Supporting Information

Lin et al. 10.1073/pnas.1110271109

SI Materials and Methods

Plasmid Construction. The DNA fragments that code L12 and L10 proteins were amplified by PCR from the cDNA of *Mycobacterium tuberculosis* H37Rv. To construct the yeast two-hybrid system, the resulting PCR fragments were inserted into pGADT7 (activation domain, AD) after digestion with NdeI and BamHI, thereby the DNA fragments for L12 and L10 were fused in frame with the Gal4 transcription activating domain in the resulting plasmids pAD-L12 and pAD-L10, respectively. Similarly, L12 and L10 DNA fragments were cloned into pGBKT7 (binding domain, BD) to generate pBD-L12 and pBD-L10 plasmids, in which the DNA fragments were fused in frame with the Gal4 DNA binding domain. The control plasmids pBD-53, pBD-lam, and pAD-T were obtained from Clontech. For the expression of L12 and L10 proteins in *Escherichia coli*, recombinant plasmids pET16b-L12 and pET16b-L10 were constructed, with 6× His-tag at the N terminal. All constructs were sequenced for verification.

For the overexpression of L12 protein in *Mycobacterium smegmatis* mc^2155, the eGFP (enhanced green fluorescent protein) gene was inserted into the pMV261 vector with ClaI and HpaI sites, resulting in a plasmid pMV261-eGFP. The gene encoding L12 (*M. tuberculosis*) was inserted between the promoter and the eGFP gene with the BamHI and HindIII sites in a way that L12 is in frame with eGFP. The resulting pMV261:L12-eGFP plasmid was amplified using *E. coli* and then transformed into competent *M. smegmatis* mc^2155. PCR was used to screen strains expressing eGFP, which indicates the expression of L12 protein. A similar strategy was used for the construction of plasmids for the overexpressing L10 and Icl. The expression of these proteins is further confirmed by fluorescence microscopy.

Yeast Two-Hybrid Assay. The Matchmaker Gal4 Two-Hybrid system was used to verify protein–protein interaction. The pAD-L12 plasmid was cotransformed with pBD-L10 into yeast AH109 cells to generate AH109 (pAD-L12 + pBD-L10), and we obtained AH109 (pAD-L10 + pBD-L12) strain after plasmids pAD-L10 and pBD-L12 were cotransformed into the AH109 strain. Similarly, we obtained transformants AH109 (pAD + pBD-L10) and AH109 (pAD-L12 + pBD). Strains AH109 (pAD-T + pBD-53) and AH109 (pAD-T + pBD-lam) were used as positive or negative control. The transformants were streaked onto synthetic defined (SD) −Leu −Trp dropout plates and incubated at 30 °C for 2–3 d. The double dropout plates allow the growth of yeast cells with the two fusion plasmids. The transformants were further streaked onto SD −Leu −Trp −Ade −His plates, which are incubated at 30 °C for 3–4 d to examine the growth. The interaction of the two fusion proteins activates the reporter genes, resulting in the growth of yeast cells on the quadruple dropout plates.

The β-galactosidase (β-gal) activity was measured according to the Yeast Protocols Handbook (Clontech). In brief, yeast cells grown in SD −Leu −Trp dropout medium for 48 h at 30 °C were transferred onto filter paper and the cells were lysed in liquid nitrogen for 1 min. Then, 5 mL of Z buffer (0.1 M Na_2HPO_4, 35 mM NaH_2PO_4, 10 mM KCl, and 1 mM $MgSO_4$, pH 7.0) containing 83.5 μL 20 mg/mL X-gal and β-mercaptoethanol (0.27%) were added for further incubation at 30 °C to examine the color change. To quantify the β-gal activity, 4.5 mL of yeast cultures in midlog phase (OD_{600} = 0.5) were harvested by centrifugation and then resuspended in 300 μL Z buffer. A total of 100 μL of cell suspension was lysed by repeated freeze/thaw cycles and then 160 μL *o*-nitrophenyl β-D-galactopyranoside (ONPG) (4 mg/mL in Z buffer) was added to the lysate and incubated at 30 °C until we observed color change. The reaction was stopped by addition of 0.4 mL 1 M Na_2CO_3. After centrifugation at 14,000 × *g* for 10 min, the A_{420} of the supernatant was measured. As a control, yeast cells with control vectors were used. The units of β-gal activity were calculated according to the instructions (Yeast Protocols Handbook, Clontech). The experiments were repeated three times, and three sets of readings were taken each time.

