SigH stress response mediates killing of *Mycobacterium tuberculosis* by activating nitronaphthofuran prodrugs via induction of Mrx2 expression

Laura Cioetto-Mazzabò[1†], Francesca Boldrin[1†], Claire Beauvineau[2], Martin Speth[3], Alberto Marina[4], Amine Namouchi[5,6], Greta Segafreddo[1], Mena Cimino[5], Sandrine Favre-Rochex[5], Seetha Balasingham[7], Beatriz Trastoy[4,8], Hélène Munier-Lehmann[9], Gareth Griffiths[3], Brigitte Gicquel[5,10], Marcelo E. Guerin[4,8,11], Riccardo Manganelli[1,*], Noelia Alonso-Rodríguez[3,5,*]

[1] Department of Molecular Medicine, University of Padova, Padova, 35122, Italy
[2] Chemical Library Institut Curie/CNRS, CNRS UMR9187, INSERM U1196 and CNRS UMR3666, INSERM U1193, Université Paris-Saclay, Orsay, 91405, France
[3] Department Biosciences, Faculty of Mathematics and Natural Sciences, University of Oslo, Oslo, 0371, Norway
[4] Center for Cooperative Research in Biosciences (CIC bioGUNE), Basque Research and Technology Alliance (BRTA), Bizkaia Technology Park, Derio, 48160 Spain
[5] Génétique Mycobactérienne, Institute Pasteur, Paris, 75015, France
[6] Centre for Ecological and Evolutionary Synthesis (CEES), Department of Biosciences, University of Oslo, Oslo, 0371, Norway
[7] Department of Microbiology. Oslo University Hospital, Oslo, 0450, Norway
[8] Structural Glycobiology Laboratory, Biocruces Bizkaia Health Research Institute, Cruces University Hospital, Bizkaia, 48903, Spain
[9] Département de Biologie Structurale et Chimie, Institut Pasteur, CNRS UMR3523, Université de Paris, Paris, 75015, France
[10] Department of Tuberculosis Control and Prevention, Shenzhen Nanshan Centre for Chronic Disease Control, Shenzhen, 518054, China
[11] IKERBASQUE, Basque Foundation for Science, Bilbao, 48009, Spain

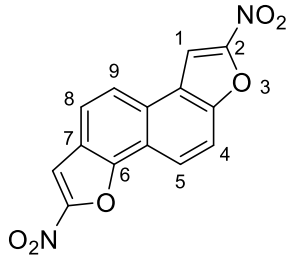
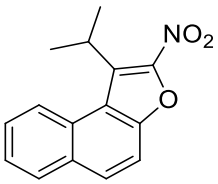
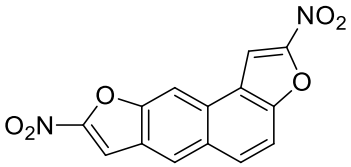
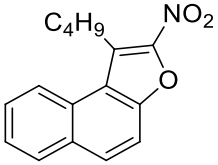
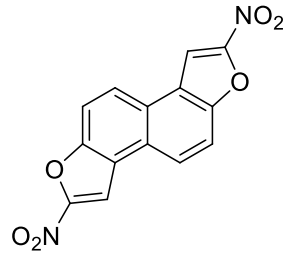
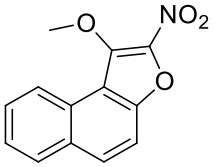
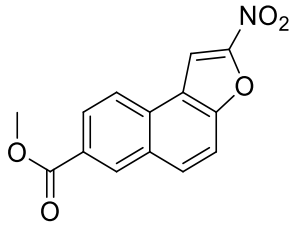
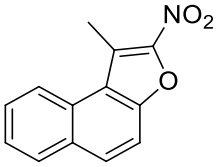
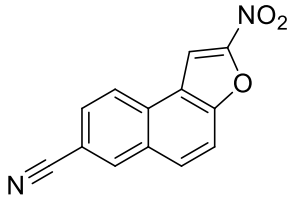
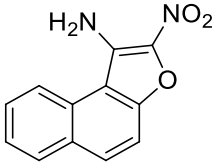
[†] These authors contributed equally to this work

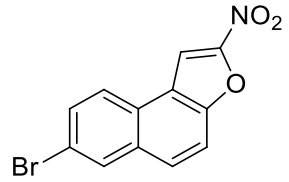
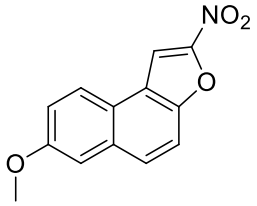
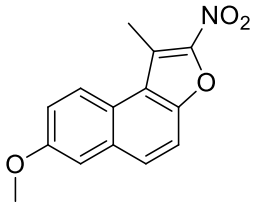
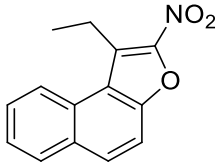
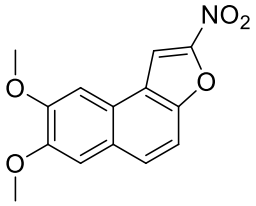
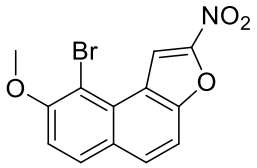
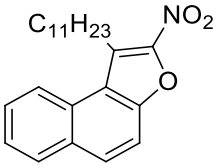
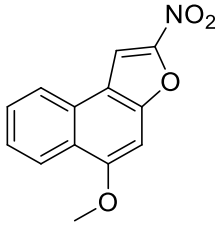
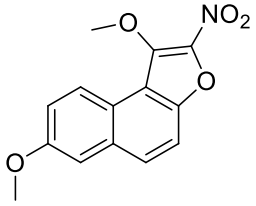
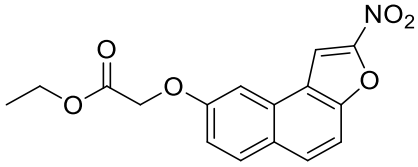
[*]To whom correspondence should be addressed: Riccardo Manganelli, riccardo.manganelli@unipd.it; Noelia Alonso-Rodriguez, n.a.rodriguez@ibv.uio.no or noelialonsor@gmail.com.

