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Biological Evaluation of Potent Triclosan-Derived Inhibitors of the Enoyl-Acyl Carrier Protein Reductase InhA in Drug-sensitive and Drug-resistant Strains of *Mycobacterium tuberculosis*

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Abstract

New triclosan (TRC) analogs were evaluated for their activity against the enoyl-acyl carrier protein reductase InhA in *Mycobacterium tuberculosis* (*Mtb*). TRC is a well-known inhibitor of InhA and specific modifications to its positions 5 and 4' afforded twenty-seven derivatives; of these compounds seven derivatives showed an improved potency in comparison to TRC. These analogs were active against both drug-susceptible and drug-resistant *Mtb* strains. The most active compound in this series, **3**, had an MIC value of 0.6 µg/mL (1.5 µM) against wild-type *Mtb*. At a concentration equal to its MIC, this molecule inhibited the purified InhA enzyme to the extent of 98%, and it showed an IC₅₀ value of 90 nM. Compounds **3** and **14** were able to inhibit the biosynthesis of mycolic acids. Furthermore, mc²4914, an *Mtb* strain overexpressing *inhA*, was resistant to the compounds **3** and **14**, supporting the notion that InhA is the likely molecular target of the TRC derivatives presented herein.

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Keywords

Mycobacterium tuberculosis; enoyl reductase; mycolic acid; triclosan scaffold; molecular docking

Introduction

Current therapies to combat the spread of tuberculosis (TB) are based on drugs developed decades ago (as early as the 1950s), and they require that multiple drugs be taken daily for a minimum of six months duration. In addition, poor management of the available chemotherapy and the nature of evolution have contributed to the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of *Mycobacterium tuberculosis* (*Mtb*), which represent a severe threat to global health. At particularly high risk of acquiring TB are populations living in medically under-served and low-income countries, as well as HIV/AIDS-immunocompromised patients.^[1, 2] The World Health Organization (WHO) has estimated that there was a total of 8.6 million new and 12 million prevalent TB cases in 2012, with around 1.3 million deaths (~ 1 million deaths among HIV-negative individuals and 0.3 million deaths among HIV-positive individuals) associated with this infectious disease.^[3] Although men are the most profoundly affected by TB, the number of TB cases and deaths among women and children is still relatively high.^[3] Thus, to diminish both the health and the socioeconomical burdens caused by TB, the discovery and development of new and effective antitubercular drugs still remains an area of high medical need.

The high lipid content in the *Mtb* cell wall is unique and associated with its virulence. The major constituents of the thick, waxy cell wall are mycolic acids, β -hydroxy fatty acids possessing a long α -alkyl side chain.^[4] One of the key enzymes involved in the biosynthesis of the mycolic acids in *Mtb* is the enoyl-acyl carrier protein (ACP) reductase, InhA, which promotes the elongation of the fatty acid by catalyzing the NADH-dependent stereospecific reduction of 2-*trans*-enoyl-ACP.^[5-7] Inhibition of InhA leads to the death of mycobacterial cells,^[8, 9] therefore validating this enzyme as an excellent target for the discovery of new anti-TB drugs.

The current first-line drug against *Mtb*, isoniazid (INH), was introduced to the chemotherapy repertoire in 1952,^[10, 11] but only recently was its antitubercular action linked with the inhibition of InhA.^[12] The mode of action of this drug is complex^[13] and requires enzymatic activation by the catalase-peroxidase KatG^[14-16] to generate an isonicotinoyl radical, which subsequently forms a covalent INH–NAD adduct^[17] that in turn acts as an effective inhibitor of InhA.^[18]

The second-line antitubercular drug ethionamide (ETH) also targets the InhA enzyme, however, this agent is activated by an alternative enzyme, the flavin monooxygenase (EtaA).^[19, 20] Resistance of *Mtb* to INH and ETH predominantly stems from mutations in their respective activating enzymes KatG and EtaA.^[21-25] Therefore, promising approaches to successfully treat INH- and ETH-resistant *Mtb* strains rely on the development of direct InhA inhibitors, which do not require prior activation by KatG or EtaA. Triclosan (TRC), a known antimicrobial agent widely used in household products,^[26-28] was shown to inhibit

Mtb growth at sub-micromolar concentrations by inactivating InhA.^[29] Subsequent modifications of the TRC scaffold led to the synthesis of a wide range of direct inhibitors of this enzyme.^[30-32] Additionally, molecules belonging to the class of pyrrolidine carboxamides,^[33] isoniazid-NAD adducts,^[34] 2,4-diaryloxazolones,^[35] triazoles,^[36] proline amides (GSK)^[37] and thiazoles (AZ)^[38] have been reported to effectively inhibit InhA. In our efforts to discover direct and effective inhibitors of the bacterial and parasitic enoyl-acyl reductases, we designed and synthesized a broad range of TRC analogs. These analogs were previously evaluated for biological activity against *Bacillus anthracis*^[39] and *Toxoplasma gondii*.^[40, 41]

Herein, we report the testing of 27 compounds from this small library against drug-susceptible and drug-resistant *Mtb* strains and provide some evidence that these compounds target InhA. One compound (**3**) had high antitubercular activity and is a viable starting point for further development of agents effective against *Mtb*.