The protein expression in yeast was verified by Western blot. Protein extracts were obtained using trichloroacetic acid (TCA) method (Yeast Protocols Handbook, Clontech). Protein expression in yeast cells was verified by Western blot using anti-myc and anti-HA monoclonal antibodies (Clontech).

Compound Library. The compound library used for this screening is a combination of synthetic (synthesized by Enamine) and natural products from the Institute of Medicinal Biotechnology.

Expression and Purification of Recombinant Proteins. We used *E. coli* BL21(DE3) to express recombinant His-tagged L12 and L10 proteins. Bacteria harboring pET16b-L12 or pET16b-L10 plasmid were grown at 37 °C to OD_{600} = 0.8 in LB media containing 100 μg/mL ampicillin. The expression of L12 was induced by addition of 0.5 mM isopropyl-β-D-thiogalactoside for 10 h at 28 °C. His-tagged L10 was obtained in auto-inducible ZYM-5052 media (1). *E. coli* BL21(DE3) cells containing pET16b-L10 was grown in LB, and then inoculated into fresh ZYM-5052 media and incubated with shaking at 20 °C overnight.

The ÄKTA explorer system (GE Healthcare) was used for the purification of the recombinant L12 and L10 proteins by immobilized metal affinity chromatography technique. Proteins were separated by 15% (wt/vol) SDS/PAGE followed by Coomassie blue staining, and the purity of each fraction was also evaluated. The purified proteins were then pooled and concentrated using an Amicon-15 device (Millipore) with a 10 K membrane cutoff and spun at 5,000 × *g* for 10 min. Protein concentrations were determined by the Bradford method. The purified recombinant proteins were confirmed by Western blotting using anti-His antibody.

Surface Plasmon Resonance Assays. The measurements were performed using a BIAcore 3000 (Biacore) at 25 °C in a running buffer PBS-T (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, and 140 mM NaCl, pH 7.4, 0.05% Tween 20, and 0.1% DMSO). Purified His-tagged L12 (50 μg/mL) was immobilized onto a flow cell of CM5 sensor chip using an NHS/EDC [N-hydroxysuccinimide/1-ethyl-3-(3-dimethylperpyl)-carboiimide] amine coupling kit in 10 mM sodium acetate buffer at pH 4.5 and with a ligand density of ∼2,000 response units (RU). Purified His-tagged L10 (50 μg/mL) was immobilized on another flow cell using the same buffer, with a ligand density of ∼800 RU.

To determine the binding affinity of His-tagged L12 and L10, diluted His-tagged L10 was injected into the L12-immobilized channel at various concentrations ranging from 0.03 to 0.93 μM. The surface of the chip was regenerated by washing with 50 mM NaOH. His-tagged L12 was injected into the L10-immobilized chamber at concentrations ranging from 0.004 to 0.128 μM. The interaction of L12 and L10 was represented by RU. Both association (K_a) and dissociation (K_d) values were determined with BIAcore evaluation 3.1 software package (Biacore).

For the binding affinity assays, solutions of compounds T766 and T054 (0.1% DMSO) at different concentrations (2.5 μM to

40 μM) were injected into the L12 and L10 immobilized chambers. The surface was regenerated by 12.5 mM NaOH. For competitive binding studies, 40 μM T766 or T054 was injected (10 μL/injection) to saturate ligand L12 that is immobilized on sensor surface. Subsequently, His-tagged L10 (0.18 μM) was injected (20 μL/injection). As a control, PBS-T was injected at first and followed with the injection of L10 (0.18 μM).