Key words: *Mycobacterium tuberculosis*, SigH, sigma factor, transcription factor, overexpression, Mrx2, nitroreductase, nitronaphthofurans, nitric oxide

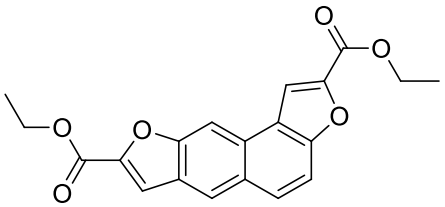
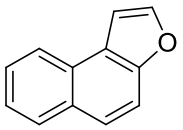
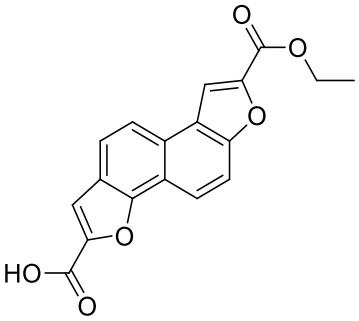
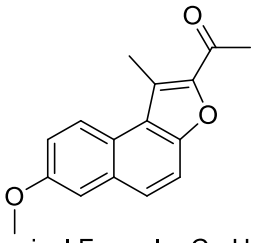
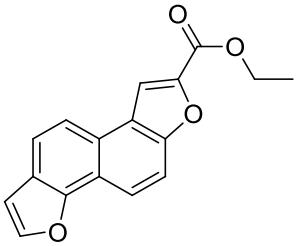
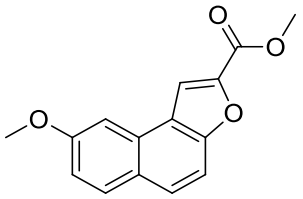
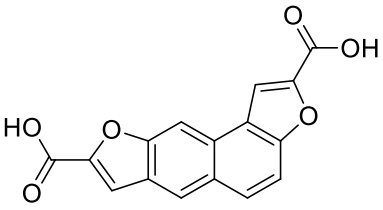
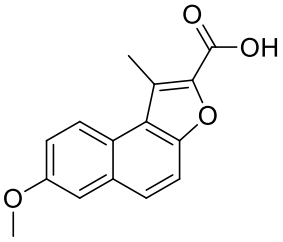
Table of Contents

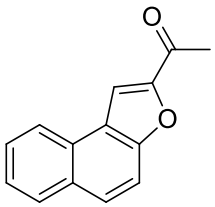
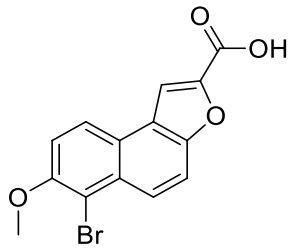
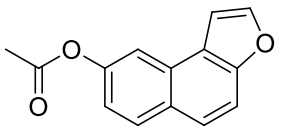
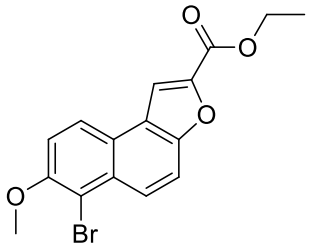
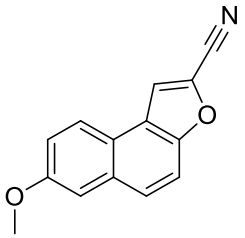
Supplementary Table S1. Nitronaphthofuran (nNF) derivatives. Twenty different derivatives (nNF-C1 - nNF-C20) belonging to the nitronaphthofuran chemical group were studied in this work. Chemical structure, chemical formula and molecular weight of the 20 derivatives are displayed in the table.

nNF-C1	 Chemical Formula: $C_{14}H_6N_2O_6$ Molecular Weight: 298.2072	**nNF-C11**	 Chemical Formula: $C_{15}H_{13}NO_3$ Molecular Weight: 255.2686
nNF-C2	 Chemical Formula: $C_{14}H_6N_2O_6$ Molecular Weight: 298.2072	**nNF-C12**	 Chemical Formula: $C_{16}H_{15}NO_3$ Molecular Weight: 269.2952
nNF-C3	 Chemical Formula: $C_{14}H_6N_2O_6$ Molecular Weight: 298.2072	**nNF-C13**	 Chemical Formula: $C_{13}H_9NO_4$ Molecular Weight: 243.2149
nNF-C4	 Chemical Formula: $C_{14}H_9NO_5$ Molecular Weight: 271.2250	**nNF-C14**	 Chemical Formula: $C_{13}H_9NO_3$ Molecular Weight: 227.2155
nNF-C5	 Chemical Formula: $C_{13}H_6N_2O_3$ Molecular Weight: 238.1983	**nNF-C15**	 Chemical Formula: C12H8N2O3 Molecular Weight: 228.2035

