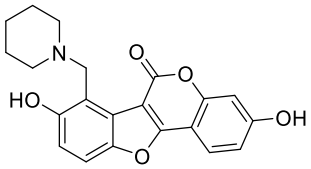
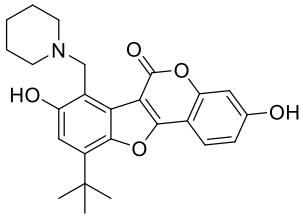
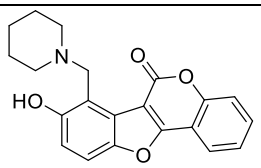
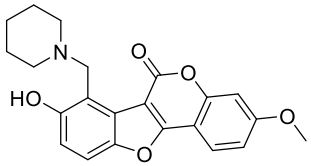
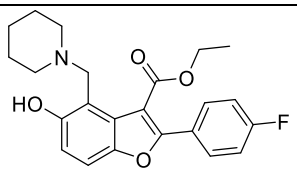
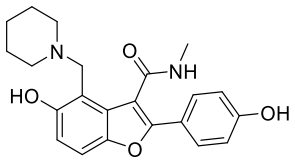
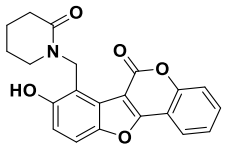
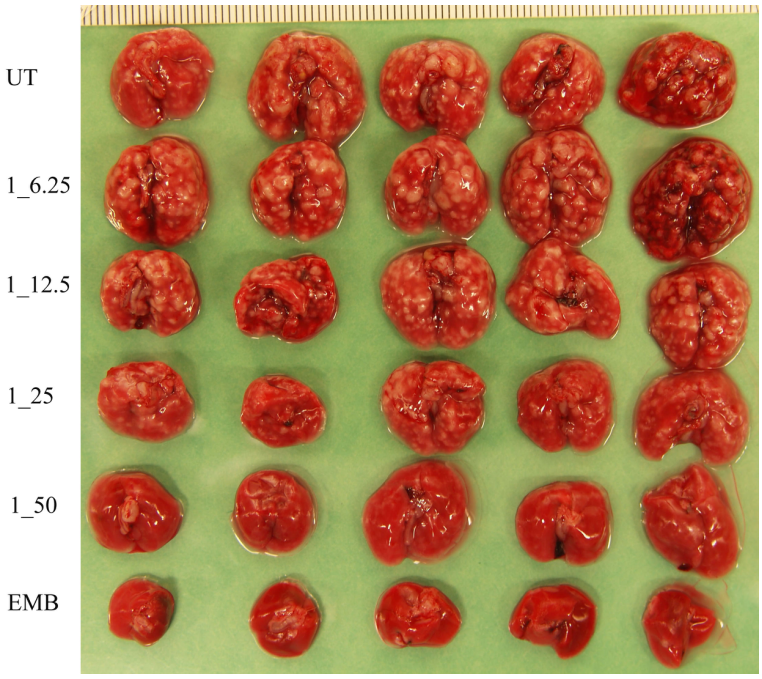
Supplemental material

Table S1. Thermal stabilization of Pks13-TE with compounds by nanoDSF.

ID	Structure	ΔT_m [a], °C		MIC [b], µg/mL
		5X	10X	
DMSO		-1.4±0.07		-
Compound 1		9.8±0.54	9.9±0.10	0.0039
Compound 2		10.1±0.04	10.4±0.01	0.0039
Compound 3		10.0±0.13	9.3±0.15	0.125-0.25
Compound 4		9.8±0.24	10.0±0.08	0.25
Compound 5		9.1±0.09	9.1±0.11	0.5

Compound 6		8.9±0.09	10.1±0.07	0.0313
Compound 7		0.4±0.16	1.0±0.043	> 64

Note: Compound 7 was a non-inhibitor control.