Anti-TB Activity of Compounds T766 and T054. Anti-TB H37Rv [American Type Culture Collection (ATCC) 27294] activity of compounds T766 and T054 were determined by using the microplate alamar blue assay (MABA). The activities against clinically sensitive strains STB-960, STB-825, STB-9102, drug-resistant clinical isolates MDR-699, MDR-843, MDR-6233, and XDR-83, XDR-164, and XDR-926 were analyzed using the same method. The first-line anti-TB drugs, rifampin, isoniazid, and ethambutol, were used as reference. The final concentrations for each compound ranged from 0.0625 to 64 μg/mL. All *M. tuberculosis* strains were cultured at 37 °C in Middlebrook 7H9 broth (Difco) supplied with 0.2% glycerol and 10% oleic acid-alumin-dextrose-citric acid (OADC) until log phase. The cells were diluted into Middlebrook 7H9 broth to 10^6 cfu/mL with various concentrations of antibiotics. The minimum inhibitory concentration (MIC) was measured in sterile 96-well microplates and the final volume in each well was 100 μL. The visual MIC was defined as the lowest drug concentration that prevents the color change of alamar blue reagent from blue to pink.

Sensitivity of Other Bacterial Strains to T766 and T054. The MICs of compounds T766 and T054 against ATCC strains and clinical isolates were determined by using the agar dilution method recommend by the National Committee for Clinical Laboratory Standards (2). Inoculations were adjusted to yield ~10^4 cfu/spot using a multipoint inoculator (Bolney) and incubated at 35 °C for 18 h. The MICs were determined as the lowest concentration of the compound that inhibits the growth of bacteria on the plate. Levofloxacin was used as the reference drug.

Testing the Mode of Action: Bacteriostatic vs. Bactericidal Mode. *M. smegmatis* mc^{2}155 is a strain close to *M. tuberculosis* but exhibits fast growing and is noninfectious. This strain was grown to early log phase in Middlebrook 7H9 broth at 37 °C. The culture was diluted to 5 × 10^5 cfu/mL in fresh media containing various concentrations of T766 (0.5–32 μg/mL) or T054 (1–64 μg/mL). Bacteria were collected at different time points, serially diluted, and plated. After incubation 48 h at 37 °C, the number of colonies was counted.

In Vitro Translation Inhibition by Compounds T766 and T054. Translation inhibition by compounds T766 and T054 was assessed by using an in vitro cell-free translation system supplied with ribosomes from either *E. coli*, rabbit reticulocyte, or *M. smegmatis* as well as a luciferase reporter plasmid. Translation assays with *E. coli* S30 extract or rabbit reticulocyte ribosomes were carried out according to instructions (Promega). *M. smegmatis* ribosome extract was prepared as described by Kigawa et al. (3) followed by addition of a mixture of nucleotide triphosphates, amino acids, and a luciferase reporter plasmid. Light emission was recorded with a luminescence counter (Perkin-Elmer). Compounds T766 and T054 were tested at concentrations ranging from 1 to 400 μg/mL. The IC_{50} was determined on the basis of the ratio of light emission units over the concentration of compounds (log plots) that fits to a variable-slope dose–response equation.

Examination of Ribosome-Dependent Bactericidal Activity of T766 and T054. According to the method described previously (4), *M. smegmatis* mc^{2}155 was grown in 7H9 broth to midlog phase and then diluted 10,000-fold into fresh prewarmed media. Before the addition of test compounds, the cultures were preincubated with 16 μg/mL of thiostrepton (Tsr) for 5 min at 37 °C. The compounds T766, T054, and INH (isoniazid) were added into the medium at 64-fold of MICs. Aliquots from each culture were removed at the indicated times, serially diluted, plated, and incubated at 37 °C for 48 h to count the number of colonies.

Determining the Antibacterial Activity of Compounds T766 and T054 Against *M. smegmatis* mc^{2}155 Strains Overexpressing L12, L10, or Icl (Isocitrase). *M. smegmatis* strains overexpressing L12-eGFP, L10-eGFP, or Icl-eGFP fusion proteins were isolated using fluorescence microscopy based on the intensity of GFP signal and the strains with obvious GFP signal were used for the experiment. The MICs of compounds T766 and T054 against *M. smegmatis* mc^{2}155 strains with a control vector or strains overexpressing L12, L10, and Icl were determined using conventional plate dilution method.

