Identification of antituberculosis agents that target ribosomal protein interactions using a yeast two-hybrid system

Yuan Lin[a,b,1], Yan Li[a,1], Yuanjun Zhu[a,2], Jing Zhang[a], Yongzhen Li[a], Xiao Liu[a], Wei Jiang[a], Shishan Yu[b], Xue-Fu You[a], Chunling Xiao[a], Bin Hong[a], Yanchang Wang[c,3], Jian-Dong Jiang[a,b,3], and Shuyi Si[a,3]

[a]Key Laboratory of Biotechnology of Antibiotics of Ministry of Health, Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China; [b]State Key Laboratory of Bioactive Substances and Function of Natural Medicine, Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing 100050, China; and [c]Department of Biomedical Sciences, College of Medicine, Florida State University, Tallahassee, FL 32312

Edited by Ada Yonath, Weizmann Institue, Rehovot, Israel, and approved September 13, 2012 (received for review June 29, 2011)

Mycobacterium tuberculosis **kills about 2 million people annually and antibiotic resistance is a cause of increased mortality. Therefore, development of new antituberculosis drugs is urgent for the control of widespread tuberculosis infections. For this purpose, we performed an innovative screen to identify new agents that disrupt the function of ribosomes in *M. tuberculosis*. Two bacterial ribosomal proteins L12 and L10 interact with each other and constitute the stalk of the 50S ribosomal subunit, which recruits initiation and elongation factors (EFs) during translation. Therefore, the L12–L10 interaction should be essential for ribosomal function and protein synthesis. We established a yeast two-hybrid system to identify small molecules that block the interaction between L12 and L10 proteins from *M. tuberculosis*. Using this system, we identified two compounds T766 and T054 that show strong bactericidal activity against tuberculosis but with low toxicity to mice and other bacterial strains. Moreover, using surface plasmon resonance (SPR) assay, we have demonstrated that these compounds bind specifically to L12 to disrupt L12–L10 interaction. Overproduction of L12 protein, but not L10, lowers the antibacterial activity of T766 and T054, indicating that the ribosome is likely the cellular target. Therefore, our data demonstrate that this yeast two-hybrid system is a useful tool to identify unique antituberculosis agents targeting the ribosomal protein L12–L10 interaction.**

Infections caused by *mycobacterium tuberculosis* (TB) are responsible for close to 2 million deaths each year worldwide and this situation is becoming worse due to the HIV/AIDS pandemic and the emergence of multidrug-resistant (MDR) TB strains (1). Currently, the standard treatment for tuberculosis infection is the combination of four first-line antibiotics: isoniazid, rifampin, pyrazinamid, and ethambutol. The last drug with a new mechanism of action approved for TB was rifampin. The World Health Organization data from 2007 indicate that 4.8% of all new and subsequent cases of tuberculosis are resistant to both isoniazid and rifampin. Therefore, antituberculosis (anti-TB) drugs with new molecular targets are necessary to control this widespread infectious disease.

The ribosome is the factory where protein synthesis occurs. The structure of the ribosome in bacteria and human cells differs significantly and this difference allows some antibiotics to specifically kill bacteria (2). For example, streptomycin inhibits protein synthesis in bacteria by causing the misreading of codons during translation (3). Chloramphenicol binds to the 23S rRNA of the 50S ribosomal subunit to prevent peptide bond formation in bacteria (4). The ribosome is a multiprotein complex and we speculate that the protein–protein interactions of the ribosomal subunits could be attractive targets for new antibiotics because of the different ribosome structure between bacteria and human cells.

In bacteria, elongation factors (EFs) EF-G and EF-Tu are large, multidomain GTP-binding proteins essential for protein synthesis. They interact with the stalk of the large ribosomal subunit (50S), and this interaction enhances the GTPase activity, suggesting a critical role of this structure domain of the ribosome in protein synthesis (5). In some bacteria species, such as *Escherichia coli*, the stalk consists of a single molecule of 23S rRNA as well as an L10 protein and two L12 protein dimers, whereas in other species, such as *Mycobacterium smegmatis*, the ribosome stalk contains three dimers of L12 in addition to an L10 protein and a 23S rRNA (6). It has been shown that the C-terminal α-helix of L10 anchors two or three L12 dimers by associating with the N-terminal domains of L12 (7). The C-terminal domain of L12 facilitates the recruitment of elongation factors EF-G and EF-Tu to a ribosome (8). Consistently, a ribosomal stalk lacking L12 is unable to interact with elongation factors (9). Truncation of the C-terminal region of L10 abolishes the binding of L12 to ribosomes and causes the loss of ribosomal GTPase activity (10). Therefore, the L12–L10 interaction is essential for ribosomal function and could be an ideal target for new antibiotics.

The yeast two-hybrid system is widely used to detect protein–protein interactions (11). To test the potential interaction of two proteins, they are fused to either the DNA binding domain (BD) or the activation domain (AD) of a transcription factor. The interaction of these two proteins brings the AD and BD domains together to activate the transcription of downstream reporter genes. With this technique, genome-wide protein interaction networks in different organisms have been established (12). The yeast two-hybrid system is not only a powerful tool to identify protein–protein interaction, but also useful to screen compounds that can disrupt a given protein–protein interaction. Several modified yeast two-hybrid systems were developed for this purpose (13–16). For example, a counter selection reporter system was used to identify calcium channel modulators (14), whereas a modified yeast two-hybrid system was used to screen inhibitors of Ras/Raf-1 interaction (13). However, the potential for the yeast two-hybrid system in drug discovery remains largely unexplored.

We have developed a screening system based on the interaction between *M. tuberculosis* ribosomal proteins L12 and L10. First, we adopted the yeast two-hybrid system to confirm the L12–L10 interaction; this has enabled us to identify compounds that specifically inhibit this interaction in *M. tuberculosis*. Through

Author contributions: Yuan Lin, Y.W., J.-D.J., and S.S. designed research; Yuan Lin, Yan Li, Yongzhen Li, X.L., X.-F.Y., and C.X. performed research; W.J., S.Y., and B.H. contributed new reagents/analytic tools; Y.Z. and J.Z. analyzed data; and Yuan Lin, Y.W., J.-D.J., and S.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]Yuan Lin and Yan Li contributed equally to this work.