UT
1_6.25
1_12.5
1_25
1_50
EMB

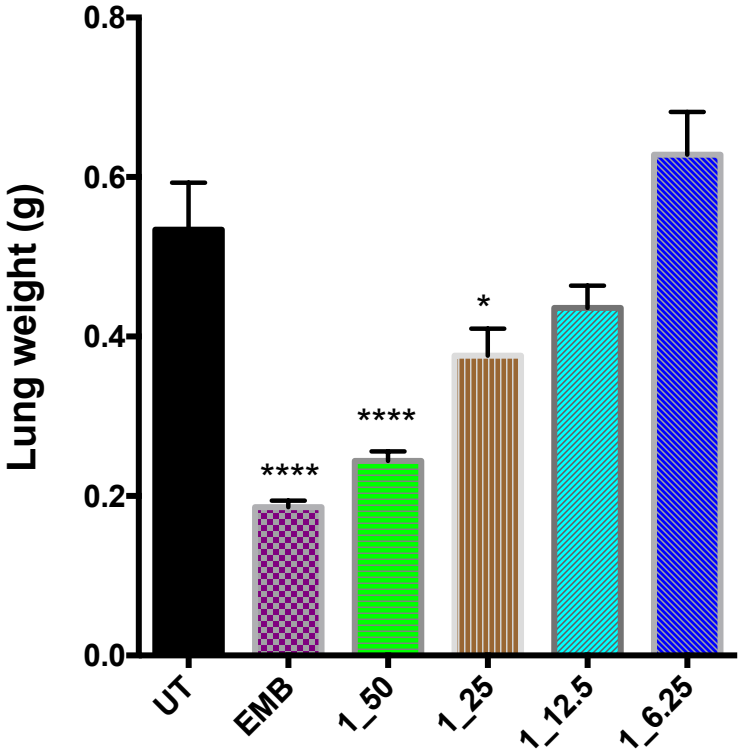
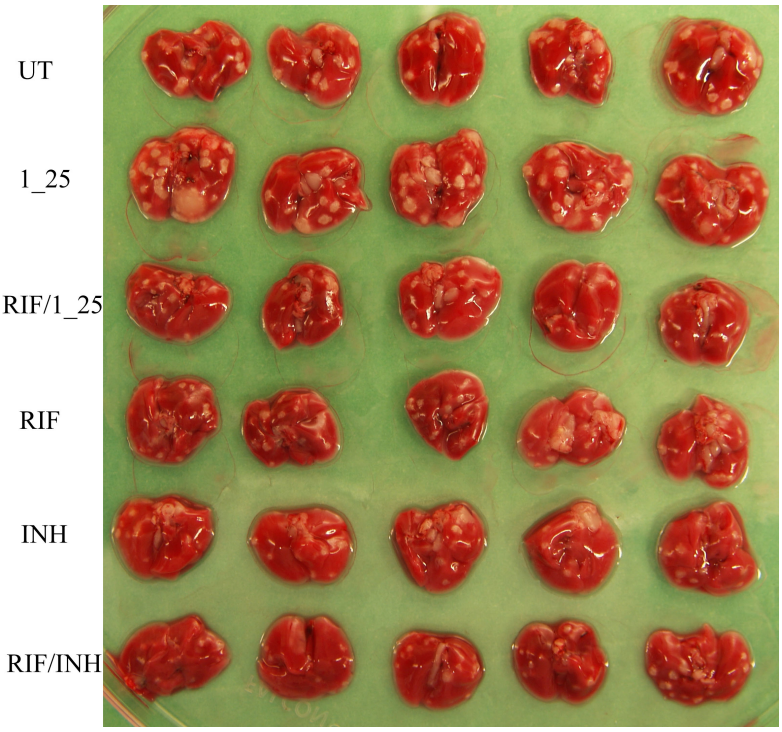
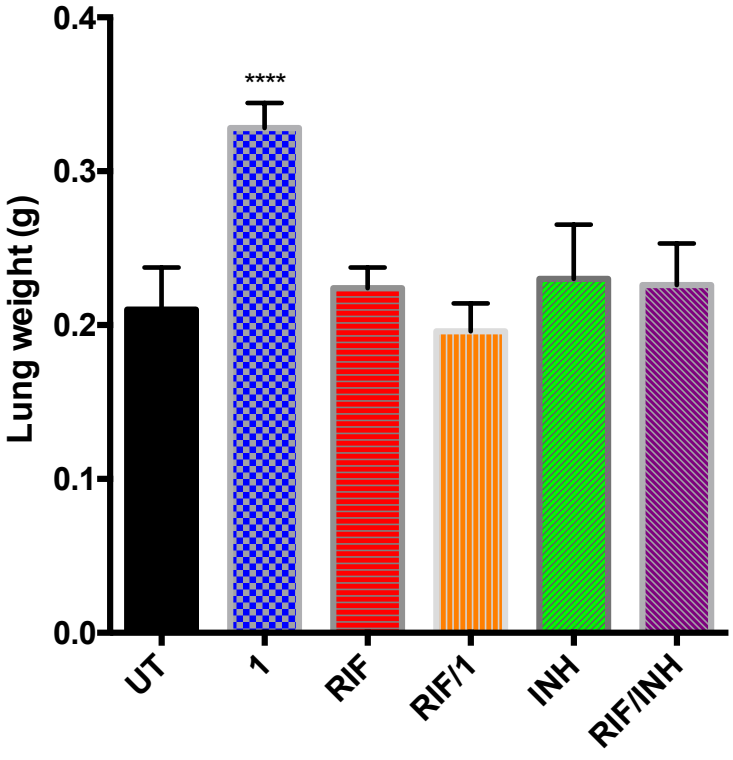