nNF-C6	 Chemical Formula: $C_{13}H_8BrNO_3$ Molecular Weight: 306.1115	**nNF-C16**	 Chemical Formula: $C_{13}H_9NO_4$ Molecular Weight: 243.2149
nNF-C7	 Chemical Formula: $C_{14}H_{11}NO_4$ Molecular Weight: 257.2414	**nNF-C17**	 Chemical Formula: $C_{14}H_{11}NO_3$ Molecular Weight: 241.2420
nNF-C8	 Chemical Formula: $C_{14}H_{11}NO_5$ Molecular Weight: 273.2408	**nNF-C18**	 Chemical Formula: $C_{13}H_8BrNO_4$ Molecular Weight: 322.1109
nNF-C9	 Chemical Formula: $C_{23}H_{29}NO_3$ Molecular Weight: 367.4813	**nNF-C19**	 Chemical Formula: $C_{13}H_9NO_4$ Molecular Weight: 243.2149
nNF-C10	 Chemical Formula: $C_{14}H_{11}NO_5$ Molecular Weight: 273.2408	**nNF-C20**	 Chemical Formula: $C_{16}H_{13}NO_6$ Molecular Weight: 315.2775

Supplementary Table S2. Des-nitro analogues of nNF derivatives. Chemical structure, chemical formula and molecular weight of 13 different des-nitro analogues of nNFs (C1B - C13B) included in the study. MIC_{90} was determined by REMA in *M.tuberculosis* H37Rv and none of the 13 derivatives showed anti-tuberculosis activity at the maximum concentration assayed (MIC_{90} >32 μg/ml).

C1B	 Chemical Formula: $C_{20}H_{16}O_6$ Molecular Weight: 352.3374	**C8B**	 Chemical Formula: $C_{12}H_8O$ Molecular Weight: 168.1913
C2B	 Chemical Formula: $C_{18}H_{12}O_6$ Molecular Weight: 324.2843	**C9B**	 Chemical Formula: $C_{16}H_{14}O_3$ Molecular Weight: 254.2806
C3B	 Chemical Formula: $C_{17}H_{12}O_4$ Molecular Weight: 280.2748	**C10B**	 Chemical Formula: $C_{15}H_{12}O_4$ Molecular Weight: 256.2534
C4B	 Chemical Formula: $C_{16}H_8O_6$ Molecular Weight: 296.2311	**C11B**	 Chemical Formula: $C_{15}H_{12}O_4$ Molecular Weight: 256.2534

C5B	 Chemical Formula: $C_{14}H_{10}O_2$ Molecular Weight: 210.2280	**C12B**	 Chemical Formula: $C_{14}H_9BrO_4$ Molecular Weight: 321.1229
C6B	 Chemical Formula: $C_{14}H_{10}O_3$ Molecular Weight: 226.2274	**C13B**	 Chemical Formula: $C_{16}H_{13}BrO_4$ Molecular Weight: 349.1760
C7B	 Chemical Formula: $C_{14}H_9NO_2$ Molecular Weight: 223.2268		

Supplementary Table S3. Activity of nNFs against sensitive and multidrug resistant (MDR) clinical *M. tuberculosis* isolates. MIC_{90} values for the set of 20 nNFs included in this study were determined by REMA on the clinical Beijing strain GC1237. The derivative nNF-C2 was also tested in two different MDR clinical strains isolated from TB patients at Padova University Hospital.

		MIC_{90} *M. tuberculosis* CLINICAL STRAINS		
	COMPOUND	GC1237 Beijing	MDR- strain A	MDR- strain B
		[μg/ml]	[μg/ml]	[μg/ml]
High activity	**nNF-C1**	0.062		
	nNF-C2	0.062	0.062	0.031
	nNF-C3	0.5		
	nNF-C4	0.062		
	nNF-C5	0.5		
	nNF-C6	0.5		
	nNF-C8	0.062		
	nNF-C16	0.031		
	nNF-C18	0.25		
	nNF-C20	0.25		
Moderate activity	**nNF-C7**	8		
	nNF-C11	8		
	nNF-C12	2		
	nNF-C14	2		
	nNF-C17	2		
Low/No activity	**nNF-C9**	>32		
	nNF-C10	>32		
	nNF-C13	>32		
	nNF-C15	16		
	nNF-C19	16		

Supplementary Table S4. Antimycobacterial activity of nNFs against *M. bovis* BCG and non-tuberculous mycobacteria (NTM). MIC_{90} was determined by REMA in different mycobacterial species: *M. smegmatis*, *M.bovis* BCG, *M. aurum*, *M. avium*, *M. marinum* and *M. abcessus*. Non active compounds according to MIC_{90} values ≥ 16 μg/ml are highlighted with grey background.