[2]Present address: Department of Molecular and Cellular Pharmacology, School of Pharmaceutical Sciences, Peking University Health Science Center, Beijing 100191, China.

[3]To whom correspondence may be addressed. E-mail: sisyimb@hotmail.com, yanchang.wang@med.fsu.edu, or jiang.jdong@163.com.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1110271109/-/DCSupplemental.

high-throughput screening (HTS), we identified two compounds (T766 and T054) that inhibit this interaction and show anti-TB activity. Surface plasmon resonance (SPR) was used to confirm that T766 and T054 inhibit L12–L10 interaction by binding to L12 protein. In contrast to the strong growth inhibition of TB by these two compounds, they can only inhibit the growth of other bacterial strains at very high concentrations. Consistently, the protein synthesis inhibition in *M. smegmatis*, a strain close to TB, by these compounds is much more robust than that in other bacteria (*E. coli*) and mammalian cells. T766 and T054 show bactericidal activity against *M. smegmatis*, and this activity is abolished when treated with the protein synthesis inhibitor thiostrepton. Our observation that overexpression of L12, but not L10, in *M. smegmatis* increases the minimum inhibitory concentration (MIC) of these two compounds, indicates that the primary target is likely the ribosome protein L12. Therefore, using the yeast two-hybrid assay, we successfully identified unique anti-TB agents targeting L12–L10 interaction with high selectivity. In addition, our data indicate that L12–L10 interaction is an ideal target for anti-TB agents that disrupt ribosomal function.

Results

Detection of the Interaction Between *M. tuberculosis* Ribosomal Proteins L12 and L10 by Yeast Two-Hybrid Assay. It has been previously shown that two ribosomal proteins L12 and L10 interact with each other (17, 18); this interaction has been further confirmed by the crystal structure of the ribosome stalk (8, 19). To identify compounds that inhibit L12–L10 interaction, we first adopted the yeast two-hybrid system to confirm this interaction. For this purpose, we constructed plasmids pAD-L12 and pAD-L10, in which the L12 and L10 encoding genes from *M. tuberculosis* were fused in frame to the activating domain of Gal4 transcription factor. We also constructed plasmids pBD-L12 and pBD-L10, in which they were fused to Gal4 DNA binding domain. A pair of plasmids of pAD-L12 and pBD-L10, or pAD-L10 and pBD-L12 was introduced to AH109 yeast strain (20). The interaction between L12 and L10 leads to the activation of three reporter genes *ADE2*, *HIS3*, and *LacZ* in AH109 cells. Thus, the interaction between L12 and L10 can be determined on the basis of the growth of yeast cells on plates lacking adenine and histidine [synthetic defined (SD) −Ade −His], as well as the change to blue color after incubation in the solution containing *LacZ* substrate X-Gal or strong β-gal activity using *o*-nitrophenyl β-D-galactopyranoside (ONPG) as the *LacZ* substrate (Fig. 1*A*).

As a positive control, AH109 yeast cells with plasmids pAD-T and pBD-53 grew well on a SD −Ade −His dropout plate and could activate the β-gal activity because the expressed proteins p53 and SV40-T interact with each other (21) (Fig. 1 *B* and *C*). In contrast, AH109 cells with pAD-T and pBD-lam (human lamin C) did not grow on the dropout plate, and we used this strain as a negative control. AH109 cells with plasmids pAD-L12 and pBD-L10 grew well on a SD −Ade −His plate. Moreover, this strain is positive for β-gal activity, indicating the interaction between L12 and L10. Surprisingly, AH109 with pAD-L10 and pBD-L12 plasmids neither grew on the dropout plates nor showed β-gal activity. Because the N-terminal domain of L12 is responsible for the interaction with L10 protein (7), we speculate that fusion of the DNA binding domain of Gal4 at the N terminus of L12 protein might affect its interaction with L10. Yeast cells harboring either pAD-L12 or pBD-L10 alone did not show growth and were negative for β-gal activity, excluding the possibility of self-activation (Fig. 1 *B* and *C*). We also used Western blot analysis to validate the expression of L12 and L10 proteins in yeast cells (Fig. 1*D*). Therefore, we confirmed the TB L12–L10 interaction with the yeast two-hybrid system and this assay can be used for drug screening.

HTS for Compounds That Block L12–L10 Interaction. To screen for compounds that can block the interaction between *M. tuberculosis* L12 and L10 proteins, the growth of AH109 yeast cells with pAD-L12 and pBD-L10 plasmids in SD −Ade −His medium was analyzed in 96-well plates in the presence of various compounds at