Lung weight (g)
0.8
0.6
0.4
0.2
0.0
****
****
*
UT
EMB
1_50
1_25
1_12.5
1_6.25

Fig. S1. Mouse lung gross pathology (upper panel) and lung weight (lower panel) in the acute model. Mice were infected with 3.2-$\log_{10}$ CFU by the aerosol route and treatment was initiated on day 1 after infection. Lungs were removed after four weeks of monotherapy. UT, untreated; EMB, ethambutol (100 mg/kg); **1**_50, compound **1** (50mg/kg). Data represent mean lung weight of five BALB/c mice as mean±SEM. One-way ANOVA and Tukey's multiple comparisons test was applied and significant differences from untreated control were indicated (*, $p<0.05$; ****, $p<0.0001$).


UT
1_25
RIF/1_25
RIF
INH
RIF/INH

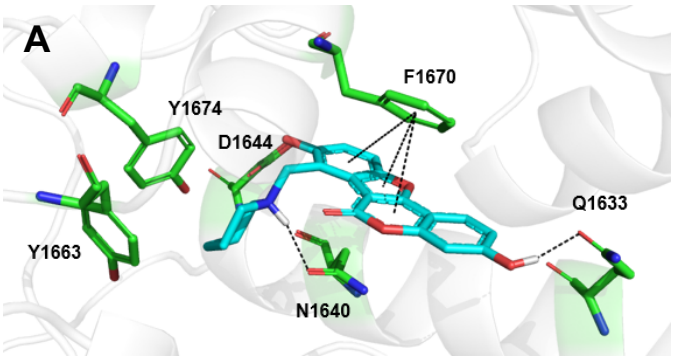
24

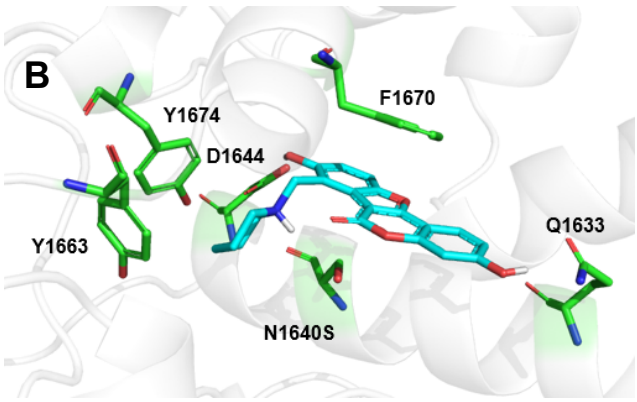
25


0.4
0.3
0.2
0.1
0.0
Lung weight (g)
****
UT
1
RIF
RIF/1
INH
RIF/INH

26

Fig. S2. Mouse lung gross pathology (upper panel) and lung weight (lower panel) in the chronic model. Mice were infected with 2.0-$\log_{10}$ CFU by the aerosol route and treatment was initiated 28 days after infection. Lungs were removed after eight weeks of combination therapy. UT, untreated; **1**, compound **1** (25 mg/kg); RIF, rifampin (10 mg/kg); INH, isoniazid (10 mg/kg). Data represent mean lung weight of five BALB/c mice as mean±SD. One-way ANOVA and Tukey's multiple comparisons test was applied and significant differences from untreated control were indicated (****, $p<0.0001$).