1. Studier FW (2005) Protein production by auto-induction in high density shaking cultures. *Protein Expr Purif* 41(1):207–234.
2. National Committee for Clinical Laboratory Standards (1997) *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically* (National Committee for Clinical Laboratory Standards, Wayne, PA), 4th Ed, approved standard.
3. Kigawa T, et al. (2004) Preparation of Escherichia coli cell extract for highly productive cell-free protein expression. *J Struct Funct Genomics* 5(1–2):63–68.
4. Zhou Y, et al. (2005) Structure-guided discovery of novel aminoglycoside mimetics as antibacterial translation inhibitors. *Antimicrob Agents Chemother* 49(12):4942–4949.

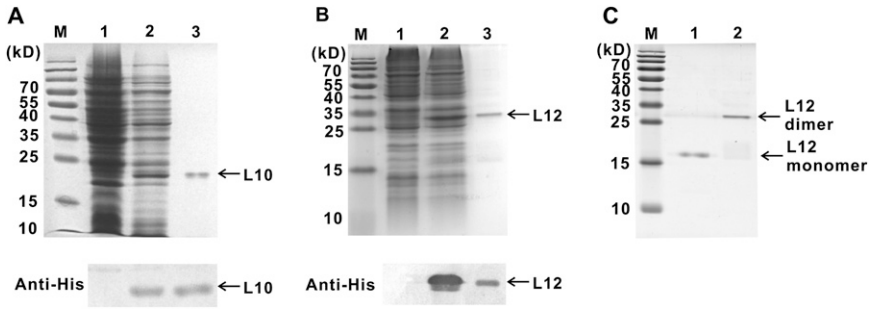

Fig. S1. Expression and purification of *M. tuberculosis* L12 and L10 proteins from bacteria *E. coli*. BL21(DE3) cells with L12 or L10 plasmid were induced as described in *Materials and Methods*. Protein samples were separated by SDS/PAGE and the protein bands are shown after Coomassie blue staining. *A* is for L10 and *B* is for L12. Lane M represents prestained protein markers. Total cell proteins before induction are shown in lane 1; whereas the total proteins after induction are shown in lane 2. Purified His-tagged L12 or L10 proteins are shown in lane 3. Only one single band appeared for both L12 and L10 after purification. Lower panels show the Western blot analysis of His-tagged L12 and L10 with anti-His antibody. Western blotting assay with anti-His antibody confirmed that the induced bands are His-tagged L12 and L10. (*C*) Dimerization of purified L12 protein. Purified His-tagged L12 protein was incubated with 5% H_2O_2 at 30 °C for 1 h. H_2O_2-treated (lane 1) and untreated (lane 2) L12 protein was resolved by SDS/PAGE and the protein bands were visualized after staining with Coomassie blue. Lane M shows prestained protein markers. Purified L12 protein showed a molecular weight at ~34 kDa, which is close to twice the size of L12 protein, suggesting that L12 forms a stable homodimer even in the presence of SDS. After treatment of purified L12 protein with 5% H_2O_2, a band of ~17 kDa appeared. This observation is consistent with a previous observation that dimerized L12 is extremely stable even in the presence of SDS, but the treatment with H_2O_2 disrupts the L12 dimer.

