Supplementary Information for:

An amiloride derivative is active against the F_1F_o-ATP synthase and cytochrome *bd* oxidase of *Mycobacterium tuberculosis*

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Supplementary Table 1: Bacterial strains and plasmids used in this study

Strain or Plasmid	Description	Source
E. coli strains		
ATCC 10536	*E. coli* quality control strain	ATCC
S. aureus strains		
ATCC 6538	*S. aureus* quality control strain	ATCC
E. faecalis strains		
JH2-2	Laboratory strain, plasmid-free; rif^r , fs^r	[1]
M. smegmatis strains		
mc^2155	Electrocompetent wild-type strain of *M. smegmatis*	[2]
Δ*cyd*	mc^2155 with a markerless in frame deletion in the *cydAB* gene	[3]
Δ*qcr*	mc^2155 with a markerless in frame deletion in the *qcrB* gene	[4]
M. tuberculosis strains		
H37Rv	*M. tuberculosis* reference strain	
mc^26230	Avirulent auxotrophic *M. tuberculosis* mutant (Δ*RD1* Δ*panCD*). Wild-type for this study.	[5]
mc^26206	Avirulent auxotrophic *M. tuberculosis* mutant (Δ*leuCD* Δ*panCD*).	[6]
AtpE(A63P)	mc^26206 with a G to C SNP at position 187 bp *atpE.*	This study
Rv0678(G65fs)	mc^26206 with a single nucleotide deletion at position 193 bp of *rv0678*. Resulting protein is frameshifted.	This study
Rv3006(F134S)	mc^26230 with a X to X SNP at position X of Rv3006.	This study

Rv3006(R36S)	$mc^2$6230 with a X to X SNP at position X of Rv3006.	This study
Mycobacterium bovis BCG	Pasteur 1173P2 (NC_008769.1)	[7]
Plasmids		
pJLR965	CRISPRi cloning vector for *M. tuberculosis*	[8]
pCi73	pJLR965 containing sgRNA targeting *atpB* of *M. tuberculosis*	[9]
pCi7	pJLR965 containing sgRNA targeting *mmpL3* of *M. tuberculosis*	[10]
pYUB28b	Episomal expression vector for mycobacteria	[11]
pLH*cyd*	pYUB28b with *rv_1623c-rv1620c* (*cydABDC*), containing C-terminal FLAG tag on *cydB*.	[12]

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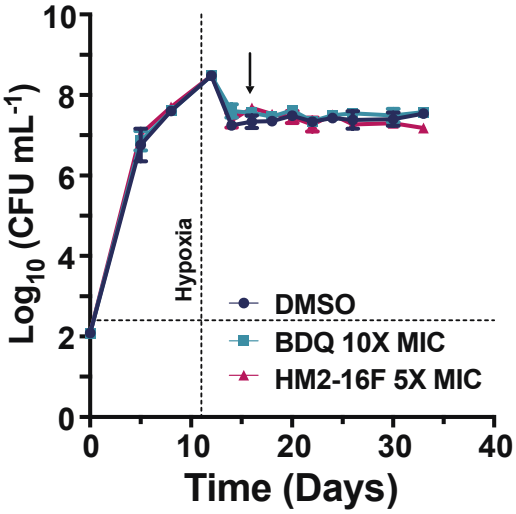
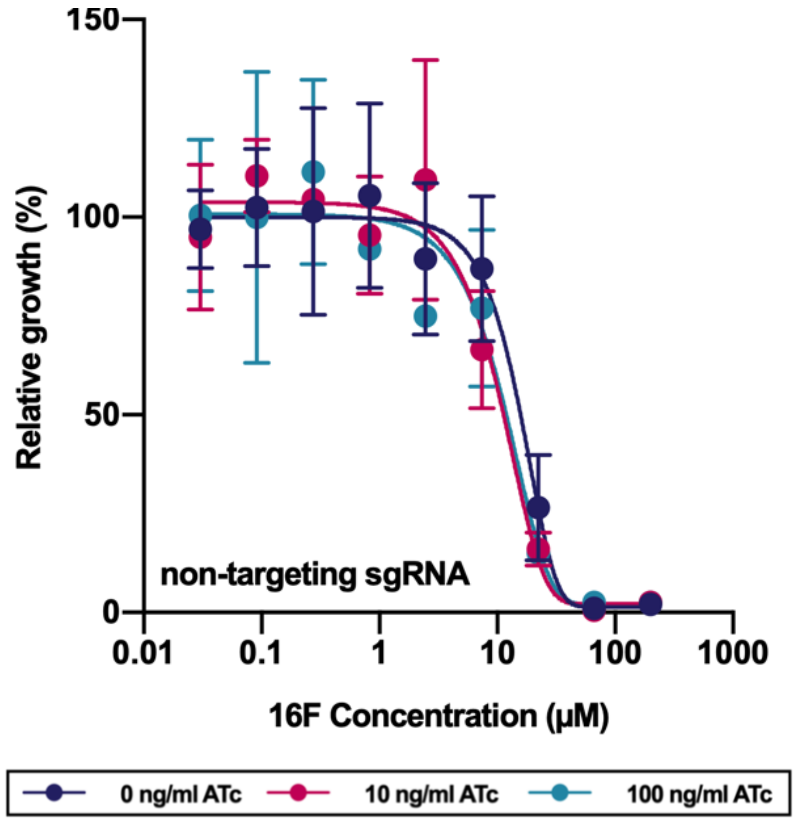
Supplementary Table 2: IC_{50} values of the indicated compounds towards *M. tuberculosis* cytochrome *bd* oxidase.

