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Anti-tuberculosis drug target discovery by targeting the higher in-degree proteins (HidPs) of the pathogen's transcriptional network

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Abstract

With the emergence of multiple-drug resistant bacteria pathogens, it has become increasingly important to develop new strategies for exploring alternative drug targets and new bactericides. However, new drug targets with a different antimycobacterial spectrum than current clinical drugs are lacking. In this study, based on a transcriptional regulation (TR) network of Mycobacterium tuberculosis, more than 60 higher in-degree proteins (HidPs) from the network were first characterized. Targeting HidPs of the TR network was further shown to be an efficient strategy for potential anti-tuberculosis drug target discovery. Top 5 HidPs, whose crystal structures have been resolved, were selected as hypothetical targets for docking-based virtual screen against a library containing 10,756 compounds. Seventy-nine docking compounds were used for further antibacterial screen. Six compounds, which target 4 HidPs, could specifically inhibit the growth of both M. tuberculosis H37Rv and a multi-drug resistant strain isolated from a clinic. Furthermore, the specific interaction of a newly characterized inhibitor with the Rv3290c protein could be confirmed. Thus, we have characterized new anti-tuberculosis candidate targets and bactericides, and the identified HidPs can serve as an important resource for further anti-TB drug discovery efforts. Our strategy should be applicable for combating a wide range of pathogens.

Introduction

The emergence of drug resistant strains of many bacterial pathogens is one of the major challenges faced by efforts aimed at controlling infectious diseases [1]. In some cases, this concern has been amplified with the emergence of multi-drug resistant (MDR) strains [2]. As a result, there is a dire need for development of new strategies and identification of effective, alternative drug targets that may be used to combat these pathogens. Mycobacterium tuberculosis, the causative agent of tuberculosis, continues to be a leading cause of mortality world wide and about two million people die from it annually [3]. Despite the availability of the Bacillus-Calmette Guerin (BCG) vaccine and current chemotherapeutic drugs, neither of these approaches has proven completely effective in the prevention or treatment of TB. Emergence of MDR and extensively drug-resistant (XDR) tuberculosis [2] raise the need for identification of new drug tar-

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gets and discovery of molecular scaffolds to fight this pathogen.

Antibacterial drugs have traditionally been designed to inhibit the essential components of the pathogen to disrupt its survival. Therefore, ongoing searches for new drug targets have focused mostly on discovery of essential genes. The search for such essential genes have been performed by varied experimental procedures such as single gene knockouts [4], conditional knockouts [5], RNA interference [6], antisense RNA [7] and transposon mutagenesis [8]. However, each of these techniques requires a large investment of time and resources, and is not always feasible. With the development of 'omic' biology in recent years, various experimental and computational methods have been developed to detect and predict the interactions among molecules such as protein-protein and protein-DNA interactions in a cell. These advances have allowed us to construct large-scale biological networks and to analyze cellular processes at the network level. The search for drug targets from these biological networks has garnered much attention in recent years [9]. For example, various approaches have been introduced to identify drug targets from protein-protein interaction networks [10,11] and metabolic networks [12]. Unlike traditional methods, these new methods can allow rapid high-throughput identification of targets and avoidance of organism-specific limitations.

Transcriptional regulation (TR) is essential for the survival of a pathogen and its interactions with its host. Therefore, a comprehensive analysis of transcriptional regulation in pathogens can improve our understanding of pathogenesis and provide the opportunity to identify new and effective drug targets. Several studies have implied the importance of TR networks for the pathogenesis and survival of M. tuberculosis [13-16]. In a recent study, using a high-throughput bacterial one-hybrid technique [16], a genome-wide transcriptional factor-gene interaction network for *M. tuberculosis* has been successfully constructed [17].

In this study, we developed a new strategy that combines in-degree analysis of the bacterial TR network and rational structure-based drug design pipelines to identify candidate drug targets against *M. tuberculosis* (**Figure 1**). A group of higher in-degree proteins (HidPs) from the network were identified. Then, potential compounds with bacteriostatic activity against M. tuberculosis were screened for and characterized. Finally, the specific interaction between a newly characterized inhibitor and its target protein was further confirmed.