	MIC_{90} [μg/ml]					
COMPOUND	***M. smegmatis***	***M. bovis* BCG**	***M. aurum***	***M. avium***	***M. marinum***	***M. abscessus***
nNF-C1	>32	0.0625	0.5	0.25	0.0625	>32
nNF-C2	4	0.03	0.125	0.125	0.03	32
nNF-C3	>32	0.5	16	16	1	>32
nNF-C4	>32	0.125	1	2	0.0625	>32
nNF-C5	>32	0.25	>32	>32	0.25	>32
nNF-C6	>32	0.5	>32	>32	0.25	>32
nNF-C7	>32	4	>32	>32	0.016	>32
nNF-C8	>32	0.125	1	0.25	0.016	>32
nNF-C9	>32	>32	>32	>32	>32	>32
nNF-C10	>32	>32	>32	>32	>32	>32
nNF-C11	32	4	8	>32	0.5	32
nNF-C12	16	4	2	16	0.25	32
nNF-C13	>32	>32	>32	>32	2	>32
nNF-C14	16	2	0.5	4	0.0625	4
nNF-C15	>32	>32	>32	>32	4	>32
nNF-C16	2	0.125	0.25	0.25	0.016	0.5
nNF-C17	8	2	1	4	0.0625	16
nNF-C18	2	0.25	0.25	1	0.0625	8
nNF-C19	16	16	0.5	4	0.125	>32
nNF-C20	0.25	0.125	0.25	0.0625	0.016	4
RIF	32	0.01	1.25	0.05	0.1	>32

Supplementary Table S5. Antimicrobial activity of nNFs against other bacterial (non-mycobacteria) species. MIC_{90} was determined by REMA in different bacterial species: *E. coli*, *K. pneumoniae*, *S. enterica*, *E. cloacae*, *E. aerogenes*, *S. aureus*, and *P. aeruginosa*. Non active compounds according to MIC_{90} values ≥ 16 μg/ml are highlighted with grey background.

	MIC_{90} [μg/ml]						
COMPOUND	***E. coli***	***K. pneumonie***	***S. enterica***	***E. cloacae***	***E. aerogenes***	***S. aureus***	***P. aeruginosa***
nNF-C1	>32	>32	<0.25	>32	>32	>32	>32
nNF-C2	>32	2	<0.25	>32	>32	4	>32
nNF-C3	>32	8	<0.25	>32	>32	4	>32
nNF-C4	>32	>32	>32	>32	>32	>32	>32
nNF-C5	>32	0.25	<0.25	32	>32	2	>32
nNF-C6	>32	>32	>32	>32	>32	>32	>32
nNF-C7	>32	>32	>32	>32	>32	>32	>32
nNF-C8	>32	>32	>32	>32	>32	>32	>32
nNF-C9	>32	>32	>32	>32	>32	>32	>32
nNF-C10	>32	>32	>32	>32	>32	>32	>32
nNF-C11	>32	>32	4	>32	>32	>32	>32
nNF-C12	>32	1.25	0.5	>32	>32	>32	>32
nNF-C13	>32	>32	32	>32	>32	>32	>32
nNF-C14	2	0.25	<0.25	4	1.1	1	>32
nNF-C15	>32	16	16	>32	>32	>32	>32
nNF-C16	1.5	0.125	<0.25	1.5	>32	<0.25	>32
nNF-C17	1	0.5	1	1	2	2	>32
nNF-C18	0.1	0.016	<0.25	0.25	0.09	<0.25	>32
nNF-C19	>32	>32	>32	>32	>32	>32	>32
nNF-C20	>32	0.25	0.5	>32	>32	4	>32

Supplementary Table S6. Bactericidal activity of selected nNF derivatives against replicating *M.tuberculosis*. The minimum bactericidal concentration (MBC_{99}) represents the minimum concentration needed to kill 99% of the initial *M. tuberculosis* inoculum in the specific assays. *A*. MBC values were determined for the 10 nNFs classified as "Highly Active" by enumeration of CFU after serial dilutions plated in complete 7H10 agar plates. *B*. MBC_{99} values obtained in the Charcoal Agar Resazurin assay (CARA) and by plating and enumerating CFU in charcoal containing agar plates. C. Resazurin readouts of charcoal containing agar plates from the CARA. The nNF derivatives nNF-C2 and nNF-C4, and the anti-tuberculosis drugs Pretomanid (PA-824) and Isoniazid (INH) (as positive and negative control, respectively), were tested using the charcoal agar assay.

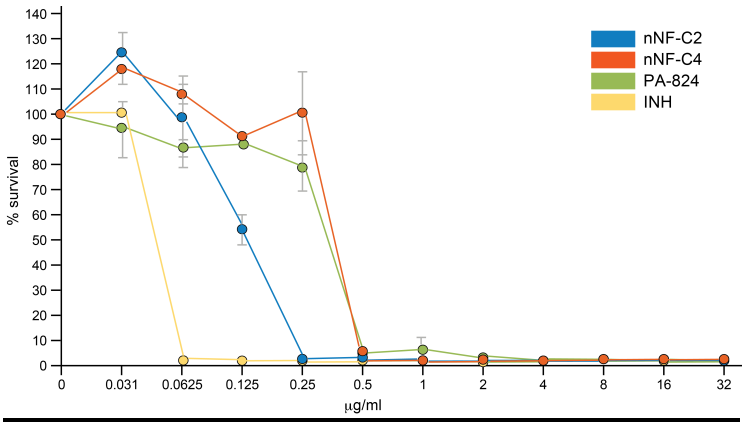
A.

