular and competition experiments bacteria were washed with PBS and the pellet was stored at -80°C over night. Pellet was suspended in 1 mL PBS supplemented with 80 µL protease inhibitor (stock: 1 tablet solved in 2 ml ddH_2O). Samples were sonicated (duty cycle: 50; output 10 (100%)) at 4°C for 20 min each, centrifuged (15 000 xG, 4°C, 30 min) and the supernatant was centrifuged over 0.22 µm Spin-X Centrifuge tube filter (Costar) (15 000 xG, 4°C, 15 min). Samples were stored at -80°C.

Two identical samples each derived from 1 mL OD_{600}=40 cultures were combined and treated with 120 µL gel-free ABPP Mix (40 µL Biotin-PEG_3-N_3 (Jena Bioscience, CLK-AZ104P4-100; 10 mM in DMSO), 20 µL fresh TCEP (50 mM in ddH_2O), 60 µL TBTA Ligand (1.667 mM in 80 % tBuOH and 20 % DMSO)). The final concentrations were 233 µM Biotin-PEG_3-N_3, 581 µM TCEP and 58.2 µM TBTA Ligand. The lysates were mixed by vortexing and 20 µL $CuSO_4$ solution (50 mM in ddH_2O) were added. The lysates were mixed by vortexing again and incubated for 1h at RT in the dark. After the click-reaction the lysates were transferred to 15 mL falcon tubes and 8 mL of cold acetone (-80°C, MS grade) were added. Proteins were precipitated ON at -80°C.

The precipitated proteins were thawed on ice, pelletized (16900 xG, 15 min, 4°C) and supernatant was disposed. Falcon tubes were stored on ice during the following washing procedure: The proteins were washed two times with 1 mL cold methanol (-80°C). Resuspension was achieved by sonication (15 sec at 10 % intensity) and proteins were pelletized via centrifugation (16900 xG, 10 min, 4°C). Only MS grade water was used for the following procedures. After two washing steps supernatant was disposed and the pellet was resuspended in 500 µL 0.2 % SDS in PBS at RT by sonication (15 sec at 10 % intensity). Avidin beads were thawed on ice and resuspended by carefully inverting. Then 50 µL of bead suspension were transferred into Protein LoBind Eppendorf tubes using wide bore pipette tips and washed three times with 1 mL 0.2 % SDS in PBS (resuspension: carefully inverting 10 times, pelleting: 400 xG, 3 min, RT). 500 µL protein solution from the 15 mL falcon tubes were transferred to the Protein LoBind Eppendorf tubes with washed avidin beads and incubated under continuous inverting (20 rpm, 1 h, RT). Beads were washed 3 times with 1 mL 0.2 % SDS in PBS, 2 times with 1 mL 6 M urea in water and 3 times with 1 mL PBS (resuspension: carefully inverting 20 times, pelleting: 400 xG, 3 min, RT).

The beads were resuspended in 200 µl denaturation buffer (7 M urea, 2 M thiourea in 20 mM pH 7.5 HEPES buffer). Proteins were reduced through addition of dithiothreitol (DTT, 1 M, 0.2 µL), the tubes were mixed by vortexing shortly and incubated in a thermomixer (450 rpm, 45 min, RT). Then 2-iodoacetamide (IAA, 550 mM, 2 µL) was added for alkylation, the tubes were mixed by vortexing shortly and incubated in a thermomixer (450 rpm, 30 min, RT, in the dark). Remaining IAA was quenched by the addition of dithiothreitol (DTT, 1 M, 0.8 µL). The tubes were shortly mixed by vortexing and incubated in a thermomixer (450 rpm, 30 min, RT). LysC (0.5 µg/µL, Wako) was thawed on ice and 1µL was added to each microcentrifuge tube, the tubes were shortly mixed by vortexing and incubated in a thermomixer (450 rpm, 2 h, RT, in the dark). TEAB solution (600 µL, 50 mM in water) and then trypsin (1.5 µL, 0.5 µg/µL in 50 mM acetic acid, Promega) were added and tubes were shortly vortexed after each addition. The reaction was incubated in a thermomixer (450 rpm, 13-15 h, 37 °C). The digest was stopped by adding 6 µL formic acid (FA) and vortexing. After centrifugation (100 xG, 1 min, RT), the supernatant was transferred to a new Protein LoBind Eppendorf tube. FA (50 µL, aqueous 0.1 % solution) was added to the

beads and after vortexing and centrifugation (100 xG, 1 min, RT) the supernatant was added to the supernatant collected before. Again FA (50 µL, aqueous 0.1 % solution) was added to the beads and after vortexing and centrifugation (16200 xG, 3 min, RT) the supernatant was transferred to the combined supernatants.