Experimental Procedures

Strains, Enzymes, Plasmids and Chemicals

Escherichia coli: BL21 strains and the pET28a expression vector were purchased from Novagen. All the enzymes including DNA polymerase, restriction enzymes and DNA ligase were purchased from TaKaRa Biotech. Deoxynucleoside triphosphates (dNTPs) and all antibiotics were purchased from TaKaRa biotech as well. PCR primers were synthesized by Invitrogen (Supplementary Table S1).

Expression and purification of recombinant TF proteins of M. tuberculosis

The *M. tuberculosis*: Rv3290c and its mutant genes were cloned into the over expression vectors of pET28a to produce corresponding recombinant vectors. E. coli BL21 (DE3) cells transformed with the recombinant plasmid were grown at 37°C in 200 ml of LB medium containing 50 mg/ml kanamycin. Protein expression was induced by the addition of 1 mM isopropyl β-D-1- thiogalactopyranoside (IPTG) at 37°c for 2 h. Proteins were purified on Ni2+ affinity columns as described previously [16]. The purified proteins were determined by SDS-PAGE. Protein concentrations were determined by spectrophotometric absorbance at 260 nm.

In silico inhibitor screen

DOCK (18) (version 6.4) was used for in silico docking base virtual screen. The compound library was obtained from the ZINC database [19]. Compounds used for in vitro assays were purchased from Specs Company. Molecular graphics images were produced using the UCSF Chimera package [20] and PyMOL (http://www.pymol.org/).

L-Lysine ε-aminotransferase assay

Standard aminotransferase assay was performed as previously described [21]. Enzyme incubations contained Rv3290c (0.5 µM, 1.0 ml), 40 µmol of L-lysine, 40 µmol of a-ketoglutarate, and 0.15 µmol of pyridoxal phosphate in a final volume of 2.0 ml. Serial two-fold dilutions of the compounds, ranging from 400 µM to 25 µM with 5 different concentrations, were used for the inhibition assays of enzyme activity. The reaction was run at 37°c for 60 min and stopped by the addition of a 1:10 mixture of 50% trichloroacetic acid and absolute ethanol (1.0 ml).

Formation of piperideine carboxylate was monitored with o-aminobenzaldehyde (o-AB), which forms an orange dihydroquinazolinium complex with piperideine carboxylate. The amount of 1-piperideine-6-carboxylate formed was calculated by using the extinction coefficient of 2,800 liters mol-1 cm-1 previously reported by Fothergill and Guest [22]. The specific activity of the aminotransferase represents micrograms of a-aminoadipic-8-semialdehyde (AAS) formed per milligram of protein per hour.

Inhibition assay of mycobacterial growth

The susceptibility of several different mycobacterial species to test compounds were evaluated by determining their growth trends in the presence of the test compounds and comparing them to growth with rifampicin. The mycobacterial species tested were M. tuberculosis H37Rv, M. bovis BCG, M. smegmatis mc2 155, and a MDR strain M. tuberculosis strain (MDR10093006, Wuhan) that is resistant to 250 µg/ml rifampicin, 100 µg/ml streptomycin, 1 µg/ml isoniazid and 5 µg/ml ethambutol. Briefly, a 500 µl 7H9 broth, yielding a final inoculum of 10 4 - 10 5 colony forming units (CFU), was added to each vessel. Serial two-fold dilutions of each compound were prepared and added directly into the medium ranging from 0.03 to 32 µg/ml. After 7 days of incubation at 37 °C in a normal atmosphere, the optical densities of mycobacterial growth were measured at OD 600 nm. The minimal inhibitory concentrations (MICs) of the drug or compounds were determined using a previously described procedure [23]. An average of three individual measurements was presented.