Results and Discussion

The chemical synthesis and analytical data for all TRC analogs bearing triazole, ketone, amide, isoxazole, or secondary amine substituents at the 5 (ring A) or 4' (ring B) positions (Figure 1) of the diaryl ether scaffold were reported in an earlier manuscript describing the action of these compounds against *T. gondii*.^[41]

All compounds were screened against wild type *Mtb* (strain H37Rv). Compounds with an MIC comparable to or lower than that of TRC (12.5 µg/mL) were then tested against the *Mtb* strain mc²4914 (H37Rv *P_{mabA}inhA* c-15t), and against the recombinant InhA enzyme. The structures, activity, and cytotoxicity data are presented in Table 1.

The 5-cyano analog **1** was as potent as TRC indicating that simple substitution of chlorine by the cyano group had little impact on the activity (Table 1). Introduction of a more bulky aromatic triazole group at the 5-position gave analog **2**, which was 3-fold more active than the reference compound, thus demonstrating that a larger, heteroaromatic group can be accommodated at this position. Following this lead, we introduced an additional *n*-butyl chain at the 4-position of the triazole ring, which provided the most active derivative **3** in this set. The MIC value of **3** was found to be 0.6 µg/mL against wild type *Mtb*, which is approximately 29-fold lower than that of TRC (1.5 µM vs 43 µM). On the other hand, preparation of an analog with a 4-phenyl group on the triazole ring gave a completely inactive compound **4**, indicating possible limits in the size of the binding pocket and that a flexible aliphatic chain on the triazole group is preferred for activity.

Between the propan-1-one and (phenyl)methanone derivatives **5** and **6**, respectively, only the former showed very modest activity against the H37Rv strain of *Mtb* (Table 1). Next, we examined phenyl, heterocycloalkyl, and *n*-alkyl substitutions attached at position 5 via an amide bond (**7-10**). Disappointingly, none of those derivatives were active, which strongly suggests that the rigid, polar amide bond might result in a steric clash with the enzyme's binding pocket. This notion is supported in part by analogs **11-13**, which possess a rigid isoxazole ring attached to ring A, all of which were again inactive. On the other hand,

introduction of a benzylamide linker between ring A and the 5-methylisoxazole group provided compound **14**, which showed over 3-fold increase in potency (5 µg/mL, 13 µM) against wild type *Mtb* when compared to TRC. This result clearly suggests that a flexible linker between an aromatic substituent and the 5 position of the TRC phenolic ring (ring A) is needed for good antitubercular activity. A similar observation based solely on the inhibition of InhA by 5-substituted triclosan analogs was previously reported.^[32] This finding is also supported by the improved activity found for the triazole analogs **2** and **3**, which both possess a methylene linker between the triazole group and the TRC scaffold. Introduction of a (phenylamino)methyl substituent afforded analog **15** that is 3-fold more potent than the lead compound. This compound was slightly more active than its counterpart bearing the *N*-cyclohexyl substituent (**16**), and nearly two times more active than the analog modified with the (hexylamino)methyl group (**17**). For compounds bearing modifications at the 4' position of the diaryl ether skeleton, the results were largely unremarkable. Among all of the 4'-modified compounds (**18-28**), only the compound with a (hexylamino)methyl group (**18**) showed improved potency relative to TRC, whereas the analog bearing a (phenylamino)methyl substituent (**19**) displayed only weak activity against *Mtb*. Disappointingly, all the remaining analogs bearing triazole (**20-22**), isoxazole (**23, 24**), and amide (**24-28**) substituents turned out to be inactive.

All triclosan analogs exhibiting an *Mtb* MIC equivalent or better to that of triclosan were assayed^[44] for their percent inhibition of purified InhA at their respective MIC concentration (Table 1). Five out of the eight analogs exhibited 97% enzyme inhibition at their MIC. These results are consistent with the antibacterial efficacy deriving largely from the inhibition of InhA. Compounds **17**, **16**, and **18** displayed 73%, 39% and 38% enzyme inhibition, respectively, at their MIC, which would suggest that modulation of other targets may be responsible for the observed whole-cell activity. Compound **3** demonstrated the most potent antitubercular efficacy with an MIC of 0.6 µg/mL (1.5 µM) and an InhA IC₅₀ of 90 nM (Figure 1, Supplementary Materials). The results for this new analog demonstrate significant gains in whole-cell activity and InhA inhibition over those found for triclosan (MIC = 43 µM; IC₅₀ = 1400 nM; Figure 2, Supplementary Materials).