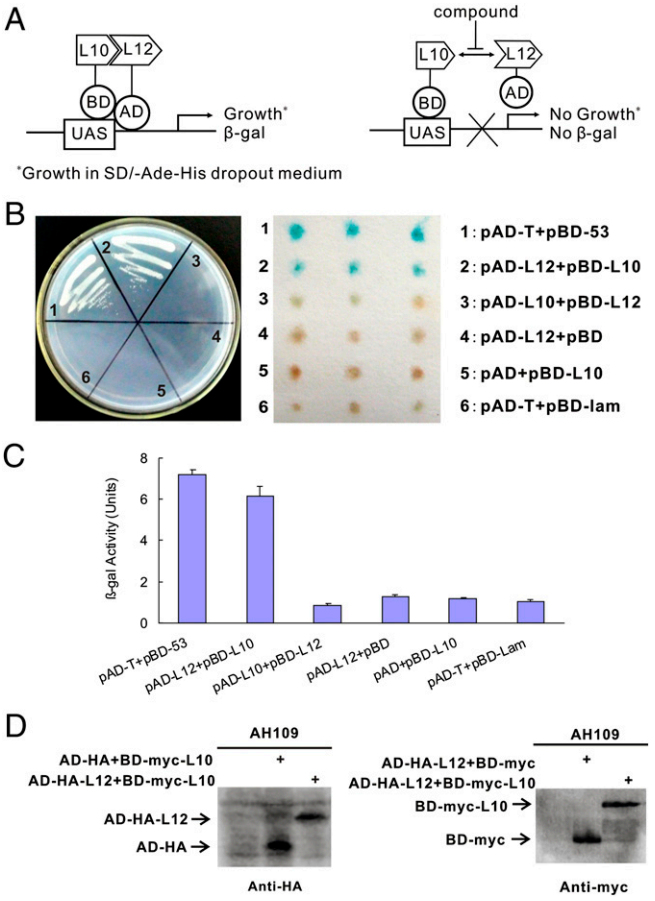

Fig. 1. Establishment of a yeast two-hybrid assay to detect the L12–L10 protein interaction. (*A*) Strategy for the high throughput screen using the yeast two-hybrid system. Interaction of L12 and L10 proteins reconstitutes the function of the Gal4 protein and results in the expression of the reporter genes, *ADE2*, *HIS3*, and *LacZ*. Compounds that disrupt L12–L10 interaction prevent the growth of yeast cells in SD −Ade −His dropout medium and avoid the production of β-gal. (*B*) Growth and β-gal activity of yeast cells expressing various combinations of BD and AD fusions. The combination of plasmids in AH109 strains is indicated on the right. Among them, strains 1 and 6 are used as positive and negative controls, respectively. The left panel shows the growth of yeast cells with indicated plasmids on a SD −Leu −Trp −Ade −His dropout plate. The right panel shows the β-gal activity of indicated strains. (*C*) Quantification of β-gal activity in yeast cells containing various combinations of plasmids. Results shown are the average units from triplicate assays. (*D*) Expression of L12 and L10 proteins in the yeast cells. Yeast cells expressing L12 and L10 fusion proteins were used to prepare protein samples and the expression was detected using anti-HA and anti-myc antibodies.

10 μg/mL. From a compound library, we found some compounds that inhibited the growth of AH109 (pAD-L12 + pBD-L10) in SD −Ade −His medium. One possibility is that these positive compounds disrupt the Gal4 expression system. If that is the case, the growth of AH109 (pAD-T + pBD-53) should also be inhibited by the compounds. Therefore, we examined the growth of AH109 (pAD-T + pBD-53) in the presence of selected compounds and only those that show specific growth inhibition for AH109 (pAD-L12 + pBD-L10) were selected. Another possibility is that the selected compounds are antifungal agents. To exclude this possibility, we also examined the growth of AH109 yeast cells in YPD (a rich medium for yeast cells) in the presence of the selected compounds. A representative set of plates from the HTS is presented in Fig. 2*A*. The compounds that only exhibit inhibition on the growth of AH109 (pAD-L12 + pBD-L10) in the dropout medium are likely the candidates that disrupt L12–L10 interaction.

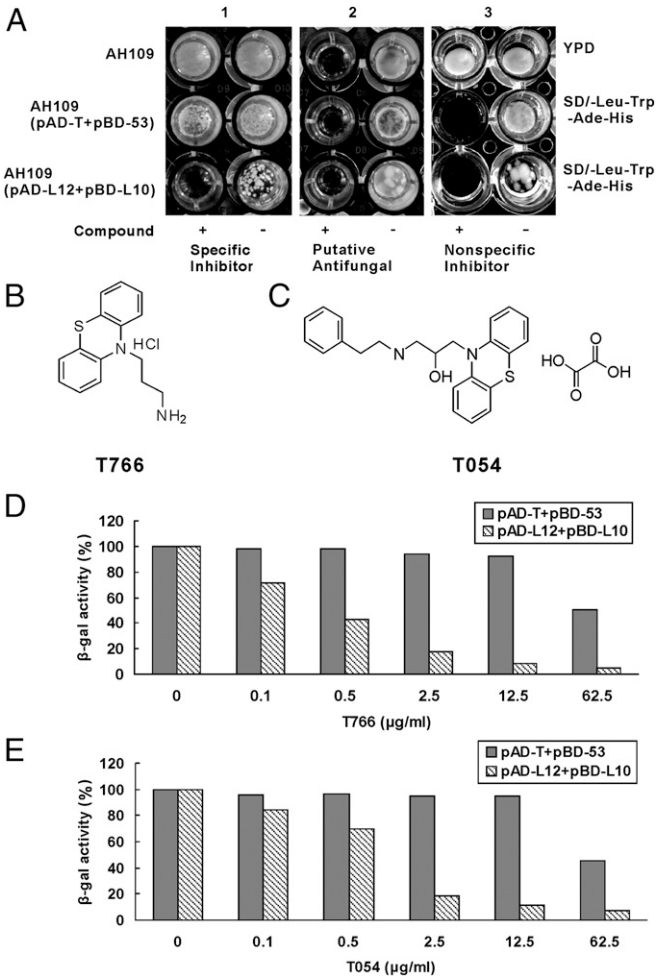

Fig. 2. Identification of compounds that block the L12–L10 interaction. (*A*) Growth inhibition of yeast cells by some representative compounds. AH109 strains with indicated plasmids were inoculated into SD −Leu −Trp −Ade −His dropout medium in 96-well plates (*Bottom* and *Middle*). AH109 was also inoculated into YPD medium (*Top*). The compound was added into one of the two wells for each strain at the concentration of 10 μg/mL and growth was examined after 24 h at 30 °C. (*B* and *C*) Structure of compounds T766 and T054. (*D* and *E*) Inhibition of β-gal activity of AH109 (pAD-L12 + pBD-L10) cells by T766 (*D*) and T054 (*E*) at various concentrations. Strain AH109 (pAD-T + pBD-53) was used as a control. Values represent the percentage of β-gal activity in cells treated with the compounds over that in untreated cells. β-Gal activity was examined in the presence of T766 and T054 at the concentrations of 0.1, 0.5, 2.5, 12.5, and 62.5 μg/mL.