Surface plasmon resonance (SPR) analysis

The interactions between the drugs and Rv3290c series were assayed using SPR. The 6×His Rv3290c protein was bound to the NTA chip (BIAcore) via a previously published procedure (24). Drugs were dissolved in 100% DMSO and diluted in 1.0×PBS to obtain a 100 mM stock solution in 10 mM PBS and 5% DMSO. The stock was then further diluted in DMSO running buffer. Concentration series from 0.2 mM to 1 mM, including control and repeated samples, were used for assays. All experiments were

carried out at 25 °C at a flow speed of 30 µl/min. The effect of DMSO was also corrected according to a previously reported method [25].

Scanning electron microscopy (SEM) observation

Mycobacterial cells prepared for SEM observation were grown in 7H9 broth for 2-7 d in the presence of either 50 µg/ml compound or rifampicin. The cells were harvested by centrifugation. The bacterial pellets were resuspended and incubated at 4 °C for 24 h in 2.5% glutardialdehyde solution. The cells were washed twice in double-distilled water then dehydrated for 15 min treatments in 30%, 50%, 75%, 85%, 95% and 100% ethanol. The incubation in 100% ethanol was repeated to ensure a complete dehydration. Samples were critical-point dried, sputter-coated with gold, and observed using a scanning electron microscope (S570; Hitachi, Tokyo, Japan).

Results

Characterization of higher in-degree proteins

We calculated the number of TFs binding to the promoter (in-degree) of the M. tuberculosis PDI network which we had recently constructed [17]. As shown in Supplementary Table S2, the in-degree ranges from 1 to 142 and the top proteins with in-degree of more than 50 were listed. The highest in-degree promoter Rv1219c_up can be bound by 142 regulators. The top 10 high in-degree promoters are involved in the expression of membrane transport system, metabolism enzymes and two regulators whib2 and lsr2. By mapping each promoter in the PDI network to genes, a large group of higher in-degree genes or proteins were further characterized from the TR network.

In silico structure-based screen for inhibitors targeting HidPs

HidPs in the TR network may be the proteins that are indispensable for bacterial survival and are thus good candidates for a screen for inhibitors. To test this possibility and explore new strategies for inhibitor design, we employed docking based virtual screen methods to perform an in silico inhibitor screen. First, potential targets were selected with consideration for in-degree and structure information. Five proteins for which three-dimensional structures have already been experimentally resolved in the structure database PDB (http://www.pdb.org) were selected as potential candidate targets (**Table 1**) and are as follows: Rv3290c (in-degree: 136), Rv2780 (in-degree:104), Rv0233 (in-degree:64), Rv0674 (in-degree:60) and (Rv2498c, in-degree:51). Except for Rv0674, whose function is unknown, the other 4 proteins are catalytic enzymes (**Table 1**).

For these five potential candidate targets, the known ligand-binding cavity for small molecule (**Table 1**) was selected as an active site. These included the pyridoxal phosphate and alpha-ketoglutarate binding cavity in Rv3290c, the NAD binding cavity in Rv2780c, the myristic acid binding cavity in Rv0233 and the oxaloacetate ion binding cavity in Rv2498c (Supplementary Figure S1). However, only a hypothetical active site for Rv0674 was defined by manual analysis of protein surface structure because both its function and the identity of its ligand-binding pocket remain unclear. The docking screen for each target was performed against a library including 10,756 compounds using the DOCK program [18]. In order to obtain compounds with high interaction specificity and stability, the top-scored compounds were further filtered by restraining the Van der Waals (VDW) force and internal energy. In total, 79 docking compounds for all five target proteins were selected for further evaluation (Supplementary Table S3).