Subsequently we examined the ability of the most active compounds to inhibit the growth of selected monodrug-resistant and MDR strains of *Mtb*. We tested compounds **1-3** and **14-18** against *Mtb* strains that were resistant to first-line and second-line TB drugs such as INH, rifampin ofloxacin, ETH, streptomycin, or ethambutol (Table 2). These TRC analogs were as active against INH-resistant (mc²4977) and MDR (mc²5855, CII2081) *Mtb* strains as against drug-susceptible *Mtb* (Table 2). Interestingly, while analogs **1-3** and **14-15** showed the same levels of activity against the INH-resistant *Mtb* strains where the resistance was solely due to *katG* mutations, the analogs were less active against a KwaZulu-Natal (South Africa) isolate (V2475), which is resistant to INH due to mutations in *katG* (S315T) and in the promoter region of *inhA* (t-8a)^[43] (Table 2), suggesting that these analogs do target InhA. This trend, however, was not observed for compounds **16-18**, which again may indicate an off-target activity of these molecules. Furthermore, the high activity of compound **3** against drug-susceptible and drug-resistant *Mtb* strains highlights this analog as one of the most potent TRC derivatives inhibiting InhA enzyme reported to date. Such

information provides additional support to advance derivative **3** through further rounds of lead optimization.

To further investigate the mode of action of the synthesized compounds, analogs with the best potency against wild type *Mtb* were selected for evaluation versus mc²4914,^[9] a strain of *Mtb* with a mutation in the promoter region of *inhA* ($P_{mabAinhA}$ c-15t) (Table 1). The *inhA* mRNA level in mc²4914 is about 20 times higher than in wild-type *Mtb*, resulting in an increased InhA protein level, which leads to increased resistance to drugs targeting InhA.^[9] Curiously, the level of resistance to one subset of the TRC analogs (compounds **1**, **2**, **15**, **16**, **17** and **18**) was relatively low (1-5 fold), while the MIC for compound **3** was 130-fold higher for the mc²4914 strain, again pointing to InhA as the likely target of this compound. Surprisingly, the MIC of TRC against mc²4914 also did not vary much from the wild-type (20 µg/mL for mc²4914 vs. 12.5 µg/mL against wild-type *Mtb*), suggesting that the lethal effects of TRC also stem from disrupting other mycobacterial targets.^[45, 46]

On the other hand, compounds **3** and **14** showed a larger increase in their MIC values when tested against the mc²4914 strain (133- and 16-fold, respectively) (Table 1). This result strongly suggests that the main target of these two analogs is InhA. Interestingly, the resistance pattern of the TRC analogs observed in the mc²4914 strain matches that obtained using the MDR clinical isolate V2475 (Table 2), which has a different mutation in the promoter region of *inhA* ($P_{mabAinhA}$ t -8a).

To provide additional evidence that the TRC analogs target InhA, mycolic acid biosynthesis was analyzed in *Mtb* cultures treated with the TRC derivatives at 5 to 16 times the MIC concentration and then labeled with ¹⁴C-acetate. Mycolic acids were extracted from treated *Mtb* cultures, esterified to form mycolic acid methyl esters (MAMEs), and analyzed by thin layer chromatography (Figure 2). Mycolic acid biosynthesis was inhibited by all the analogs tested. In addition to the inhibition of the FAS II system, compounds **16** and **18** also inhibited the FAS I system as seen by the total inhibition of the biosynthesis of fatty acids (FAMES). The experiment was repeated for compounds **16**, **18**, and the reference compound TRC (at 5 and 10 times the MIC) and again the inhibition of both FAS II and FAS I systems was observed. It is interesting to note that some of the TRC analogs appear to be weaker FAS I inhibitors than the parent compound. At 10 times the MIC, inhibition of mycolic acids and therefore FAS II was total in *Mtb* treated with compounds **2**, **3**, **14** and **15**, but fatty acid biosynthesis was still occurring. This suggests that these compounds are more selective inhibitors of InhA than TRC. Fatty acid and mycolic acid biosynthesis inhibition was also studied in mc²4914 treated with the most active compounds **3** and **14** (Figure 2). The same concentrations of inhibitors **3** and **14** that resulted in the total inhibition of mycolic acid in wild-type *Mtb* were only partially able to inhibit mycolic acid biosynthesis in the mc²4914 strain (Figure 2). Moreover, the apparent accumulation of FAS I products (FAMES) in these samples was further analyzed by HPLC, which revealed an increase in the FAS I end product (C26:0 fatty acid) (Figure 3). The accumulation of the FAS I end product has been shown to be directly correlated to the inhibition of InhA.^[8]