From the library of 4,450 compounds, 10 were selected after the HTS assay.

To further confirm that the identified compounds selectively block L12–L10 interaction, yeast strains AH109 (pAD-L12 + pBD-L10) were subjected to a liquid β-gal assay in the presence of different concentrations of the selected compounds. Two of them, T766 and T054, inhibited the β-gal activity of this strain almost completely at 2.5 μg/mL, but not that of AH109 (pAD-T + pBD-53). Moreover, both compounds inhibited the β-gal activity of AH109 (pAD-L12 + pBD-L10) in a dose-dependent manner (Fig. 2 *D* and *E*). T766 and T054 showed a clear inhibition of β-gal activity at 0.1 and 0.5 μg/mL, respectively. Therefore, T766 (3-(10H-phenothiazin-10-yl) propan-1-amine hydrochloride) and T054 (1-phenethylamino-3-phenothiazin-10-yl-Propan-2-ol; compound with oxalic acid) are considered to be ideal candidates for further study and the structures of T766 and T054 are shown in Fig. 2 *B* and *C*.

Confirmation of L12–L10 Interaction by SPR Assay. SPR has been widely used for quantitatively measuring intermolecular interactions in real time, including the interaction of small molecules with proteins (22). To examine the interaction of purified *M. tuberculosis* L12 and L10 proteins, we successfully induced expression of 6× His-tagged L12 and L10 proteins in *E. coli* (Fig. S1). To detect the interaction of the purified L12 and L10 proteins with SPR, BIAcore sensor chips were coated with L12 and then exposed to L10 at various concentrations. L10 was able to bind to L12 as evidenced by a measurable change in response units, and this change was dose dependent (Fig. 3*A*). Similarly, BIAcore sensor chips were coated with L10 and then exposed to L12 protein. The result showed that L12 also interacts with immobilized L10 (Fig. 3*B*). The equilibrium dissociation constant (K_D) is 50 nM. The association (K_a) of the interaction is $1.2 \times 10^5\ M^{-1}{\cdot}s^{-1}$, and the dissociation ($K_d$) is $6.0 \times 10^{-3}{\cdot}s^{-1}$. There is a difference in the association and dissociation kinetics with coated L12 or L10 proteins (Fig. 3 *A* and *B*). One reason could be that the binding ratio between L12 and L10 is not 1:1. As mentioned before, one single L10 protein associates with two or three L12 dimers. Therefore, the SPR data verifies that L12 and L10 proteins from *M. tuberculosis* interact directly with each other.

Disruption of L12–L10 Interaction by T766 and T054. To determine whether compounds T766 and T054 bind to L12, L10, or both, we exposed these compounds to L12- or L10-coated sensor chips. Both T766 and T054 were able to bind to L12 as indicated by the changes in response units (Fig. 3 *C* and *D*). The K_D is 10 μM for T766 and 21.8 μM for T054. However, these two compounds failed to bind to L10 (Fig. 3 *E* and *F*), suggesting that L12 but not L10 is their binding target. To determine whether T766 and T054 abolish L12–L10 interaction, we first exposed them to an L12-coated sensor chip to saturate the immobilized L12 protein molecules. We then injected purified L10 protein and examined the change in response units. The pretreatment of L12-coated sensor chip with either T766 or T054 blocked the binding of L10 to L12 [T766 <5 response units (RU); T054 <20 RU], but the control buffer had no effect (Fig. 3*G*). These data collectively demonstrate that compounds T766 and T054 disrupt L12–L10 interaction by binding to L12 protein.

Antituberculosis Activity of T766 and T054. We speculate that the L12–L10 interaction is essential for ribosomal function and disruption of this interaction will inhibit the growth of *M. tuberculosis*. Indeed, both T766 and T054 showed anti-TB activity with a MIC at 0.312 μg/mL and 1.25 μg/mL for standard strains, respectively (Table 1). The MICs for these two compounds are comparable to the first-line antituberculosis drugs: rifampin (0.156 μg/mL), isoniazid (0.156 μg/mL), and ethambutol (1.25 μg/mL) (23). In wild-type clinical strains (STB-960, -825, and -9102), the MIC range was 0.25–2.0 μg/mL for T766, and 1.0–4.0 μg/mL for T054; the potency appeared not as strong as that of isoniazid (0.0625–0.125 μg/mL) and rifampin (0.0625–0.125 μg/mL). For the clinical MDR strains (MDR-699, -843, and -6233), however, the MICs were 2–4 μg/mL for T766 and 2–8 μg/mL for T054, similar to that of isoniazid but more potent than that of rifampin. For extensively drug resistant (XDR) clinical strains (XDR-83, -164, and -926), the compounds showed a potency with a range of 16–64 μg/mL, similar to that of rifampin. Isoniazid exhibited the highest activity against the three XDR strains with MICs from 4 to 8 μg/mL (Table 1). Multidrug resistant tuberculosis (MDR-TB) develops frequently in the course of TB treatment (24). We speculate that T766 and T054 inhibit the growth of MDR-TB strains by targeting L12–L10 interaction, which is different from those of the first-line anti-TB antibiotics. The higher MIC of these compounds against the tested XDR strains might be attributed to the highly active efflux pump or the decreased permeability of the mycolic acid-containing cell wall in these strains, which prevents the accumulation of compounds inside the cell (25).