Inhibition activity screen of docking compounds on mycobacterial growth

Bacteriostasis activity is one of the most important criteria for the success of a lead compound screen. We examined the inhibitory activity of all of the above 79 docking hit compounds on mycobacterial growth (**Figure 2**). As shown in Fig. 2a, compound #14 was designed to target Rv3290c and it substantially inhibited the growth of both M. tuberculosis H37Rv (OD 600= 0.1) and M. bovis BCG (OD 600= 0.1), but not M. smegmatis mc2 155 (OD 600= 0.3), when compared with the untreated control group (OD 600= 0.3) (CK-1). This result is very similar to that of rifampicin (CK-2) (OD 600= 0.1), a first-line anti-TB drug. In contrast, compound #80 (CK-3) (OD 600= 0.28), an unrelated compound used as a negative control, did not have any obvious effect on the growth of three mycobacterial species. Similarly, compounds #23 and #24, both of which were designed to target Rv2780, were found to inhibit the growth of M. tuberculosis H37Rv and M. bovis BCG (**Figure 2b**). Notably, these two compounds showed better inhibition of M. tuberculosis H37Rv than of M. bovis BCG, and had no obvious effect against M. smegmatis. Compound #51 targeting Rv0233 (**Figure 2c**), and compounds #66 and #70 targeting Rv2498c (**Figure 2d**) were also shown to specifically inhibit the growth of M. tuberculosis H37Rv and M. bovis BCG. However, based on the hypothetical active site of Rv0674, no compound was successfully identified (**Figure 2e**).

Thus, six compounds (**Figure 3a**) from 79 in silico structure-based candidate inhibitors were characterized to have obvious inhibitory activity on the growth of M. tuberculosis H37Rv and M. bovis BCG, but not on that of M. smegmatis. Those compounds were designed for 4 targets: Rv3290c, Rv2780, Rv0233 and Rv2498c (**Figure 3b**). Minimal inhibitory concentrations (MICs) of compounds were further measured against several mycobacterial species, including a MDR M. tuberculosis strain isolated from a clinic. Importantly, these compounds had MICs ranging from 0.5 µg/ml - 8 µg/ml against M. tuberculosis H37Rv (**Figure 3b**). However, a much higher concentration was needed to sterilize M. smegmatis. Interestingly, six compounds displayed similar activity against clinical MDR isolates of M. tuberculosis, implying that those targets are excellent candidates. Potential binding modes of the compounds with their targets were generated for six compounds (**Figure 4**).

Specific interaction between compound #14 and the target protein Rv3290c

The interaction between compound #14 and the Rv3290c protein was further studied to validate the specificity of the characterized potential inhibitors. In a surface plasmon resonance (SPR) assay, 6×His-tagged Rv3290c was first associated with the NTA chip (GE Healthcare). As shown in (**Figure 5a**), when an increasing concentration of compound #14 (0.1-1 mM) was passed over the chip surface, a corresponding increase in response value was observed, indicating a concentration-dependent change in the interaction of the compound with Rv3290c. The trend line was generated by using the equation ($y = 144.5x0.721$, $R^2 = 0.999$) (**Figure 5b**). In contrast, an unrelated compound (#80) showed no obvious binding activity (**Figure 5c**). Using computer simulations, the interaction residues were chosen to perform site-directed mutagenesis. Three mutants, R170A, K300A, and R170A/K300A, were designed and their proteins were purified (**Figure 5d**). The interactions of compound #14 with these mu-

tant proteins were examined by SPR. Notably, compound #14 had reduced binding with all three mutant proteins compared with that of the wild-type protein, suggesting that it interacted specifically with the substrate binding pocket of Rv3290c (**Figure 5e**).We further performed the L-Lysine ε-aminotransferase assay of Rv3290c to test the specific inhibitory role of compound #14 on its enzyme activity. As shown in (**Figure 5f**), the degree of inhibition increased rapidly with the compound concentration, even though the compound had only been through primary screening and could be further optimized. In contrast, an unrelated compound (#80) did not show any inhibitory activity under the same conditions. The IC50 value [26] for compound #14 or the concentration at which 50% of the enzyme activity of Rv3290c could be inhibited was 200 μM.

Time-lapse imaging of the M. bovis BCG culture done by scanning electron microscopy (SEM) revealed that upon exposure to compound #14, the growth rate rapidly decreased, which was followed by cell lysis after 192h. This effect was similar to that of the first-line anti-TB drug, rifampicin. In contrast, compound #80, used as negative controls, showed no effect on bacterial growth (**Figure 5g**). Next, all four cultures were transferred to a drug-free 7H10 slant medium and maintained for an additional month. Unlike the cultures that had been treated with compound #80, not a single colony grew for the cultures that had been previously exposed to RFP and compound #14 (Supplementary Figure S2).