To further test the efficacy of the best TRC analog against *Mtb*, a culture of *Mtb* was treated with TRC or its derivative **3** at a concentration equivalent to 5 times its MIC (206 µM or 7.5

μM , respectively) (Figure 4). Viability of the *Mtb* culture was assessed by taking samples at different time points and plating for colony-forming-units. At a concentration 27 times lower than TRC, compound **3** achieved the same killing activity of *Mtb* as the reference compound, demonstrating again that **3** represents a good scaffold for further drug development.

Encouraged by the promising *in vitro* results of compound **3**, we decided to evaluate its *in vivo* efficacy in serum inhibition titration assay.^[47] Dissapointingly, this compound (**3**) turned out to be only weakly active at both 100 and 300 mg/kg dose (see Supplementary Materials Figures 3 and 4, respectively). These results suggest that derivative **3** has very low oral bioavailability, which most likely is associated with its poor water solubility and fast Phase II metabolism. The TRC analogs were also tested for activity against Vero cells to assess their toxicity against eukaryotic cells.^[48] All of the tested compounds were found to have single-digit $\mu\text{g/mL}$ Vero cell IC_{50} values (Table 1), which consequently results in low (<10) SI indices. Improvements in the SI values will be needed in order to advance these compounds.

To better understand the activity data, we modeled the most active analogs **3** and **14** into the active site of the InhA enzyme. In lieu of X-ray crystal structures, we offer the docking calculations provide an understanding of reasonable inhibitor binding modes to InhA, given the assumptions made by the software. Since InhA is a flexible enzyme which contains a substrate-binding loop that samples multiple distinct conformations when it forms a lid on the active site,^[49] several conformations of InhA from multiple chains of four different ligand-bound crystal structures of InhA were utilized as targets for these docking studies. 2X23^[50] and 3FNH^[32] are both bound to triclosan derivatives, while 4COD^[37] and 4BQP^[38] are new crystal structures of InhA bound to structurally distinct lead compounds from GlaxoSmithKline and AstraZeneca, respectively. To help validate the modeling protocols, positive control docking calculations were performed. The positive control re-docking experiments were successful (see Supplementary Materials Figure 5): when compound **25** (from 3FNH) was docked to the conformation of InhA in 3FNH, it produced an RMSD = 0.80 Å. Similarly, when the GSK inhibitor (from 4COD) was docked to the conformation of InhA from 4COD (chain F), it afforded an RMSD = 0.18 Å. When the triclosan derivative PT70 was re-docked against 2X23, it produced an RMSD = 0.49 Å (data not shown). The more challenging cross-docking experiment with the triclosan derivative PT70 (from 2X23) was also successful (see Figure 5.A): when PT70 was docked against 3FNH, it afforded a binding mode that overlapped with the conformation of PT70 obtained by superimposing 2X23 onto 3FNH (using the alpha carbons).

Docking compounds **3** and **14** versus these ten models of InhA produced three different predicted binding modes. The docking results were analyzed according to the estimated free energy of binding value (FEB) calculated by AD Vina^[51] and, most importantly, by visual inspection: a predicted binding mode passed visual inspection if the docked mode displayed some similarity to the previous crystallographic binding modes of known InhA inhibitors, and if the conserved region of compounds **3** and **14** docked consistently against a given target, that mode was given extra weight. Mode 1 displayed good agreement with the crystallographic binding modes of known triclosan derivatives (see Figure 5.B and

Supplementary Materials Figure 6.A). However, mode 1 was only observed for compound **3** when docked against 3FNH (the FEB scores and details regarding all of the InhA target models that produced each particular mode may be found in Supplementary Materials Table 1). Mode 1 of compound **3** displayed a hydrogen bond and favorable electrostatic interactions with the 2'-hydroxyl of the ribose adjacent to the nicotinamide ring of the NAD cofactor, favorable electrostatic interactions with the backbone nitrogen of Met98, and the alkyl tail docked into the hydrophobic pocket into which the *n*-hexyl moiety of PT70 packs. Favorable electrostatic interactions can occur when a group with a full or partial positive charge (such as a hydrogen bond donor) is near a group with a full or partial negative charge (such as a hydrogen bond acceptor), but those groups are too far away (*i.e.*, greater than 3.4 to 3.7 Å, depending on the atom types) to form a hydrogen bond or salt bridge; the strength of these interactions are proportional to the magnitude of the two partial charges and are inversely proportional to the interatomic distance and the dielectric constant of the local environment. However, the nature of the spherical hydrogen bond potential in AD Vina^[51] and the presence of the amino group of the nicotinamide moiety of the cofactor could have caused the triazole ring to dock incorrectly (based on previous crystallographic analyses and currently unpublished docking studies of other novel InhA inhibitors; see Supplementary Materials Figure 6 for additional details).