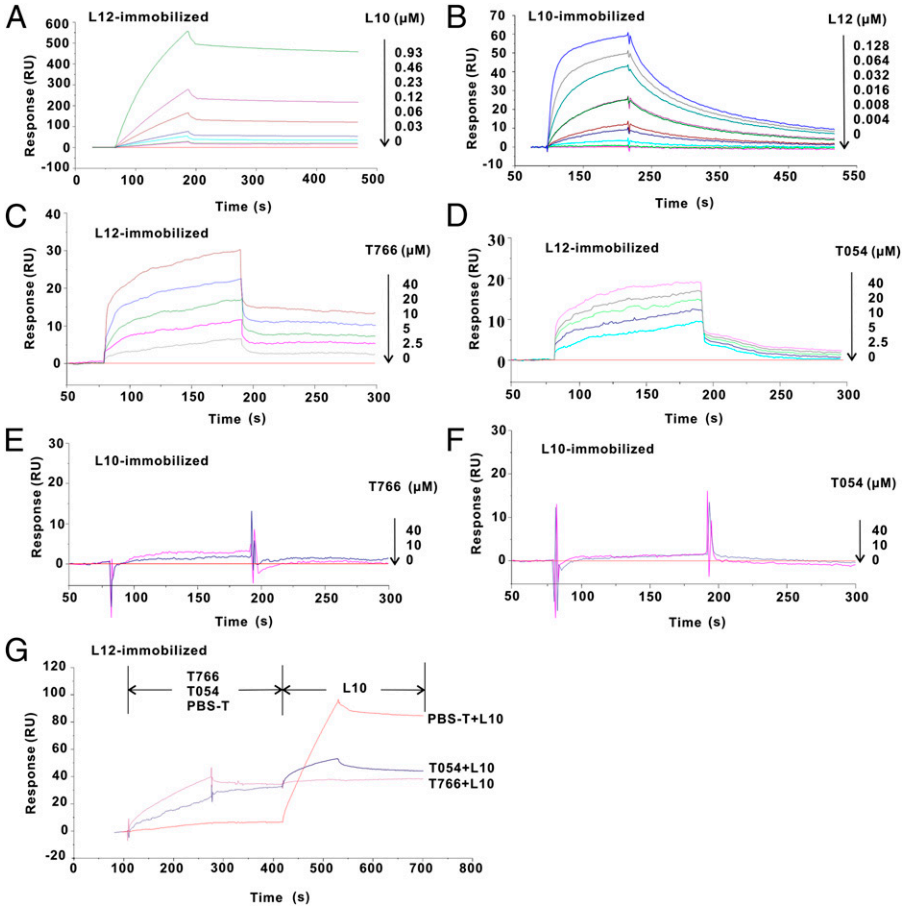

Fig. 3. SPR analysis for the binding of T766 and T054 compounds to L12 and L10 proteins and their effect on L12–L10 protein interaction. (*A*) Demonstration of L12–L10 interaction by SPR. A sensor chip coated with purified L12 was exposed to various concentrations of L10, ranging from 0.03 to 0.93 μM. The change in response units is shown. (*B*) To detect L12–L10 interaction using immobilized L10, various concentrations of purified L12 (0.004–0.128 μM) were injected into a chamber with a L10-coated sensor chip. Kinetic parameters were obtained using BIA-evaluation software. (*C* and *D*) Binding of the compounds T766 and T054 to L12 protein. Solutions with series concentrations (2.5–40 μM) of T766 (*C*) or T054 (*D*) were injected into the chamber with a L12-coated sensor chip. The change of response units is shown. (*E* and *F*) Binding of the compounds T766 and T054 to L10 protein. (*G*) Compounds T766 and T054 block L12–L10 interaction. Compound T766 (40 μM) or T054 (40 μM) was first injected into a chamber with a L12-coated sensor chip. Purified L10 protein (0.18 μM) was subsequently injected (20 μL/injection). As a control, PBS-T was injected at first and followed by the injection of L10 (0.18 μM). The change of response units was measured over time.

Because we used L12 and L10 proteins from *M. tuberculosis* for the drug screen, the identified compounds may show stronger growth inhibition of this species. Thus, we examined the growth inhibition of 37 bacterial strains from the American Type Culture Collection (ATCC) and clinical isolates by T766 and T054. Interestingly, the two compounds showed high MICs on these strains (T766 MIC ≥ 64 μg/mL; T054 MIC ≥ 32 μg/mL) (Table S1). Moreover, the MICs of the two compounds on budding yeast *Saccharomyces cerevisiae* were 25 μg/mL. The IC_{50} for human HeLa cells was 10 μg/mL for both of the compounds. Less growth inhibition on other organisms suggests that T766 and T054 could selectively kill *M. tuberculosis*. Furthermore, the LD_{50} value of compound T766 to uninfected C57BL/6 mice was 650 mg/kg (orally), indicating the safety of these compounds in animals.

Mechanism of *M. tuberculosis* Growth Inhibition by T766 and T054. T766 and T054 may inhibit the growth of TB through bacteriostatic or bactericidal effect. As *M. tuberculosis* grows very slowly, we chose *M. smegmatis*, a nonpathogenic strain biologically close to *M. tuberculosis*, to examine the viability after treatment with these compounds. The MICs for T766 and T054 for this nonpathogenic strain were 0.5 and 1 μg/mL, respectively. Interestingly, both compounds showed bactericidal activity against this strain at the MIC and the activity was increased dramatically when the concentration of the compounds were at 4-, 16-, or 64-fold of the MIC. After 3 h incubation, almost all of the bacteria were killed in the presence of 64× MIC (Fig. S2 *A* and *B*). The results suggest a strong bactericidal activity of T766 and T054 against mycobacteria.