These results show that the HidP Rv3290c is a potential target protein for designing inhibitors against M. tuberculosis. Additionally, we found that compound #14 can specifically target Rv3290c and inhibit the growth of both M. tuberculosis H37Rv and a MDR M. tuberculosis strain isolated from a clinic even at a low concentration.

Discussion

The identification of new drug targets and discovery of molecular scaffolds have become a priority with the emergence of MDR and XDR strains of M. tuberculosis [27]. Search for new efficient molecular targets to eradicate M. tuberculosis remains an important and challenging task.

Search of new drug targets usually focus on identification of essential genes required for the pathogen's survival. Some recent strategies [7,28] have identified several potential drug targets. However, these current strategies have their own limitations. For example, expensive facilities and expertise for further data analysis are required. In the current study, by integrating TR network analysis pipeline and rational drug design pipeline, we developed a new strategy for efficient discovery of candidate drug targets for combating M. tuberculosis. We confirmed the feasibility and efficiency of this new pipeline and characterized candidate anti-tuberculosis targets and potential bactericides.

HidPs in the TR network are regulated by a large group of TFs. At least two scenarios can be imagined why such high in-degree proteins may be needed by pathogens. Firstly, the protein needs to be expressed in response to various environmental signals and thus needs to be regulated by multiple different response regulators. Secondly, the protein needs to play roles in different periods such as during cell growth and division, and thus needs to be regulated by different regulators at different times. Under this view, higher in-degree translates to more frequent demand for bacterial survival. Therefore, in-degree analysis based on a network may provide an alternative approach for identifying potential essential genes and drug targets. In the present study, we provide evidence to show that targeting HidPs in a TR network is an efficient strategy for exploring potential targets and inhibitors. We showed that four higher in-degree proteins (Rv3290c, Rv2780, Rv2498c and Rv0233), were candidate targets for developing new bactericides. The necessity of some characterized high in-degree proteins for pathogen survival has been implied in previous studies. For example, Rv3290c was found to be substantially up-regulated in nutrient-starved or non-replicating persistence models (29,30), and down-regulated during long-term latency (31). The expression profile of Rv3290c is consistent with that of high in-degree proteins in a TR network. The crystal structures of other higher in-degree proteins characterized from the current TR network, which include Rv1219c (ABC transporter), Rv2011c (hypothetical protein), Rv3682c (bifunctional membrane associated penicillin-binding protein 1a/1b Pona2), and Rv3177 (possible peroxidase), remain unsolved. These proteins may be potential targets for drugs against M. tuberculosis and the resolution of their structures should promote the development of new lead compounds.

M. tuberculosis continues to be a leading cause of mortality worldwide and MDR tuberculosis has become a major public health concern. However, new drug targets with a different antimycobacterial spectrum than current clinical drugs are lacking. In the present study, we selected a relatively small library containing 10,756 compounds for in silico screen and only 15-20 compounds were further selected for each target for further growth inhibition assays. Despite the small number of candidate compounds we worked with, we were able to identify new compounds that inhibited the growth of M. tuberculosis at low MICs ranging from 0.5 to 8 μg/ml. Interestingly, each new target protein corresponded to at least one inhibitor and their inhibitory effects were specific for M. tuberculosis (**Figure 2**). In particular, these compounds also displayed similar activity against clinical isolates of the MDR M. tuberculosis strain. The inhibitor-target interaction was direct and specific, as exemplified by the assay of the interaction between compound #14 and the Rv3290c protein.

In summary, a large group of HidPs were characterized from the newly constructed TR network of M. tuberculosis. We showed the feasibility and efficacy of using HidPs in the TR network as anti-bacterial targets. Although we screened a relatively small compound library and focused on just a handful (five) of potential targets, six new compounds, which together target 4 HidPs, were successfully identified to have inhibitory activity on the growth of both M. tuberculosis H37Rv and a MDR M. tuberculosis strain isolated from a clinic. The HidPs we identified from the TR network of M. tuberculosis can serve as an important resource for further anti-TB drug discovery efforts. Additionally, our work shows that targeting HidPs of a TR network can be an efficient strategy for drug discovery and should be applicable for combating a wide range of pathogens.