Compound	IC_{50} [95% CI] (µM)	IC_{50} (fold MIC)	OCR at 1xMIC (%)
HM2-16F	21.16 [10.88-39.09]	2.11	58.86
BDQ	11.71 [4.57-27.03]	58.44	83.04
Aurachin D*	0.15 [0.091-0.27]	0.019	3.70

* The MIC of Aurachin D is determined from MIC testing against *M. smegmatis* Δ*qcr* mutant (supplementary Table 1).



Supplementary Figure 1: Survival of *M. tuberculosis* after treatment with HM2-16F (20 μM, 5× MIC) and bedaquiline (BDQ 2 μM, 10× MIC). Hypoxia was achieved as cultures exhausted oxygen in the sealed serum vials as indicated by the decolorization of methylene blue (vertical dotted line) [12]. Compounds were added at the indicated arrow. The horizontal dotted line indicates the limit of detection for all, but the first time point, where the limit of detection was 10 CFU mL^{-1}. Error bars indicate standard deviation from n = 3 biologically independent experiments.



Supplementary Figure 2: Control gene knockdown with a non-targeting sgRNA (pJLR965) was induced with the indicated amounts of ATc and the MIC of HM2-16F was determined. Error bars indicate standard deviation from n = 3 biologically independent experiments.

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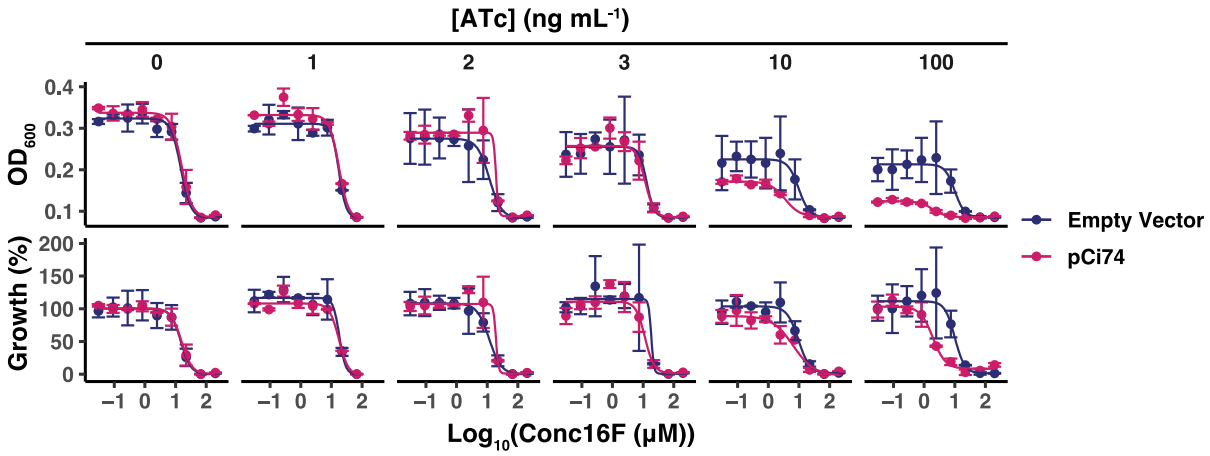