We speculate that the L12–L10 interaction is essential for ribosomal function. Therefore, we took advantage of an in vitro system to determine whether T766 and T054 inhibit ribosome-mediated protein synthesis in rabbit, *E. coli*, and *M. smegmatis*. Compound T766 showed an inhibitory effect on protein translation at IC_{50} of 16.93 μg/mL for *M. smegmatis*, whereas the IC_{50} was 117.64 μg/mL and 118.06 μg/mL for *E. coli* and rabbit reticulocyte, respectively. Compound T054 also showed stronger inhibition of protein synthesis in *M. smegmatis* (Table 2). Therefore, both compounds exhibited selective translation inhibition for *M. smegmatis*, but the selectivity of T766 is better than that of T054, possibly due to structural difference. We noticed that the IC_{50} in the translation extracts are much higher than the MICs for cultures. We speculate that these compounds inhibit protein synthesis by blocking the L12–L10 interaction. Because this interaction is stable as evidenced by the slow exchange rate (Fig. 3), these compounds are likely less effective in the in vitro translation system with already-assembled ribosomes. We propose they are more potent in blocking ribosome assembly, hence yielding a lower MIC in the case of cell culture.

Table 1. MICs of T766 and T054 against various *M. tuberculosis* strains

Compound	MIC, μg/mL				
	T766	T054	Isoniazid	Rifampin	Ethambutol
H37Rv	0.312	1.25	0.156	0.156	1.25
STB-960	0.25	1	0.125	0.125	—
STB-825	1	4	0.0625	0.0625	—
STB-9102	2	4	0.0625	0.125	—
MDR-699	4	8	64	64	—
MDR-843	2	2	2	64	—
MDR-6233	2	4	4	64	—
XDR-83	64	32	8	64	—
XDR-164	32	32	4	32	—
XDR-926	16	32	8	16	—

MDR, multidrug resistance; STB, a clinically sensitive strain of *M. tuberculosis*; XDR, extensive drug resistance. Isoniazid and rifampin were used as reference drugs. STB-960, STB-825, STB-9102, MDR-699, MDR-843, MDR-6233, XDR-83, XDR-164, and XDR-926 strains were all clinical isolates.

To further clarify the mode of the anti-TB action, we examined the bactericidal activity of T766 and T054 when protein synthesis is blocked. Thiostrepton (Tsr) exhibits bacteriostatic activity as it blocks protein synthesis by inhibiting the function of elongation factor G (26, 27). We found that Tsr inhibits the growth of *M. smegmatis* with a MIC at 2 μg/mL. Thus, we treated the growing bacteria with 16 μg/mL Tsr for 5 min at 37 °C before adding T766 or T054. Although T766 or T054 alone killed *M. smegmatis* cells rapidly (Fig. S3*A*), a significant portion of cells were viable when pretreated with Tsr (Fig. S3*B*). In contrast, INH (isoniazid), which acts on the bacterial cell wall, retained its bactericidal activity despite the pretreatment with Tsr (Fig. S3*B*). Therefore, the blockage of protein synthesis in *M. smegmatis* with Tsr abolishes the bactericidal activity of both T766 and T054. One possibility is that disruption of the ribosome function by Tsr directly interferes with the action of T766 and T054 on ribosomes. Alternatively, the growth inhibition by Tsr may suppress the bactericidal activity of T766 and T054 through an indirect mechanism. Further investigation is needed to precisely demonstrate the primary target of these compounds.

If T766 and T054 inhibit the growth of mycobacteria by binding to L12 protein and disrupting ribosome function, a high level of expression of L12 protein should lower the antibacterial activity of these compounds. Therefore, we constructed expression plasmids containing eGFP-tagged L12, L10, or a ribosome-irrelevant protein isocitrase (Icl), respectively. These expression plasmids were introduced into *M. smegmatis* and their expression was confirmed by the appearance of GFP signal (Fig. S4). Interestingly, the MICs for T766 and T054 against the *M. smegmatis* strain overexpressing L12 are 2 and 4 μg/mL, respectively, which are fourfold higher than their MICs against the strain with a vector control (Table S2). In contrast, expression of L10 or Icl in *M. smegmatis* did not increase the MIC of T766 and T054. Collectively, these data suggest that the ribosome protein L12 is likely the in vivo target of these compounds.

Table 2. In vitro inhibition of protein translation by compounds T766 and T054

Compound	IC_{50}, μg/mL		
	Mycobacteria	*E. coli*	Eukaryote
T766	16.93	117.64	118.06
T054	25.91	34.23	60.16

IC_{50} values were determined by transcription–translation assays using mycobacterial ribosome extract, *E. coli* extract, and rabbit reticulocyte ribosomes.

Discussion

The ribosome is essential for protein synthesis. Because the ribosome structure differs between bacteria and human cells, many antibiotics target the ribosome for the selective killing of bacteria. The interaction of L12 and L10 proteins is critical for ribosomal function, thus compounds that inhibit this interaction could kill bacteria by disrupting protein translation. We first used the yeast two-hybrid system to confirm the interaction of L12 and L10 proteins from *M. tuberculosis*. With this system, we identified two compounds T766 and T054 and further proved that these compounds bind to L12 to block L12–L10 interaction by using SPR assay. More importantly, these two compounds inhibit protein synthesis and show anti-TB activity with MICs comparable to other first-line anti-TB drugs. Therefore, we have identified unique anti-TB compounds that appear to target ribosomal function.

The yeast two-hybrid system has been extensively used to detect protein–protein interactions (11, 28, 29), but there are few reports for its application in drug screening. One concern is the low permeability of yeast cells to compounds due to the rigid yeast cell wall (13). As our interest is to screen for new anti-TB drugs, the yeast screening system may exclude compounds that cannot enter into bacteria. By using the yeast two-hybrid system, we identified compounds that inhibit the interaction of two ribosomal proteins in *M. tuberculosis* and show strong anti-TB activity as well. Therefore, a yeast-based system may possess an advantage over in vitro screening systems for antibacterial agents. If a compound can enter into yeast cells to block a protein–protein interaction, it might also cross the bacterial cell wall and kill bacteria.

In this study, we used a more advanced SPR technique to further demonstrate the target specificity of the identified compounds. Using SPR, we first showed the direct interaction between L12 and L10 proteins from *M. tuberculosis*. We further convincingly demonstrated that compounds T766 and T054 disrupt L12–L10 interaction through their specific binding to L12, but not L10. Because L12 protein may also interact with elongation factors, we cannot exclude the possibility that T766 and T054 also abolishes the association of L12 with other ribosomal proteins.