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Note: These two authors Tao Cui, Jumei Zeng are equally contributed to this work.

Figures

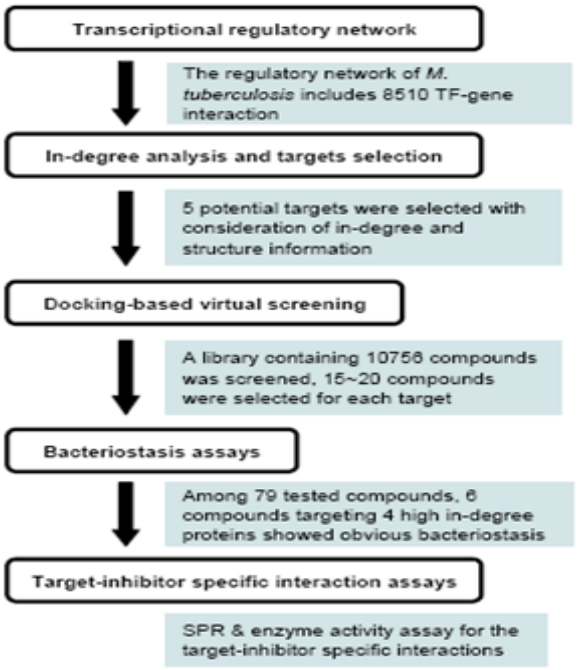


Figure 1: An outline of the strategy used for transcriptional network analysis. A scheme of the strategy developed for potential drug target discovery.

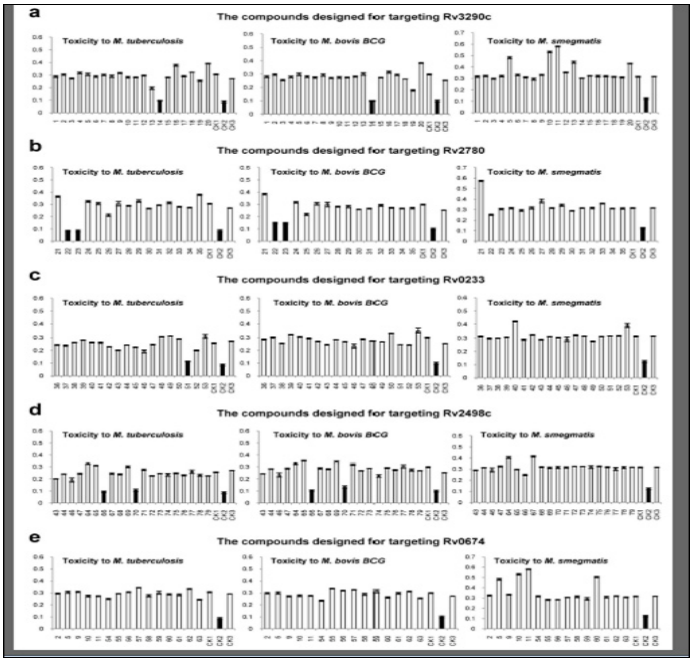
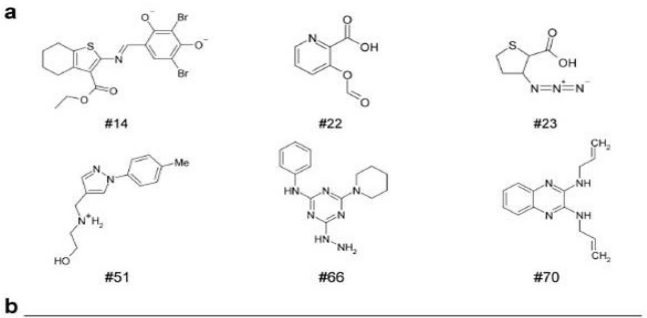