It appears likely that the triazole ring could flip to enable the nitrogen atoms to hydrogen bond with Tyr158 and the 2'-hydroxyl of the ribose adjacent to the nicotinamide ring of the cofactor, in addition to forming base-stacking interactions with NAD.

In mode 2, which occurred in docking studies against 4COD chain F (and 4COD chain B), the predicted binding modes for the region conserved between compounds **3** and **14** (*i.e.*, the dichlorophenyl, the ether linker, and the phenol group) superimposed (see Supplementary Materials Figure 6.B). For mode 2 of compound **3**, hydrogen bonds were formed with a phosphate group of NAD and the backbone carbonyl of Gly96, while favorable electrostatic interactions occurred with the side chains of Arg43 and Ser200, the backbone nitrogen groups of Ala201, Ile202, the 2' hydroxyl of the ribose adjacent to the nicotinamide moiety of NAD, and a phosphate group of NAD. Mode 2 of compound **14** (see Figure 5.C and Supplementary Materials Figure 6.B) included a hydrogen bond with the backbone carbonyl of Ala198 and favorable electrostatics interactions with the side chain of Gln100, but it lacked the aforementioned interactions with Ser200 and Ile202 that were formed by compound **3**. In mode 3 of compounds **3** and **14**, which was predicted with 4COD chain D and the four chains targeted from 4BQP (see Supplementary Materials Figure 6.C, 6.D, and Table 1), some agreement existed between the predicted binding modes for the conserved regions of these two compounds, but these regions docked much more consistently in mode 2 (against chain F). In mode 3 of compound **3**, the alkyl tail docked where the alkyl tail of the GSK inhibitor crystallized in 4COD, favorable electrostatic interactions were formed with the side chain of Arg43, the backbone nitrogen of Met98, and the backbone carbonyl of Leu197, and cation- π interactions may occur with Arg43. For mode 3 of compound **14**, hydrogen bonds were formed with the carbonyl of Leu197, the backbone nitrogen of Ala198, and the 2' hydroxyl of the ribose adjacent to the nicotinamide moiety of NAD, but it lacked the favorable interactions with Met98. Although the three predicted binding modes

were assigned a 1, 2, and 3 ranking, no particular mode was obviously significantly superior; all three modes are almost similarly plausible. Mode 1 might be slightly more probable than mode 2 (based on previous crystal structures with triclosan and its derivatives), with mode 3 being slightly less likely than mode 2 (based on how consistently the conserved regions of these compounds docked).

Conclusion

In this report we present a small series of TRC analogs bearing modifications at either positions 5 or 4' of the diaryl ether scaffold. These compounds were evaluated against wild-type *Mtb*, and seven derivatives were found to be more active than the reference compound. Moreover, the best analog in this series, compound **3**, was approximately 29-fold more potent than TRC in the whole-cell anti-*Mtb* assay. Its IC₅₀ against the purified InhA was 90 nM, and at its MIC this compound inhibited InhA to the extent of 98%. Evaluation of the TRC analogs for inhibition of mycolic acid biosynthesis and for resistance to an *Mtb* strain overexpressing *inhA* strongly suggests that the most active analogs in this series (**3** and **14**) target the InhA enzyme. Additionally, the small increase in MIC values of compounds **1**, **2**, and **15-18** against mc²4914, as well as the inhibition of the FAS I pathway by compounds **16** and **18**, suggest that these molecules most likely target additional enzymes in *Mtb*. Molecular modeling of the newly prepared TRC analogs within the active site of InhA provides additional insights into the structural requirements relevant to achieving improved activity. Overall, these studies provide at least two candidates for further research aimed at developing new and effective inhibitors, which can directly target the mycobacterial InhA enzyme. However, as noted above further improvements in the SI need to be addressed.

Experimental Section

Evaluation of the biological activity

Bacterial strains and media—All the *Mtb* strains used in this study were obtained from laboratory stocks. The strains were grown in Middlebrook 7H9 medium (Difco) supplemented with 10% (v/v) OADC enrichment (Difco), 0.2% (v/v) glycerol, and 0.05% (v/v) tyloxapol. The solid medium used was Middlebrook 7H10 medium (Difco) supplemented with 10% (v/v) OADC and 0.2% (v/v) glycerol.