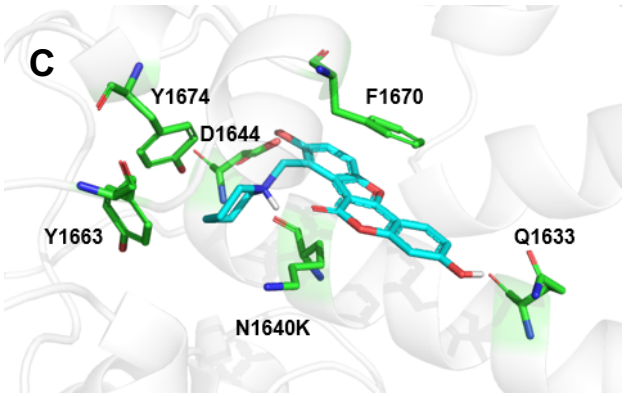


Fig S3. Proposed binding modes of compound **1** (colored cyan). Docking modes of compound **1** with Pks13-TE (A). N at 1640 was mutated into S (B) and K (C) respectively. The key amino acid residues are colored green in the active site of Pks13-TE (PDB ID 5V3Y).

Supplemental methods and data analysis:

Expression and purification of Pks13-TE

Sequence of the Mtb Pks13 gene (Rv3800c) was obtained. The wild-type Pks13-TE domain construct gene was cloned and inserted into PMCSG-19 plasmid, which was synthesized by Generay Biotechnology (Shanghai, China). The expression of the N-Mbp-His tagged Pks13-TE protein was induced with 0.5 mM IPTG in E. coli BL21(DE3)pLysS strains (Shanghai Institute of Materia Media, China), and the cells were harvested at 20 ℃ after 18 h of growth. Similar to the procedures reported previously, the Pks13 TE protein was purified by Nickle-affinity chromatography, followed by a step of TEV protease digestion to remove His-tag. Digested mixtures were loaded onto the anion exchange column (Mono Q 10/100 GL, GE Healthcare) and Superdex-75 gel filtration column (GE Healthcare), eluted with a buffer of 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 1 mM DTT. Factions were collected with > 95% purity monitored by SDS-PAGE. The Pks13 TE protein was stored at -80 ℃ for further bioassays.

NanoDSF assay.

To a solution of 1 mM of Pks13-TE protein in buffer (100 mM NaCl, 20 mM Tris-HCl pH 8.0) was incubated with different concentrations of tested compounds (5 and10 mM in DMSO) in a 30 μL reaction volume for 30 min, and drug-free proteins containing DMSO solution served as a blank control. Approximately 10 μL of the supernatant fraction was loaded to each capillary, which was then placed on the holder in the sample rack. The

thermal denaturation curves were determined by the measurement of the protein intrinsic fluorescence on label-free native nanoDSF (NanoTemper, Prometheus NT.48). The temperature was increased from 20 to 90 °C at a rate of 2°C·min^{-1}. The fluorescence intensity was recorded at 330 and 350 nm, respectively. Changes in the fluorescence ratio (F350/F330) was monitored to determine the apparent melting temperature (T_m).

Thermal Stability Analysis

To further probe their interaction on molecular level, Compound 1-6 were evaluated for thermal stability of Pks13-TE using nano differential scanning fluorimetry (nanoDSF) method. It measures the thermal unfolding transitions (Tm) of protein under native conditions, and no extra dye is required. The binding of ligands to proteins generally leads to thermal stability shift of proteins. Stabilization was evaluated by comparing the melting temperatures (T_m) of Pks13-TE (56.9 ± 0.05 ºC) in the absence and presence of the compounds and calculating the shift in the *T*m (ΔT_m). A significant increase in thermal stability of Pks13-TE was observed following the addition of the 5-fold or 10-fold compound **1-6** ($\Delta T_m > 8.0$ ℃, Table **5**), indicating binding of the compounds. Compared to the negative control compound **7** ($\Delta Tm < 3.0$ ℃), had no effect on the thermal stability of Pks13-TE. The result is in agreement with its MIC.

Molecular Docking

For the molecular docking of compound **1**, we used the solved crystal structures of Pks13-TE (PDB ID 5V3Y). First asparagine (N) at 1640 of Pks13-TE was mutated into serine (S) and lysine (K) respectively. And then the protein structure was prepared by

Protein Preparation Wizard. In this step, missing atoms and all hydrogen atoms were added to the protein according to their local environment. Compound **1** was flexibly docked into the binding pocket defined by residues Q1633, F1670, Y1674, and S1640 or K1640 using Glide. Pks13 interactions with compound **1** was pictured using PyMOL (Release 2.4.0, Schrödinger, Inc). All the molecular docking work was performed using the Maestro software (Release 2020-4, Schrödinger, LLC).