COMPOUND	MBC_{99} REPLICATING *M. tuberculosis*	
	µg/ml	µM
nNF-C1	0.25	0.82
nNF-C2	0.25	0.83
nNF-C3	2	6.71
nNF-C4	0.25	0.92
nNF-C5	1	4.20
nNF-C6	0.5	1.63
nNF-C8	0.5	1.83
nNF-C16	0.06	0.25
nNF-C18	0.5	1.55
nNF-C20	0.5	1.59

B.

COMPOUND	MBC_{99} REPLICATING *M.tuberculosis* Fluorescence CARA plates		REPLICATING *M.tuberculosis* CFU CARA plates	
	µg/ml	µM	µg/ml	µM
nNF-C2	0.25	0.83	0.25	0.83
nNF-C4	0.5	1.84	0.5	1.84
INH	0.06	0.44	0.06	0.44
PA-824	0.5	0.62	0.5	0.62

C.



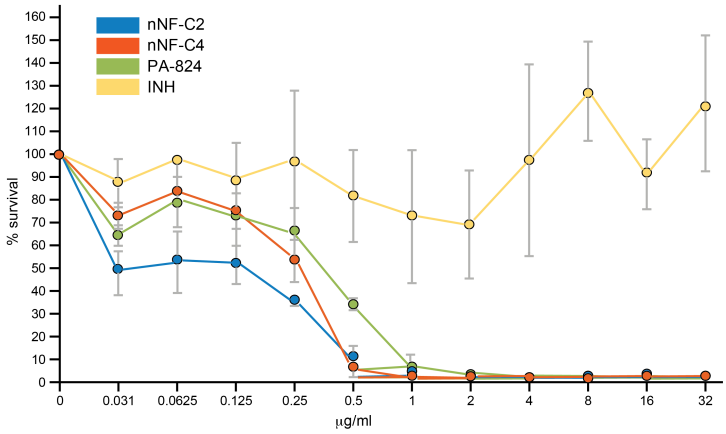
Supplementary Table S7. Frequencies of H37Rv mutants arising against nNFs derivatives. Three independent *M. tuberculosis* H37Rv cultures were grown to an OD_{600} ~ 1.5 and then plated onto 5, 10 and 20 times of the MIC of nNF derivatives discovered in our study. Frequencies refer to number of mutants obtained in the nNF-containing plates versus the bacterial inocula plated initially. No mutants were isolated against nNF-C5 and nNF-C16 in any of the concentrations assayed.

	nNF concentration at which *M. tuberculosis* mutants were isolated		
	4xMIC	**10xMIC**	**20xMIC**
nNF-C1	4.90E-07	7.20E-07	1.05E-08
nNF-C2	1.49E-06	1.40E-06	3.37E-07
nNF-C3	5.71E-06		
nNF-C4	2.14E-07	7.40E-07	
nNF-C5			
nNF-C6	1.57E-07	2.57E-07	3.16E-08
nNF-C8	5.71E-06		4.68E-06
nNF-C16			
nNF-C18	4.50E-08		
nNF-C20		4.29E-08	1.11E-08

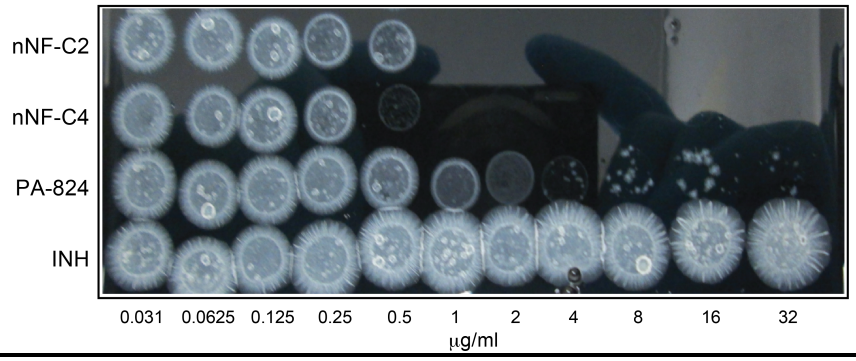
A

		MBC_{90} NON-REPLICATING *M. tuberculosis* CFU 7H10 agar plates		
		µg/ml (range)	µg/ml (median)	µM (median)
High activity	nNF-C1	0.031-0.062	0.031	0.10
	nNF-C2	0.031-0.062	0.062	0.21
	nNF-C3	2-8	4	13.44
	nNF-C4	0.125-0.25	0.25	0.92
	nNF-C5	0.125	0.125	0.53
	nNF-C6	1-2	1	3.26
	nNF-C8	0.031-1.25	0.062	0.23
	nNF-C16	0.015-0.062	0.062	0.25
	nNF-C18	0.125-0.25	0.25	0.78
	nNF-C20	0.062-0.125	0.062	0.20
TB antibiotics	INH		>20	>145
	RIF		4	11.2
	PA-284		0.5	0.62