Minimum inhibitory concentration (MIC) determination—The MTT assay^[52] was used to determine the MIC for each compound. Briefly, the MIC assay was performed in a 96-well plate in duplicate. The outside wells were filled with 0.2 mL of PBS. Each row of the inside wells contain 2-fold dilution of a compound to test (0.1 mL). The last well of each row received no compound. The strains were diluted to 10⁵ CFU/mL, and 0.1 mL was used to inoculate each inside well of the 96-well plate. The plates were incubated at 37 °C for 1 week prior to the addition of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 5 mg/mL in PBS). The plates were incubated for 24 h before the addition of a solution of 20% aqueous sodium dodecyl sulfate/50% aqueous dimethyl formamide (1/1; 0.05 mL). The plates were incubated for an additional 24 h. The MIC was determined visually as the lowest concentration of compound that prevented conversion of MTT (yellow solution) to formazan (blue/violet solution).

Analysis of fatty acid methyl esters (FAMES) and mycolic acid methyl esters (MAMES) by thin layer chromatography (TLC)—*Mtb* H37Rv cultures (10 mL) grown to early log-phase ($OD_{600\text{ nm}} = 0.2$) were treated with selected triclosan derivatives for 20 h, and then labeled with [$1\text{-}^{14}\text{C}$]-acetate for 6 h. The cultures were centrifuged and the cell pellets were saponified with a 20% solution of tetrabutylammonium hydroxide (2 mL) at 100°C for 16 h. After cooling, the suspensions were methylated with methyl iodide (0.1 mL) in methylene chloride (2 mL) for 1 h at 20°C . The organic phases were washed, dried and resuspended in methylene chloride (0.2 mL). 5 μL of each extract was spotted onto a silica gel 60 F254 TLC plate and eluted three times with hexane/ethyl acetate (95/5, v/v). FAMES and MAMES were detected by autoradiography after exposure at -80°C for 48 h.

Analysis of fatty acids by high performance liquid chromatography (HPLC)—The extracts used for the TLC were saponified with 10% potassium hydroxide in methanol for 2 h at 100°C , acidified, and extracted with chloroform. The organic phases were then esterified to UV-absorbing *p*-bromophenacyl esters, and analyzed on an HPLC coupled to a flowthrough radioisotope betagamma radiation detector as described previously.^[8]

Cloning, expression and purification of *Mycobacterium tuberculosis* InhA—*M. tuberculosis inhA* was cloned into *E. coli* BL21 as described previously.^[6] After an overnight induction at 25°C , the cells were harvested by centrifugation, resuspended in 40 mL of His-bind buffer, and lysed using sonication. Cell debris was removed by centrifugation (40,000 rpm for 60 min) and the supernatant applied to a His-bind resin column (1 mL bed volume). The His-bind column was washed successively with His-bind buffer and His-wash buffer, and the protein was eluted using 0.5 M imidazole in 20 mM Tris-HCl and 500 mM NaCl (pH 7.9). Fractions containing His-tagged InhA were immediately pooled and applied to a 2 cm 50 cm Sephadex G-25 column equilibrated with 20 mM Tris-HCl, 150 mM NaCl, and 2.5 mM CaCl_2 (pH 8.4). Fractions containing the His-tagged protein from the G-25 column were pooled, and the His tag was removed using biotinylated thrombin (1 unit/mg of protein). Subsequently, the thrombin was removed using streptavidin-agarose (20 μL /unit of thrombin) and the protein exchanged into 30 mM PIPES, 150 mM NaCl, and 1 mM EDTA at pH 6.8. The concentration of the protein was calculated from the UV absorption at 280 nm using an absorption coefficient of $37.3\text{ mM}^{-1}\text{ cm}^{-1}$.

Determination of InhA percent inhibition and IC_{50} —Enzyme kinetics, following a previously reported protocol,^[44] were determined in 150 mM NaCl and PIPES buffer at pH 6.8 with a Shimadzu UV-2550 UV-visible spectrophotometer. NADH (20 μM), InhA (20 nM) and inhibitor with different concentrations dissolved in DMSO were mixed and incubated for 5 minutes, and followed post-addition of *trans*-2-dodecenoyl-CoA (synthesized from *trans*-2-dodecenoic acid, using the mixed anhydride method as described by Parikh^[53] to initiate reaction). Assays were monitored at 25°C , following the transition from NADH to NAD^+ at 340 nm. For the determination of a molecule's percent inhibition of InhA catalytic activity, the inhibitor concentration was chosen to be its MIC versus *M. tuberculosis*. For IC_{50} determination, inhibitor concentrations were varied from 10 nM to 1000 nM as described earlier.^[32] Equation 1 was utilized to determine the NAD^+ production

curve where N_{NAD^+} was the NAD^+ concentration, A_0 and A_t were the 340 nm absorbances at time t and time 0, and C_{NADH} was the initial NADH concentration.