We reason that T766 and T054 likely kill *M. tuberculosis* by disrupting the ribosomal function based on the following observations. First, we showed that these compounds block the interaction of two ribosomal proteins L12 and L10, which is essential for protein synthesis. Furthermore, T766 and T054 showed good activity against a strain resistant to rifampin and isoniazid, which act on RNA polymerase and the cell wall, respectively (30, 31), indicating that these compounds inhibit the growth of *M. tuberculosis* via a mechanism distinctive from that of rifampin and isoniazid. Moreover, ectopic expression of the ribosome protein L12, but not L10, in *M. smegmatis* raises the MIC of both T766 and T054 by fourfold, suggesting that their target could be the L12 protein of the ribosome. Taken together, these data indicate that these compounds represent a unique class of anti-TB agents that likely target ribosomal function. However, key experiments, such as the isolation of resistant mutants or metabolic labeling, are necessary to validate the in vivo target of T766 and T054; and unfortunately, these data are not available at the present stage. The current conclusion that these compounds target L12 protein in vivo is based on indirect evidence. Investigations using L12 mutants that fail to bind to T766 and T054 are needed to demonstrate that their primary target is the ribosome.

Selectivity, rather than mere activity, is the major limiting factor in antibiotics targeting the ribosome (32). T766 and T054 show low MICs to *M. tuberculosis*, but their MICs against other bacterial strains are very high. Moreover, these two compounds are less toxic to eukaryotic cells including budding yeast and human HeLa cells. Importantly, the high mouse LD_{50} of T766 (650 mg/kg) suggests good safety in targeting the L12–L10 interaction in *M. tuberculosis*. Therefore, these compounds merit chemical optimization for further development.

In summary, we found that L12–L10 interaction is a possible unique drug target for TB and we identified two compounds

T766 and T054 that inhibit protein synthesis in mycobacteria. The high selectivity against mycobacteria and the low animal toxicity suggest the safety of this mode of action. These compounds not only inhibit the growth of TB strains from laboratory stock and clinical isolates, but also show good activity against multiple drug-resistant strains. Although the activity of the two compounds is suboptimal, we believe there is a good chance of finding potent anti-TB drug candidates after structural modification. Therefore, L12–L10 interaction appears to be a potential target for unique anti-TB agents. Our results also demonstrate that the combination of the yeast two-hybrid system and SPR assay could be a powerful tool to discover compounds that target a specific protein–protein interaction.

Materials and Methods

Materials. The yeast Gal4 two-hybrid system was obtained from Clontech. The expression vector pET-16b(+) was purchased from Novagen. Antibodies against HA, myc, and His were from Santa Cruz. The compounds T766 and T054 were purchased from J&K Chemical. Rifampin, isoniazid, ethambutol, and other chemical reagents were from Sigma.

Plasmid Construction. The PCR products of the coding sequence of L12 and L10 from *M. tuberculosis* were inserted into pGADT7 and pGBKT7 so that the resulting plasmids contain L12 or L10 fused in frame with either Gal4 AD or DNA BD. The detailed method is described in *SI Materials and Methods*.

Yeast Two-Hybrid Assay. Transformation, yeast growth, and β-galactosidase assays were performed as described (20). See *SI Materials and Methods* for a detailed description.

Compound Library Screening. Exponentially growing yeast cells AH109 (pAD-L12 + pBD-L10), AH109 (pAD-T + pBD-53), and AH109 were diluted 100 times. A total of 198 μL of diluted culture was added into each well in 96-well plates, and 2 μL of compounds were added to a final concentration of 10 μg/mL in 0.1% DMSO. A library of 4,450 compounds (*SI Materials and Methods*) was used for the screen. Growth inhibition of the yeast cells was assessed after 24 h incubation at 30 °C.

SPR Assays. The measurements were performed using a BIAcore 3000 (Biacore) at 25 °C in a running buffer PBS-T. The detailed protocol for the SPR assays is described in *SI Materials and Methods*.

Anti-TB Activity. The activities of compounds T766 and T054 against H37Rv, clinical sensitive strain, and drug-resistant clinical isolates were analyzed using the microplate alamar blue assay (MABA) (33). The detailed method is described in *SI Materials and Methods*.

In Vitro Translation Inhibition by Compounds T766 and T054. Translation inhibition 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. Detailed protocol is described in *SI Materials and Methods*.

Animal Toxicity Test. LD_{50} (lethal doses, 50%) was determined according to the method described by Behrens and Karber (see ref. 34). C57BL/6 (20 ± 2 g) mice were injected orally with compound T766 solution (0.2 mL). The number of deaths was scored over 24 h and the LD_{50} was then calculated.

ACKNOWLEDGMENTS. This work was supported by State Mega Programs (2012ZX09301-002-003, 2012ZX09301-002-001, 2009ZX09302-004, 2008ZX10003-006, and 2010ZX09401-403) and grants from the National Natural Science Foundation of China (81001461 and 81072672). Y.W. is also supported by a Research Scholar Grant RSG-08-104-010CCG from the American Cancer Society, R15GM097326-01 from National Institute of General Medical Sciences, and a research grant from College of Medicine at Florida State University.