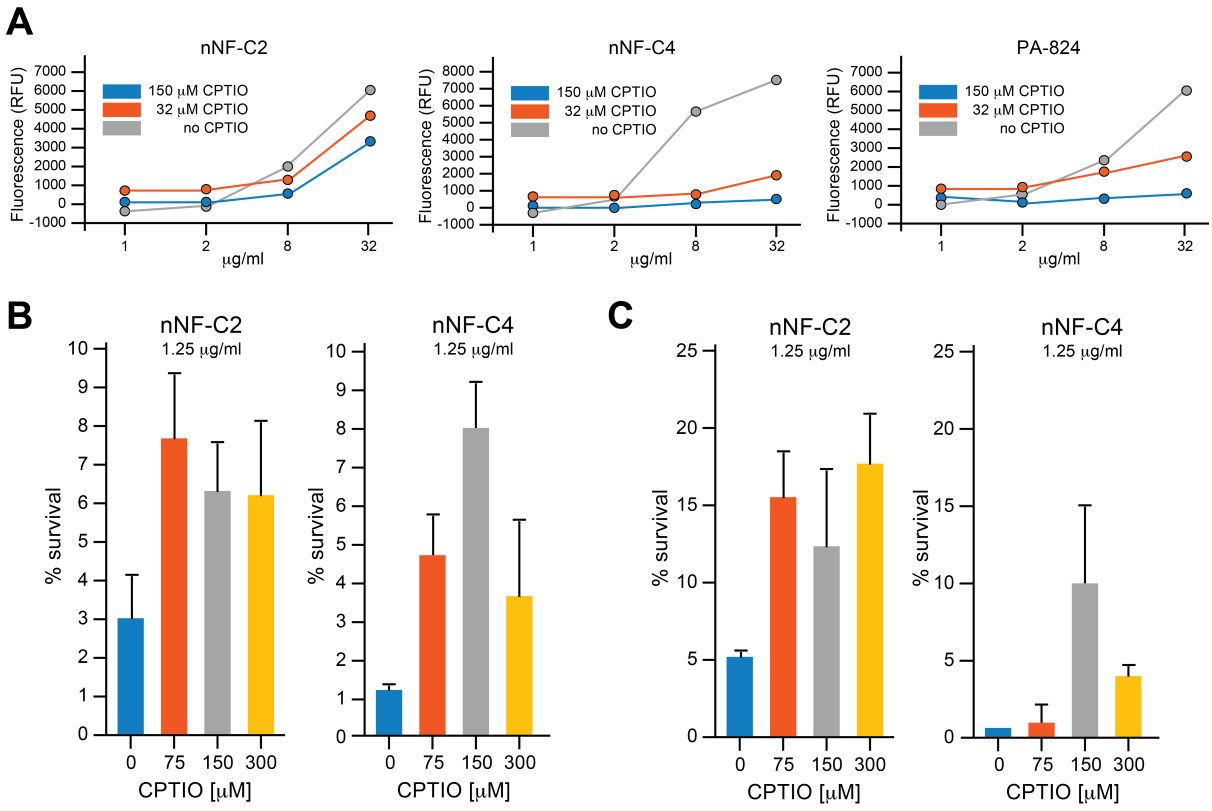
B



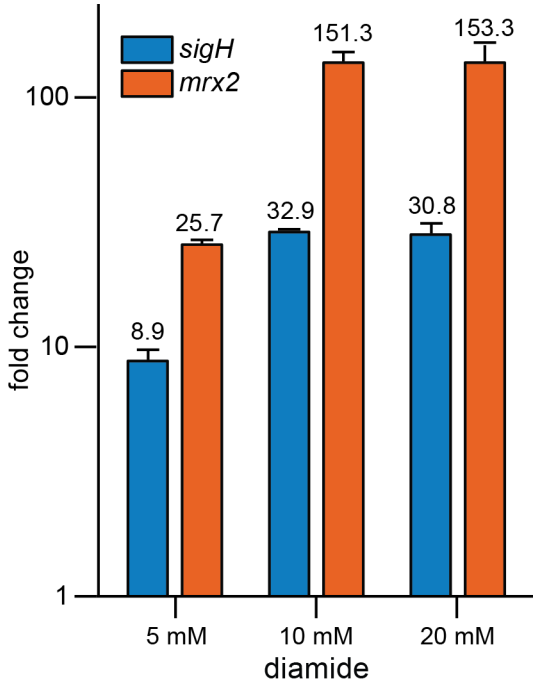
C



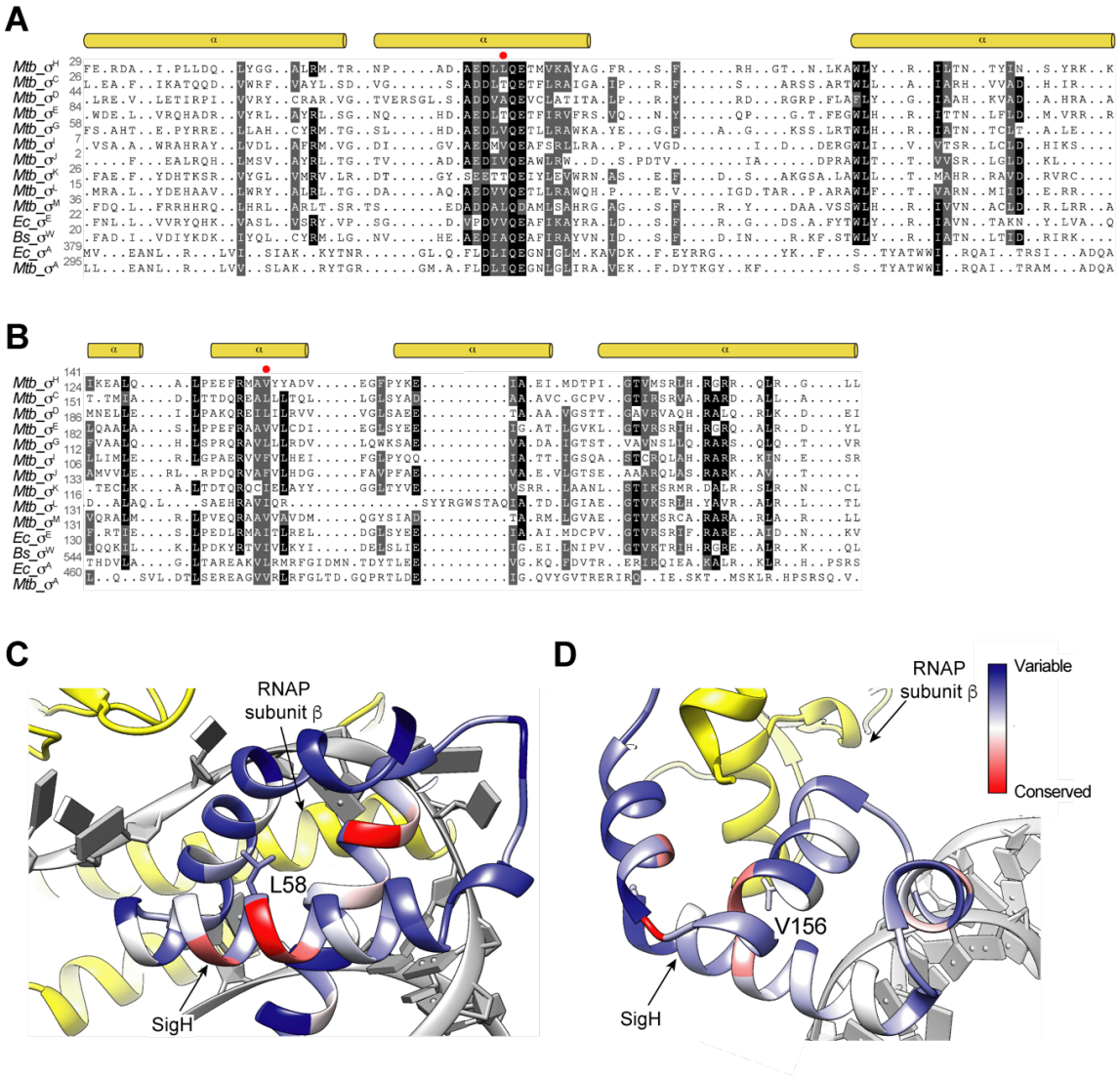
Supplementary Figure S1. Antimycobacterial activity of nNF derivatives against non replicating *M. tuberculosis*. MBC_{90} refers to the minimum bactericidal concentration needed to kill 90% of non-replicating *M. tuberculosis*. MBC_{90} was determined by two experimental approaches: *A*. Enumerating CFU of diluted cultures exposed to the tested compounds in complete Middlebrook 7H10 agar plates. The antimycobacterial activity was assessed for the 10 nNF derivatives classified as highly active against replicating *M.tuberculosis*. Other anti-tuberculosis drugs such as Rifampicin (RIF), Isoniazid (INH) and Pretomanid (PA-824) were also tested in parallel using the same experimental method. MBC_{90} values are expressed in µg/ml (range and median) and µM (median); *B, C*. Using the charcoal agar resazurin assay (CARA) as previously described (54). Resazurin readouts from CARA plates (*B)* and *M. tuberculosis* growth in 0,4% active charcoal containing agar plates (*C*) are depicted in the figure. The antimycobacterial activity against non-replicating *M. tuberculosis* was assessed for nNF-C2, nNF-C4, PA-824 and INH. The nNF-C2 and -C4 were selected from our set of compounds in the study. PA-824, a recognized antibiotic effective against NR-*M. tuberculosis* was included as a positive control. INH, non-active against NR-*M. tuberculosis* was included as a negative control.



Supplementary Figure S2. Intracellular nitric oxide release in the presence of nNF derivatives and protective role of CPTIO as NO scavenger. *M. bovis* BCG cultures were loaded with the DAF-FM fluorescent probe to detect intracellular NO release when exposed to the selected derivatives nNF-C2 and nNF-C4. The effect in NO quantification and mycobacterial viability was analyzed after treatment with or without the NO scavenger CPTIO. A) Fluorescence readouts of the DAF-FM emission in presence of 1, 2, 8, and 32 µg/ml of nNF-C2, nNF-C4, and Pretomanid (PA-824), and different concentrations of CPTIO (No CPTIO, 32 µM, or 150 µM). Values represent the average of independent measures obtained from two independent experiments; B-C). Percentage of bacteria which survived to the nNF treatment in presence or not of CPTIO. BCG bacteria were exposed to 1.25 µg/ml of nNF-C2 and 1.25 µg/ml of nNF-C4 in the presence of 0, 75, 150 or 300 µM of CPTIO. The panel S2B represents bacterial survival percentages obtained by resazurin fluorescence readouts. The panel S2C represents bacterial survival percentages obtained by enumeration of CFUs before and after the treatment. The panels B and C refer to independent experiments in which values represent the average of two independent replicates ± SD.