$$N_{\text{NAD}^+} = \frac{A_0 - A_t}{A_0} \times C_{\text{NADH}} \quad [\text{Eqn. 1}]$$

The InhA percent inhibition and/or IC_{50} for each inhibitor were calculated with Prism Graphpad version 6.0, and reported as the mean $\pm$ standard deviation.

Cytotoxicity assay—The assay was performed on Vero cells as described previously.^[48]

Serum inhibition titration assay—This assay was conducted by following the reported procedure,^[47] with BALB/c mice which were single-dosed with compound **3** at 100 and 300 mg/kg by oral gavage. Isoniazid (10 mg/kg) was used as a positive control.

Molecular docking methods

Models of compounds **3** and **14** were prepared using Avogadro.^[54] They were minimized in Avogadro using the MMFF94s force field, first by steepest descent, followed by the conjugate gradient method. The X-ray crystal structures of the targets were obtained from the RCSB Protein Data Bank^[55], PDB codes: 2X23^[50], 3FNH^[32], 4COD^[37], and 4BQP.^[38] Hydrogen atoms were added to the models of the targets using the MolProbity server.^[56] For the docking studies, four chains of 4BQP (A, B, C, and D) and four chains of 4COD (B, D, F, and H) were each utilized as separate, independent targets. The docking studies of these flexible ligand models against these 10 rigid targets were performed using AutoDock Vina (AD Vina).^[51] The center of the “grid box” was located $\sim 3 \text{ \AA}$ above the center of the amino group of the nicotinamide moiety, the carbonyl of the nicotinamide moiety, and the adjacent edge of the nicotinamide ring of the NAD cofactor. For the 2X23 and 3FNH targets, the grid box had a size of $24.0 \times 18.0 \times 20.0 \text{ \AA}^3$. For the 4COD and 4BQP targets, the grid size was increased to $24.0 \times 22.0 \times 22.0 \text{ \AA}^3$, to accommodate the larger size of their active site pocket. Due to the large size of these grid boxes, and to ensure sufficient time for these docking calculations to converge, the “exhaustiveness” setting in AD Vina was increased to 20 (from the default value of 8). Eight CPUs were used for each docking calculation on the Rutgers Univ-NJMS Linux cluster. All other run parameters in AD Vina remained at their default settings. The conformation, position, and orientation of the positive control ligands were randomized using the “randomize_only” function in AD Vina prior to docking them against these targets (to ensure that no numerical bias was caused by the models used as inputs). In the docking studies of compound **14**, three different models of this ligand were used in which its peptide bond was either trans, cis, or freely rotatable. The “pdbqt” docking inputs for the models of the ligands and targets were prepared using AutoDockTools4.2, all results were analyzed with the Python Molecular Viewer (PMV), and the molecular images were created with PMV 1.5.6.^[57] Only the top-ranked AD Vina mode per calculation for each ligand:target pair was visually inspected.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

TRC scaffold with indicated sites (R^1 , R^2) of structural modification.

Figure 2.
Inhibition of mycolic acid biosynthesis by compounds 2, 3, 14, 15, 16 and 18 in *Mtb* strain H37Rv and mc²4914.

Figure 3. Effect of compound **3** and **14** (10 and 50 mg/L, respectively) on saturated fatty acids (FA) distribution in *Mtb* strain mc²4914. The percentage of each saturated FA (from 14 to 26 carbons) was determined based on HPLC signal integration.

Figure 4.
Growth of *Mtb* H37Rv treated with TRC (206 μ M) or compound **3** (7.5 μ M) for 14 days.

Figure 5.

Results of the docking studies. (A) Positive control cross-docking study of PT70 (from 2X23) against 3FNH, with the docked result having cyan carbon atoms and the mode superimposed from 2X23 with purple carbon atoms. In all 3 panels the NAD cofactor is displayed as CPK. (B) “Mode 1” of compound **3** (with green carbons) was produced against 3FNH and sampled a conformation similar to previous triclosan derivatives (*e.g.*, PT70 from 2X23 is shown as lines with purple carbons). The key residues Met98 and Tyr158 are shown as thin sticks. (C) “Mode 2” of compound **14** (and compound **3**, see the Supplementary

Materials) was predicted against 4COD chain F. Compound **14** is shown as thick sticks with dark green carbons, while the crystallographic conformation of the GSK inhibitor is displayed as thin lines with purple carbons. The key residues Arg43, Gly96, Gln100, Tyr158, and Ala198 are shown as thin sticks with lavender carbons.