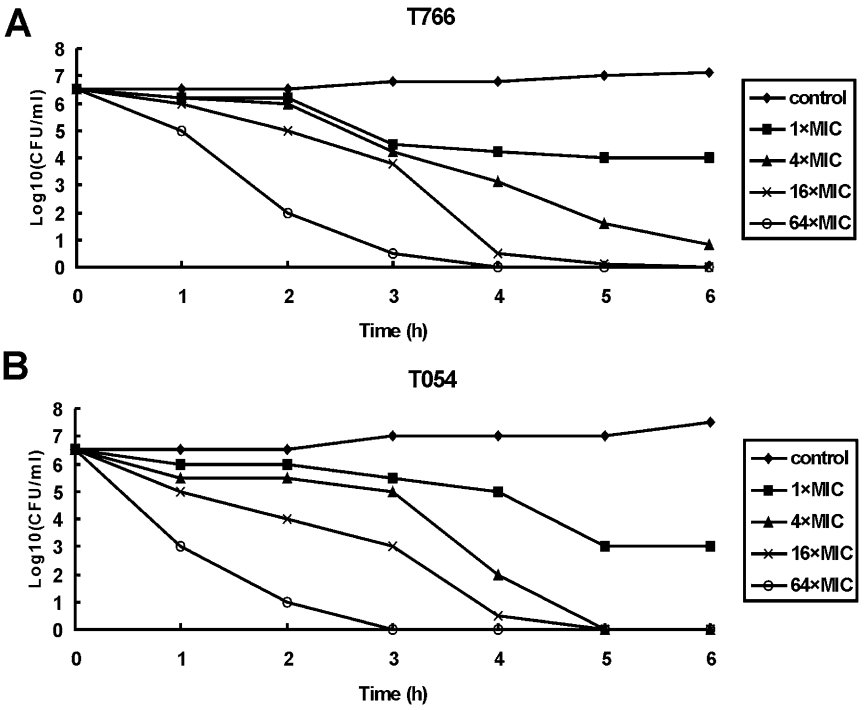

Fig. S2. Mode of action: bacteriostatic versus bactericidal mode. *Mycobacterium smegmatis* mc^2155 cells in log phase were incubated in the presence of various concentrations of T766 (*A*) and T054 (*B*). MICs for T766 and T054 against this strain is 0.5 and 1 μg/mL, respectively. Viability was counted after incubation in the presence of T766 and T054 at 1-, 4-, 16-, and 64-fold of MICs.

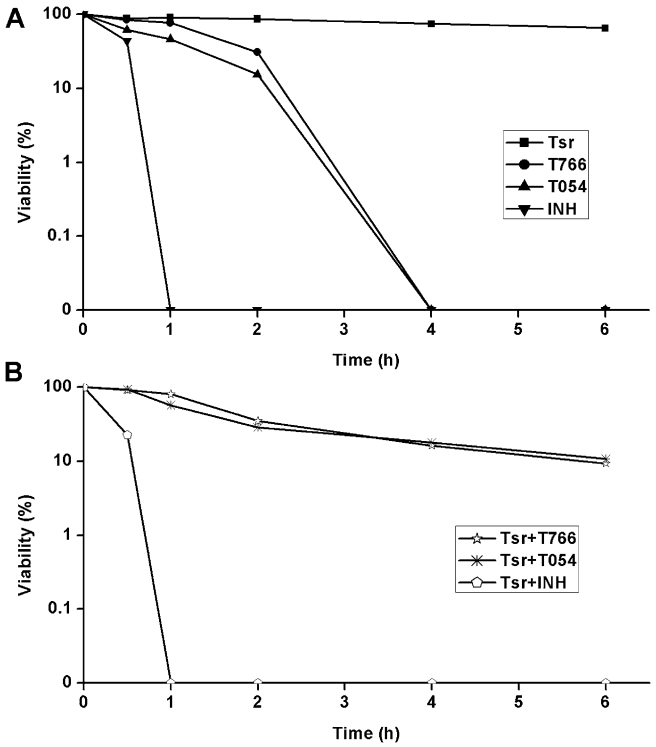

Fig. S3. Growth-dependent bactericidal activity of compounds T766 and T054. (*A*) Mode of action of tested compounds against *M. smegmatis* mc^{2}155. Growing bacteria in midlog phase were incubated in the presence of thiostrepton (Tsr) (16 μg/mL), T766 (32 μg/mL), T054 (64 μg/mL), and INH (64 μg/mL). The viability was determined every hour for 6 h. (*B*) Bactericidal activity of the tested compounds against *M. smegmatis* when preincubated with Tsr. Bacteria in midlog phase were pretreated with 16 μg/mL of Tsr for 5 min at 37 °C before the tested compounds (T766, 32 μg/mL; T054, 64 μg/mL; and INH, 64 μg/mL) were added into the cell cultures. Viability was determined every hour for 6 h.