Figure 2: Growth inhibition assays of docking compounds on several mycobacterial species. CK1 represents the control experiment without any compound added. CK2 represents the experiment with rifampicin and CK3 represents the negative control experiment with an unrelated compound (#80). The docking compounds, which were designed to target several higher in-degree target proteins including (a) Rv3290c, (b) Rv2780, (c) Rv0233, (d) Rv2498c and (e) Rv0674, were used for inhibitory assays on the growth of M. tuberculosis H37Rv (left panel), M. bovis BCG (middle panel), and M. smegmatis mc2155 (right panel). After 3~7 days of incubation at 37 °C in a normal atmosphere, the optical densities of mycobacterial growth were measured at OD 600 nm. Each growth analysis was performed in triplicate. The x-axis corresponds to compounds or drugs and the y-axis refers to OD 600. Significant inhibition of mycobacterial growth is highlighted by a black column. Compound #14 is ZINC690246 (C18H17Br2NO4S); #22 is ZINC00335084 (C7H5NO4); #23 is ZINC8383145 (C5H3N3O2S); #66 is ZINC34633360 (C14H19N7); #70 is ZINC13281923 (C14H16N4); #51 is ZINC02025622 (C13H17N3O). For information on other.



b

Inhibitors	#14	#22	#23	#51	#66	#70
Targets	Rv3290c	Rv2780	Rv2780	Rv0233	Rv2498c	Rv2498c
MICs for *M. tuberculosis.* H37Rv (μg/ml)	2	2	0.5	8	4	8
MICs for *M. tuberculosis.* MDR (μg/ml)	2	2	0.5	8	4	8
MICs for *M. bovis.* BCG (μg/ml)	2~4	8	1	>50	2	8
MICs for *M. smegmatis* (μg/ml)	>128	16~32	16~32	>128	16~32	16

Figure 3: Structures of the characterized inhibitors and their minimal inhibitory concentrations (MICs) against several mycobacterial species. (a) Structure of the inhibitors. (b) The MICs of the inhibitors. By definition, MICs are concentrations of inhibitors that inhibit 99% of the growth of different mycobacterial species.

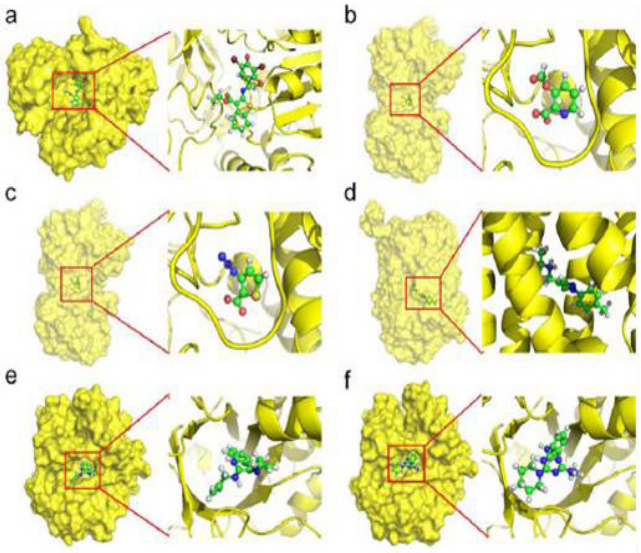


Figure 4: Predicted binding modes of inhibitors to their target proteins. Stereo view of a compound docked on the target protein (left panel, target protein was represented as surface model). Detailed view of a compound docked in the active site of the target is shown as a yellow ribbon model (right panel, target protein was represented as ribbon model). Compounds were represented as stick models. Atoms of C, N, O, Br and S were colored green, blue, red, purple and yellow, respectively. (a) Compound #14 docked in Rv3290c. (b) Compound #22 docked in Rv2780. (c) Compound #23 docked in Rv2780. (d) Compound #66 docked in Rv0233. (e) Compound #70 docked in Rv2498c. (f) Compound #51 docked in Rv2498c.