Supplementary Figure 3: Knockdown of the ATP synthase operon (*atpB* – pCi74) was induced with the indicated amounts of ATc, in the indicated strains and the growth relative to the vehicle control was determined. Raw OD_{600} absorbance values (path length = 0.33 cm) are plotted as indicated. Error bars indicate standard deviation from n = 3 biologically independent experiments.

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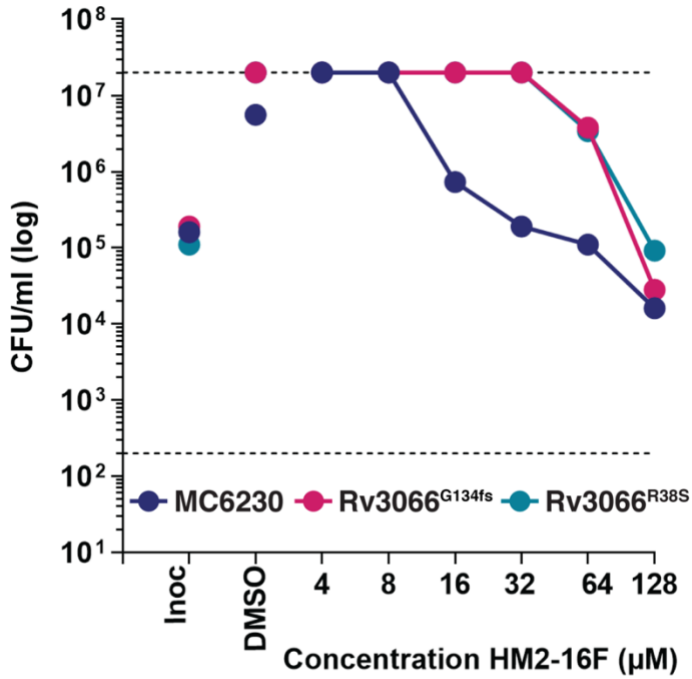
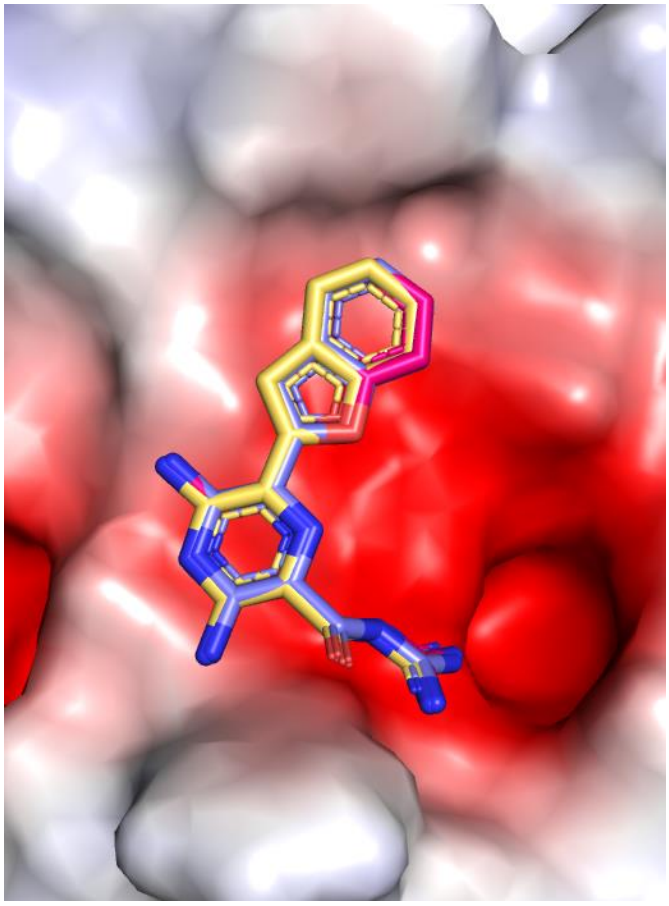
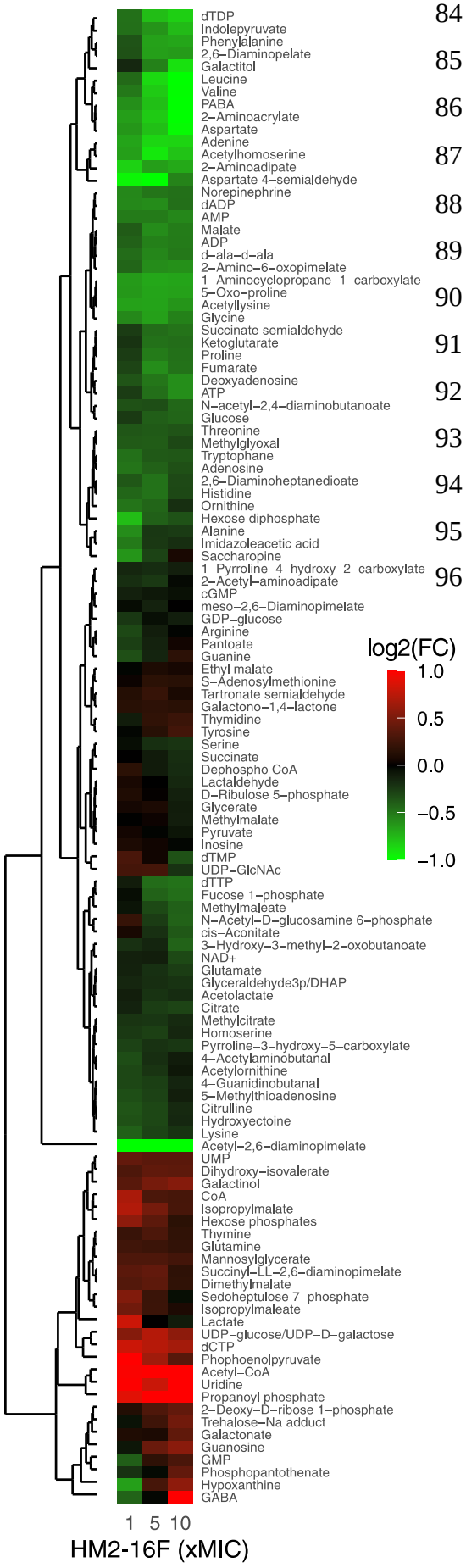


Supplementary Figure 4: Cell killing of the indicated *M. tuberculosis* strains after 10 days incubation with HM2-16F at 5x MIC. The CFU at day 0 is indicated (Inoc). MC6206 = wild-type; HM2-16-resistant mutants, transcription factor Rv3066 G134fs and R38S (Supplementary Table 1).



Supplementary Figure 5: The top three docked poses for HM2-16F (blue, yellow, pink) docked into the BDQ-binding site of mycobacterial F_1F_o-ATP synthase *c*-ring (PDB ID: 4V1F). The protein is shown as an electrostatic potential surface (red – electronegative, white – neutral, blue – electropositive; generated in PyMOL).



Supplementary Figure 6: Heatmap metabolite profile following 1d exposure of *M. tuberculosis* H37Rv to increasing concentrations of HM2-16F (1-10× MIC). Columns represent individual treatments as indicated. Rows denote individual metabolites measured. Hierarchical clustering was performed on the Euclidean distance matrix of this data and the resulting dendrogram and heatmap was visualized with ggplot2 in R. FC = Fold change.

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