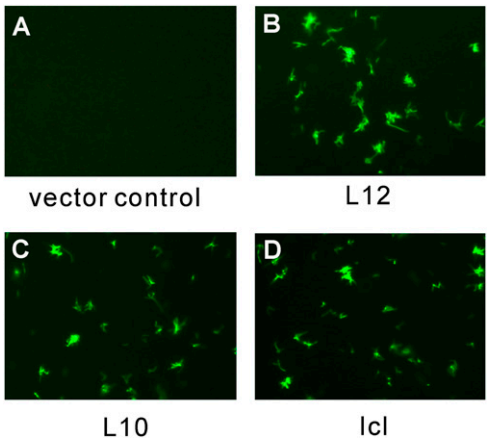

Fig. S4. Fluorescence signal in *M. smegmatis* mc^{2}155 strains with a control vector or overexpression plasmids for L12-eGFP, L10-eGFP, and Icl-eGFP. *M. smegmatis* cells carrying plasmids (*A*) pMV261, (*B*) pMV261:L12-eGFP, (*C*) pMV261:L10-eGFP, or (*D*) pMV261:Icl-eGFP were visualized by fluorescence microscopy.

Table S1. In vitro antibacterial activities of T766, T054, and the reference drug levofloxacin against various ATCC strains and clinical isolates

Strain		T766	T054	Levofloxacin
Staphylococcus aureus	ATCC29213*	128	32	0.125
	ATCC33591†	128	32	0.125
	09–6*	128	32	0.25
	09–13†	128	32	32
	15*	128	32	0.125
Staphylococcus epidermidis	ATCC12228‡	128	32	0.125
	09–3§	128	32	8
	09–9‡	64	32	0.125
Enterococcus faecalis	ATCC29212¶	128	32	2
	ATCC51299‖	128	32	2
	09–8¶	128	32	2
	09–9‖	128	32	>128
Enterococcus faecium	ATCC700221‖	64	32	128
	09–10¶	128	32	128
	09–15‖	64	32	128
Escherichia coli	ATCC25922**	64	64	<0.03
	09–1††	64	128	16
	09–20**	128	>128	0.5
	1515**	64	128	0.06
Klebsiella pneumoniae	ATCC700603††	128	>128	1
	09–8**	64	>128	0.06
	09–25††	64	128	4
	7**	64	128	0.06
	96–1**	64	64	<0.03
Pseudomonas aeruginosa	ATCC27853	>128	>128	1
	PAO1	>128	>128	4
	09–14	>128	>128	4
Acinetobacter calcoacetious	25001	64	32	0.125
Enterobacter cloacae	45301	128	128	0.125
Enterobacter aerogenes	45102	64	64	0.06
Serratia marcescens	41002	128	>128	0.125
Morganella morganii	49086	>128	>128	0.06
Providencia rettgeri	49006	>128	>128	0.125
Proteus vulgaris	56	>128	>128	<0.03
Proteus mirabilis	09–1	128	>128	4
Salmonella typhi	H901	64	32	0.06
Shigella Sonnei	51592	>128	>128	<0.03

MICs (μg/mL) were determined by the agar dilution method according to the National Committee for Clinical Laboratory Standards' recommendations.
*MSSA, methicillin sensitive staphylococcus aureus.
†MRSA, methicillin resistant staphylococcus aureus.
‡MSSE, methicillin sensitive straphylococcus epidermidis.
§MRSE, methicillin resistant straphylococcus epidermidis.
¶VSE, vancomycin sensitive enterococcus.
‖VRE, vancomycin resistant enterococcus.
**ESBLs (–), extended spectrum β-lactamases (–).
††ESBLs (+), extended spectrum β-lactamases (+).

Table S2. Antibacterial activity of T766 and T054 against the *M. smegmatis* strains with a control vector or overexpressing plasmids for L12, L10, and Icl

	MIC, μg/mL	
Strain	T766	T054
Vector control	0.5	1
Overexpressing L12	2	4
Overexpressing L10	0.5	1
Overexpressing Icl	0.5	1