Table 1

Structures and activity data for the new TRC-based inhibitors of InhA.

Compound	Structure	MIC [$\mu\text{g/mL}$] <i>Mtb</i> Strain H37Rv	InhA Inhibition ^{[b],[c]} [%]	IC ₅₀ Cytotoxicity ^[d] [$\mu\text{g/mL}$]	SI ^[e]
TRC		12.5 (43 μM) ^[f]	96 $\pm$ 0.65	<12.5 ^[g]	<1
1		12.5 (45 μM)	97 $\pm$ 0.06	4	0.3
2		5 (15 μM)	97 $\pm$ 0.14	1	0.2
3		0.6 (1.5 μM)	98 $\pm$ 0.22	1-2	1.7 – 3.3
4		> 100	nd	nd ^[h]	
5		50-100	nd	nd	
6		100	nd	nd	
7		> 100	nd	nd	

Compound	Structure	MIC [$\mu\text{g/mL}$] <i>Mtb</i> Strain H37Rv	mc ² 4914[a]	InhA Inhibition ^[b] IC ₅₀ [%]	IC ₅₀ Cytotoxicity ^[d] [$\mu\text{g/mL}$]	SI ^[e]
8		> 100	nd	nd	nd	
9		> 100	nd	nd	nd	
10		> 100	nd	nd	nd	
11		> 100	nd	nd	nd	
12		> 100	nd	nd	nd	
13		100	100	nd	nd	
14		5 (13 μM)	> 80	99 $\pm$ 0.19	8	1.6
15		5 (14 μM)	25	99 $\pm$ 0.28	8	1.6
16		6.25 (17 μM)	12.5	39 $\pm$ 0.67	4	0.6

Compound	Structure	MIC [$\mu\text{g/mL}$] <i>Mtb</i> Strain H37Rv	mc ² 4914[a]	InhA Inhibition ^[b] IC ₅₀ [%]	IC ₅₀ Cytotoxicity ^[d] [$\mu\text{g/mL}$]	SI ^[e]
17		10 (27 μM)	12.5	73 $\pm$ 0.40	4	0.4
18		5 (14 μM)	10	38 $\pm$ 0.15	8	1.6
19		50	100	nd	nd	
20		100	100	nd	nd	
21		> 100	nd	nd	nd	
22		> 100	nd	nd	nd	
23		> 100	nd	nd	nd	
24		> 100	nd	nd	nd	
25		> 100	nd	nd	nd	
26		> 100	nd	nd	nd	

Compound	Structure	MIC [$\mu\text{g/mL}$] H37Rv	MtbStrain mc ² 4914[a]	InhA Inhibition ^[b] [%]	IC ₅₀ Cytotoxicity ^[d] [$\mu\text{g/mL}$]	SI ^[e]
27		100	100	nd	nd	

[a] An *Mtb* strain overexpressing *inhA*.

[b] % Inhibition of recombinant InhA enzyme at the respective compound's MIC.

[c] Standard deviation for % inhibition of InhA.

[d] Cytotoxicity against Vero cells.

[e] SI = selectivity index calculated as IC₅₀ (Vero cells) / MIC.

[f] Concentration expressed in [μM].

[g] Reported LC₅₀ of TRC against Vero cells.[42]

[h] nd = not determined.

Table 2

Activity data for new TRC analogs against monodrug-resistant and MDR Mtb strains.

Compound	Strain			
	mc ² 4977 ^[a]	mc ² 5855 ^[b]	V2475 ^[c]	CH2081 ^[d]
	MIC [μ g/mL]			
TRC	25	25	25	12.5
1	12.5	12.5	50	12.5
2	5	5	40	5
3	0.6	0.3	> 4.8	0.3
14	5	5	40	5
15	5	10	40	5
16	6.25	6.25	12.5	6.25
17	5	5	5	5
18	10	5	5	5

^[a] Drug resistance: INH. Strain background: H37Rv. Genotype: *katG* del 371g.

^[b] Drug resistance: INH, RIF (rifampin), OF (ofloxacin). Strain background: H37Rv. Genotype: *katG* del, *rpoB* H451W, *gyrA* A90V.

^[c] Drug resistance: INH, RIF. Strain background: KZN (KwaZulu-Natal, South Africa). Genotype: Reference^[43].

^[d] Drug resistance: INH, RIF, SM (streptomycin), EMB (ethambutol), ETH. Strain background: Clinical isolate from Mexico. Genotype: not determined.