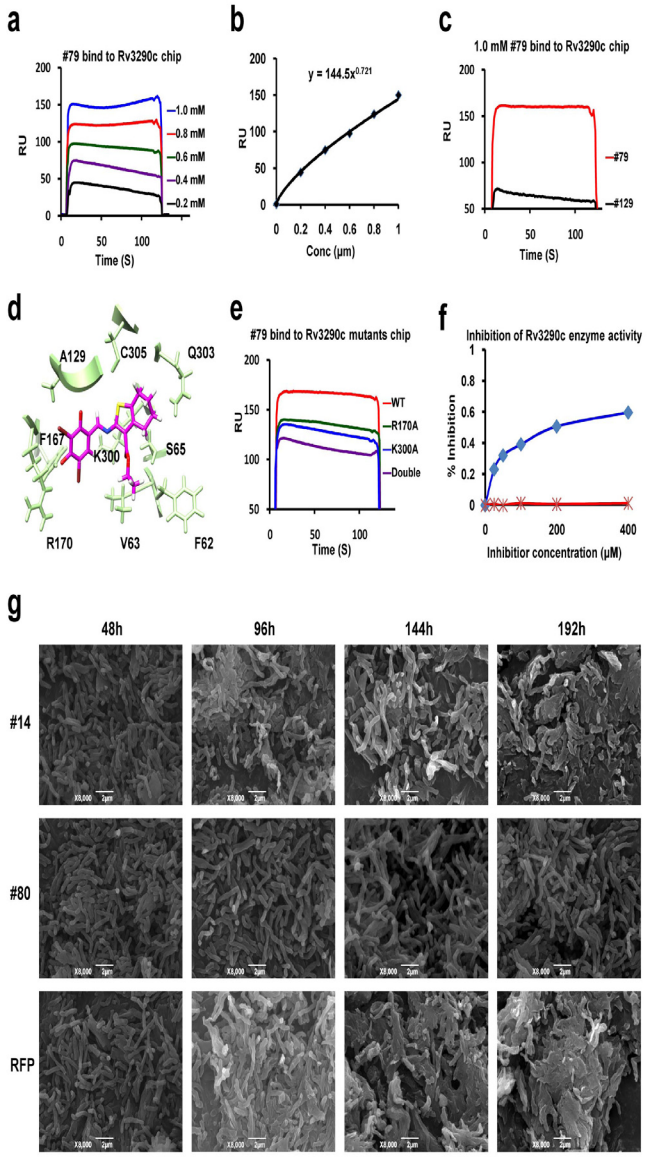


Figure 5: PSpecific interaction between compound #14 and Rv3290c. (a) SPR assay for the interaction of compound #14 with the Rv3290c protein. Different concentration of the compound (0.2 - 1 mM) was passed over the surface of the Rv3290c-associated chip. An overlay plot was produced to show the interactions. (b) Binding isotherm obtained from SPR measurements. The symbols correspond to each of the duplicate measurements. The fitted line was obtained using the equation ($y = 144.5x0.721$, $R^2 = 0.999$). (c) Comparative analysis of the interaction of 1mM compound #14 and unspecific compound #80 (ZINC04099972) with the Rv3290c protein. An overlay plot was produced to show the interactions. (d) Potential Rv3290c residues involved in interaction with compound #14. (e) Comparative analysis of the interaction between wild-type Rv3290c and its mutant variants with compound #14. R170A, K300A, and R170A+K300A double mutant proteins were associated with the NTA chip, and 1 mM of compound #14 was passed over the surface of the chip. The interaction was then examined. (f) An assay for the inhibitory effect of compound #14 on the L-Lysine ε-aminotransferase activity of Rv3290c. The experiment was carried out as described in the "Methods Section". The x-axis corresponds to increasing inhibitor concentration and the y-axis refers to the inhibition percentage, compared to control experiments with no inhibitor. Compound #80 (ZINC04099972) was used as a negative control. (g) Scanning electron microscopy was used to visualize the effect of compound #14 on cell morphology. The images were taken at 8000×magnification. Scale bars, 2 μm.

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