Supplementary Figure S3. *sigH* and *mrx2* expression levels in *M. tuberculosis* H37Rv after diamide treatment. *M. tuberculosis* H37Rv cultures were treated with 5, 10 or 20 mM of diamide for 30 min and then total RNA was isolated. Expression levels of *sigH* and *mrx2* were determined by qPCR. Fold change values are represented in the Y axis as the mean ± SD of the ratio between the transcriptional level of the gene of interest in presence of diamide and in its absence, in 3 independent replicates.



Supplementary Figure S4: Amino acid residues conservation between σ^{H}_{2} and σ^{H}_{4} domains of ECF σ factors and primary σ^{A} factors. *A-B.* Structure weighted sequence alignment of σ^{H}_{2} (*A*)and σ^{H}_{4} (*B*) domains of ECF σ factors and primary σA factors. Comparison of SigH from *M. tuberculosis* (Uniprot code, P9WGH9; PDB code, 5ZX2 (1), SigC from *M. tuberculosis* (Uniprot code, P9WGH1; PDB code, 2O8X and 2O7G (2), SigD from *M. tuberculosis* (Uniprot code, P9WGG8; Alphafold (3), SigE from *M. tuberculosis* (Uniprot code, P9WGG7; Alphafold (3), SigG from *M. tuberculosis* (Uniprot code, P9WGG5; Alphafold (3), SigI from *M. tuberculosis* (Uniprot code, P9WGH3; Alphafold (3), SigI from *M. tuberculosis* (Uniprot code, L0TCG5; PDB code, 5XE7 (4), SigK from *M. tuberculosis* (Uniprot code, P9WGH7; PDB code, 4NQW (5), SigL from *M. tuberculosis* (Uniprot code, P9WGH5; PDB code, 6TYG (6), SigM from *M. tuberculosis* (Uniprot code, O53590; Alphafold (3), SigE from *E.coli* (Uniprot code, P0AGB6; PDB code, 6JBQ (1), SigW from *B. subtilis* (Uniprot code, Q45585; PDB code, 5WUQ (7), SigA from *E. coli* (Uniprot code, P00579; PDB code, 4JKR (8), and SigA from *M. tuberculosis* (Uniprot code,P9WGI1; PDB code, 5UH5 (9). L58 and V156 are highlighted with a red circle. *C-D.* Cartoon representation of σ^{H}_{2} (*C*) and σ^{H}_{4} (*D*) domains of SigH (PDB code 5ZX2). The color-coded scales of each amino acid of SigH correspond to residue conservation as shown in the sequence alignment.

1. Li L, Fang C, Zhuang N, Wang T, Zhang Y. 2019. Structural basis for transcription initiation by bacterial ECF σ factors. Nat Commun 10:1–14.

2. Thakur KG, Joshi AM, Gopal B. 2007. Structural and Biophysical Studies on Two Promoter Recognition Domains of the Extra-cytoplasmic Function σ Factor σC from Mycobacterium tuberculosis*. J Biol Chem 282:4711–4718.

3. Jumper J, Evans R, Pritzel A, Green T, Figurnov M, Ronneberger O, Tunyasuvunakool K, Bates R, Žídek A, Potapenko A, Bridgland A, Meyer C, Kohl SAA, Ballard AJ, Cowie A, Romera-Paredes B, Nikolov S, Jain R, Adler J, Back T, Petersen S, Reiman D, Clancy E, Zielinski M, Steinegger M, Pacholska M, Berghammer T, Bodenstein S, Silver D, Vinyals O, Senior AW, Kavukcuoglu K, Kohli P, Hassabis D. 2021. Highly accurate protein structure prediction with AlphaFold. Nature 596:583–589.

4. Goutam K, Gupta AK, Gopal B. 2017. The fused SnoaL_2 domain in the Mycobacterium tuberculosis sigma factor σJ modulates promoter recognition. Nucleic Acids Res 45:9760–9772.

5. Shukla J, Gupta R, Thakur KG, Gokhale R, Gopal B. 2014. Structural basis for the redox sensitivity of the Mycobacterium tuberculosis SigK-RskA σ-anti-σ complex. Acta Crystallogr D Biol Crystallogr 70:1026–1036.

6. Li L, Molodtsov V, Lin W, Ebright RH, Zhang Y. 2020. RNA extension drives a stepwise displacement of an initiation-factor structural module in initial transcription. Proc Natl Acad Sci U S A 117:5801–5809.

7. Devkota SR, Kwon E, Ha SC, Chang HW, Kim DY. 2017. Structural insights into the regulation of Bacillus subtilis SigW activity by anti-sigma RsiW. PLoS One 12:e0174284.

8. Zuo Y, Wang Y, Steitz TA. 2013. The mechanism of E. coli RNA polymerase regulation by ppGpp is suggested by the structure of their complex. Mol Cell 50:430–436.

9. Lin W, Mandal S, Degen D, Liu Y, Ebright YW, Li S, Feng Y, Zhang Y, Mandal S, Jiang Y, Liu S, Gigliotti M, Talaue M, Connell N, Das K, Arnold E, Ebright RH. 2017. Structural Basis of Mycobacterium tuberculosis Transcription and Transcription Inhibition. Mol Cell 66:169-179.e8.