50 mg SepPak C18 columns (Waters) were equilibrated by gravity flow with 1mL acetonitrile, 1 mL elution buffer (80% ACN, 0.5% FA) and 3 mL aqueous 0.5% FA solution. Subsequently the samples were loaded by gravity flow, washed with 5 mL aqueous 0.5 % FA solution and labeled with 5 mL of the respective dimethyl labeling solution. The following solutions were used: light (L): 30 mM $NaBH_3CN$, 0.2 % CH_2O, 10 mM NaH_2PO_4, 35 mM Na_2HPO_4, pH 7.5; medium (M): 30 mM $NaBH_3CN$, 0.2 % CD_2O, 10 mM NaH_2PO_4, 35 mM Na_2HPO_4, pH 7.5; heavy (H): 30 mM $NaBHD_3CN$, 0.2 % $^{13}CD_2O$, 10 mM NaH_2PO_4, 35 mM Na_2HPO_4, pH 7.5. Labeled peptides were eluted into new 2.0 mL Protein LoBind Eppendorf tubes using two times 250 µL elution buffer. The eluates were lyophilized and stored at -20°C.

Prior to MS measurement the samples were dissolved in 30 µL 1 % FA by pipetting up and down, vortexing and sonication for 15 min (brief centrifugation after each step). Differentially labeled samples were mixed. 0.45 µm centrifugal filter units (VWR) were equilibrated with two times 500 µL water, 500 µL 0.05 N NaOH and two times 500 µL 1 % FA (centrifugation: 16200 xG, 1 min, RT). Reconstituted and mixed peptide samples were filtered through the equilibrated filters (centrifugation: 16200 xG, 2 min, RT). Samples were analyzed via HPLC-MS/MS using an UltiMate 3000 nano HPLC system (Dionex, Sunnyvale, California, USA) equipped with Acclaim C18 PepMap100 75 µm ID x 2 cm trap and Acclaim C18 PepMap RSLC, 75 µM ID x 15 cm separation columns coupled to an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). Samples were loaded on the trap and washed for 10 min with 0.1 % formic acid, then transferred to the analytical column and separated using a 120 min gradient from 3 % to 25 % acetonitrile in 0.1 % formic acid and 5 % dimethyl sulfoxide (at 200 nL/min flow rate). Orbitrap Fusion was operated in a 3 second top speed data dependent mode. Full scan acquisition was performed in the orbitrap at a resolution of 120000 and an ion target of 4E5 in a scan range of 300 – 1700 m/z. Monoisotopic precursor selection as well as dynamic exclusion for 60 s were enabled. Precursors with charge states of 2 – 7 and intensities greater than 5E3 were selected for fragmentation. Isolation was performed in the quadrupole using a window of 1.6 m/z. Precursors were collected to a target of 1E2 for a maximum injection time of 250 with “inject ions for all available parallelizable time” enabled. Fragments were generated using higher-energy collisional dissociation (HCD) and detected in the ion trap at a rapid scan rate. Internal calibration was performed using the ion signal of fluoranthene cations (EASY-ETD/IC source)

Peptide and protein identifications were performed using MaxQuant 1.5.3.8 software with Andromeda as search engine using following parameters: Carbamidomethylation of cysteines as fixed and oxidation of methionine as well as acetylation of N-termini as dynamic modifications, trypsin/P as the proteolytic enzyme, 4.5 ppm for precursor mass tolerance (main search ppm) and 0.5 Da for fragment mass tolerance (ITMS MS/MS tolerance). Searches were done against the Uniprot database for *M. tuberculosis* H37Rv (taxon identifier: 83332, downloaded on 19.5.2015). Quantification was performed using dimethyl labeling with the following settings: light: DimethLys0, DimethNter0; medium: DimethLys4, DimethNter4 and heavy: DimethLys8, DimethNter8. Variable modifications were included for quantification. The I = L and requantify options were used. Identification was done with at least 2 unique peptides and quantification only with unique peptides.