1. Cole ST, et al. (1998) Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. *Nature* 393(6685):537–544.
2. Yonath A (2005) Antibiotics targeting ribosomes: Resistance, selectivity, synergism and cellular regulation. *Annu Rev Biochem* 74:649–679.
3. Kenney TJ, Churchward G (1994) Cloning and sequence analysis of the rpsL and rpsG genes of Mycobacterium smegmatis and characterization of mutations causing resistance to streptomycin. *J Bacteriol* 176(19):6153–6156.
4. Weber MJ, DeMoss JA (1969) Inhibition of the peptide bond synthesizing cycle by chloramphenicol. *J Bacteriol* 97(3):1099–1105.
5. Savelsbergh A, Mohr D, Wilden B, Wintermeyer W, Rodnina MV (2000) Stimulation of the GTPase activity of translation elongation factor G by ribosomal protein L7/12. *J Biol Chem* 275(2):890–894.
6. Bruell CM, et al. (2008) Conservation of bacterial protein synthesis machinery: Initiation and elongation in Mycobacterium smegmatis. *Biochemistry* 47(34):8828–8839.
7. Oleinikov AV, Perroud B, Wang B, Traut RR (1993) Structural and functional domains of Escherichia coli ribosomal protein L7/L12. The hinge region is required for activity. *J Biol Chem* 268(2):917–922.
8. Wilson DN, Nierhaus KH (2005) Ribosomal proteins in the spotlight. *Crit Rev Biochem Mol Biol* 40(5):243–267.
9. Burma DP (1984) Conformational change of 23S RNA in 50S ribosome is responsible for translocation in protein synthesis. *J Biosci* 6:419–430.
10. Hagiya A, et al. (2005) A mode of assembly of P0, P1, and P2 proteins at the GTPase-associated center in animal ribosome: In vitro analyses with P0 truncation mutants. *J Biol Chem* 280(47):39193–39199.
11. Fields S, Song O (1989) A novel genetic system to detect protein-protein interactions. *Nature* 340(6230):245–246.
12. Suzuki H, et al. (2001) Protein-protein interaction panel using mouse full-length cDNAs. *Genome Res* 11(10):1758–1765.
13. Kato-Stankiewicz J, et al. (2002) Inhibitors of Ras/Raf-1 interaction identified by two-hybrid screening revert Ras-dependent transformation phenotypes in human cancer cells. *Proc Natl Acad Sci USA* 99(22):14398–14403.
14. Young K, et al. (1998) Identification of a calcium channel modulator using a high throughput yeast two-hybrid screen. *Nat Biotechnol* 16(10):946–950.
15. Zhao HF, et al. (2004) A mammalian genetic system to screen for small molecules capable of disrupting protein-protein interactions. *Anal Chem* 76(10):2922–2927.
16. Park SH, Raines RT (2000) Genetic selection for dissociative inhibitors of designated protein-protein interactions. *Nat Biotechnol* 18(8):847–851.
17. Griaznova O, Traut RR (2000) Deletion of C-terminal residues of Escherichia coli ribosomal protein L10 causes the loss of binding of one L7/L12 dimer: Ribosomes with one L7/L12 dimer are active. *Biochemistry* 39(14):4075–4081.
18. Pettersson I (1979) Studies on the RNA and protein binding sites of the E. coli ribosomal protein L10. *Nucleic Acids Res* 6(7):2637–2646.
19. Helgstrand M, et al. (2007) The ribosomal stalk binds to translation factors IF2, EF-Tu, EF-G and RF3 via a conserved region of the L12 C-terminal domain. *J Mol Biol* 365(2):468–479.
20. James P, Halladay J, Craig EA (1996) Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics* 144(4):1425–1436.
21. Li B, Fields S (1993) Identification of mutations in p53 that affect its binding to SV40 large T antigen by using the yeast two-hybrid system. *FASEB J* 7(10):957–963.
22. Löfås S (2008) Biacore systems: Leading the revolution in label-free interaction analysis. *Biointerphases* 3(3):FD2–FD3.
23. Franzblau SG, et al. (1998) Rapid, low-technology MIC determination with clinical Mycobacterium tuberculosis isolates by using the microplate Alamar Blue assay. *J Clin Microbiol* 36(2):362–366.
24. Iseman MD (1993) Treatment of multidrug-resistant tuberculosis. *N Engl J Med* 329 (11):784–791.
25. Louw GE, et al. (2009) A balancing act: Efflux/influx in mycobacterial drug resistance. *Antimicrob Agents Chemother* 53(8):3181–3189.
26. Kutscher AH, Zegarelli EV, Rankow RM, Mercadante J, Piro JD (1959) Clinical laboratory studies on a new topical antibiotic: Thiostrepton. *Oral Surg Oral Med Oral Pathol* 12(8):967–974.
27. Walter JD, Hunter M, Cobb M, Traeger G, Spiegel PC (2012) Thiostrepton inhibits stable 70S ribosome binding and ribosome-dependent GTPase activation of elongation factor G and elongation factor 4. *Nucleic Acids Res* 40(1):360–370.
28. Aronheim A, Zandi E, Hennemann H, Elledge SJ, Karin M (1997) Isolation of an AP-1 repressor by a novel method for detecting protein-protein interactions. *Mol Cell Biol* 17(6):3094–3102.
29. Allen JB, Walberg MW, Edwards MC, Elledge SJ (1995) Finding prospective partners in the library: The two-hybrid system and phage display find a match. *Trends Biochem Sci* 20(12):511–516.
30. Hui J, Gordon N, Kajioka R (1977) Permeability barrier to rifampin in mycobacteria. *Antimicrob Agents Chemother* 11(5):773–779.
31. Slayden RA, Lee RE, Barry CE, 3rd (2000) Isoniazid affects multiple components of the type II fatty acid synthase system of Mycobacterium tuberculosis. *Mol Microbiol* 38(3): 514–525.
32. Böttger EC (2007) Antimicrobial agents targeting the ribosome: the issue of selectivity and toxicity: Lessons to be learned. *Cell Mol Life Sci* 64(7–8):791–795.
33. Collins L, Franzblau SG (1997) Microplate alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against Mycobacterium tuberculosis and Mycobacterium avium. *Antimicrob Agents Chemother* 41(5): 1004–1009.
34. Gilles HJ (1974) Calculation of the index of acute toxicity by the method of linear regression. Comparison with the method of "Karber and Behrens" *Eur J Toxicol Environ Hyg*, 7(2):77–84, in French).