Statistical analysis was performed with Perseus 1.5.1.6. Putative contaminants, reverse peptides and peptides only identified by site were omitted from further processing. Dimethyllabeling ratios were $\log_2(x)$ transformed and z-score normalized. The average values of technical replicates were calculated and $-\log_{10}$(p-values) were obtained by a two sided one sample t-test over six biological replicates for standard ABPP with DMSO control or 4 biological replicates for competition experiments.

Proteins were ranked from highest to lowest $\log_2(x)$ transformed and z-score normalized dimethyllabeling ratios. They were also ranked from highest to lowest $-\log_{10}(x)$ transformed p-values. Proteins were finally ranked according to the sum of the ranking values from dimethyllabeling ratios and $-\log_{10}$(p-value) across both experiments (regular ABPP: probe / DMSO, competition experiment: probe / competition). 2% of the identified proteins with the highest final ranking (including regular ABPP and competition experiments dimethyllabeling ratios and p-values) were considered to be hits of lalistat. This cut-off was chosen as by this analysis a visual separation of enriched vs. not enriched proteins in both regular ABPP and competition experiments could be achieved.

2.7 Recombinant expression and labelling of proteins in *E. coli*

A N-terminal His_6 affinity tagged LipR construct was cloned in a pDONR201 (Invitrogen) vector and then in a pET300 expression vector via the GATEWAY cloning system using the primers shown below and genomic DNA from *M. tuberculosis* H37Rv. Expression was induced at an OD_{600} of 0.6 by addition of Isopropyl-β-D-thiogalactopyranosid (IPTG; final concentration: 0.25 mM) and carried out 4 h at 37°C in *E. coli* BL21 cells.

Construct: N-His_6-attB1-TEV-LipR-Stop-attB2

Primer 1: ggggacaagtttgtacaaaaaagcaggctttgagaatctttattttcagggcAACCTGCGCAAAAACGTCATCC

Primer 2: ggggaccactttgtacaagaaagctgggtgTCATTTGACTACTCCCCGTGG

After centrifugation (5 min, 6200 xG, 4°C) and removal of the supernatant bacteria were resuspended in PBS to get an OD_{600} of 40. To 1 mL of this suspension in a microcentrifuge tube 30 µL of La-1 solution in DMSO (or just DMSO as a control) were added. After 30 min incubation at RT in the dark the microcentrifuge tube was again mixed by vortexing and incubated for another 30 min at RT in the dark. After centrifugation (6200 xG, 2 min, 4°C) the supernatant was removed and the pellets were stored at -80°C.

Pellets were resuspended in 1 mL PBS (4°C) and transferred to a 'Precellys Glass/Ceramic Kit SK38 2.0 mL' tube. Tubes were cooled on ice for about 5 min or longer and cells were lysed with the Precellys Homogeniser using two times lysis program 3 (5400 rpm, run number: 1, run time: 20 sec, pause: 5 sec). After each lysis run the tubes were cooled on ice for 5 min. The ball mill tubes were centrifuged (16200 xG, 10 min, 4°C) and 86 µL of supernatant were transferred to new 1.5 mL microcentrifuge tubes and treated with 10 µL gel-based ABPP Mix (2 µL RhN_3 (Tetramethylrhodamine (TAMRA) Azide (Tetramethylrhodamine 5-Carboxamido-(6-Azidohexanyl)), 5-isomer (life technologies, T10182); 5 mM in DMSO), 2 µL fresh TCEP (50 mM in ddH_2O), 6 µL TBTA Ligand (1.667 mM in 80 % tBuOH and 20 % DMSO)). The final concentrations were: 104 µM RhN_3, 1.04 mM TCEP and 104 µM TBTA Ligand. The lysates were mixed by vortexing and 2 µL $CuSO_4$ solution (50 mM in ddH_2O) were added. The lysates were again mixed by vortexing and incubated for 1h at RT in the dark. Then 80 µL 2x Laemmli Sample Buffer were added, samples were mixed in a thermomixer (300 rpm, 3 min, 96°C) and analyzed via SDS PAGE (10 % agarose gel (PEQLAB Biotechnologie GmbH, Erlangen, PerfectBlue Dual Gel System, the gel was prepared according to the manual), 3.5 h, 300 V, 8 µL fluorescent protein standard) and fluorescence imaging (GE Healthcare, ImageQuant LAS-4000). After fluorescence scanning the gel was coomassie stained.

References

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