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Synthesis and anti-tubercular activity of conformationally-constrained and bisquinoline analogs of TMC207

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ABSTRACT

One of the most significant breakthroughs in the battle against tuberculosis is the recent approval of the quinoline compound, TMC207, for the treatment of drug-resistant tuberculosis. To gain insight into the molecular determinants of the activity of TMC207 and to evaluate the scope of quinoline compounds as antitubercular agents, we synthesized a series of TMC207 derivatives and evaluated their anti-tubercular activity. Making the lateral chain of the drug rigid by linking it to an adjacent phenyl substituent resulted in a decrease in activity. In contrast, replacing a phenyl substituent of TMC207 with a quinoline moiety gave bisquinolines that demonstrated potent antitubercular activity in *in vitro* experiments, in *ex vivo* mouse bone marrow macrophage assays, and also in *in vivo* mouse model of the disease. These results provide new guiding principles for modifying the TMC207 scaffold for developing efficacious anti-tubercular drugs and set the stage for the development of bisquinolines as a promising new class of antitubercular agents.

INTRODUCTION

Tuberculosis (TB) is one of the biggest threats facing human health today.[1-3] Indeed, in 2013 alone, there were more than 1.5 million deaths reported due to TB, a significant portion of which (210,000 deaths) were caused due to multi-drug resistant tuberculosis (MDR-TB).[1] In light of this extremely gloomy scenario, there is an urgent need for new anti-TB drugs that have novel mechanisms of action. One such drug, TMC207 (Figure 1a), was approved by the U.S. Food and Drug Administration in 2012 for the therapy of drug-resistant tuberculosis.[4-6] Since TMC207 functions via a unique mechanism different from those employed by other antitubercular drugs, it is effective on drug-resistant bacterial strains including those resistant to rifampicin, isoniazid, ethambutol, pyrazinamide and fluoroquinone.[7]

TMC207 acts by inhibiting the function of the mycobacterial membrane bound enzyme, ATP synthase.[7-8] The drug is believed to bind to the interface of the "a" and "c" subunits of the F_0 domain of the enzyme, which lie within the membrane at a site of the protein that plays a critical role in the proton-shuttling process which drives ATP synthesis. Docking studies based on homology models of the *M. tuberculosis* ATP synthase and structure-activity relationship (SAR) studies suggest that the tertiary OH group and the protonated tertiary amino moiety of the drug are important for binding and make hydrogen bonding interactions with Glu-61 of the c subunit, a residue that is believed to carry the protons as they are translocated through the protein.[9-12] The aromatic substituents of the drug are believed to participate in both Vander Waals' and stacking interactions with aromatic side chains of the protein.

The success of TMC207 has demonstrated the potential of quinoline compounds for anti-TB therapy. This potential was further exemplified by recent reports that demonstrate that introducing radical structural changes to the TMC207 scaffold while retaining its quinoline moiety results in potent anti-tubercular compounds. For example, replacement of the naphthyl substituent of TMC207 by an imidazole group, introduction of conformational constraints by locking the phenyl moiety with the quinoline group, and replacing the hydroxyl and amino substituents with an oxime group gave highly active antitubercular compounds.[9,13-16] These results strongly suggest that there is significant scope for further development of quinoline derivatives as anti-TB agents.

We sought to explore the scope of quinoline derivatives as anti-tubercular compounds by synthesizing TMC207 derivatives containing novel structural features and characterizing the ATP synthase-inhibition and anti-tubercular activity of these compounds. We introduced three previously unexplored structural features into the TMC207 scaffold. In one series of compounds, we locked the lateral side chain of TMC207 to the adjacent aromatic substituent (Figure 1b) to introduce conformational constraint. In a second series of compounds, we replaced the phenyl substituent of TMC207 with a quinoline group to generate bisquinoline compounds (Figure 1c). Encouraged by the potent antitubercular activity of these bisquinolines, we synthesized and tested another

bisquinoline derivative in which the two quinoline rings were linked to each other to form a macrocycle (Figure 1d).

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The syntheses of conformationally constrained analogs of TMC207 (**I** in Scheme 1a) were accomplished by treating Mannich bases (**III**) of cyclic ketones with the benzylquinoline anion of **II** which was prepared according to previously published methods.[17] Specifically, freshly prepared Mannich bases (**2a-i**) were treated with anion **4** to give a diastereomeric mixture of two conformationally constrained diarylquinolines **5a-i** containing 5-, 6-, or 7-membered rings (Scheme 1b). Conformations of these compounds were determined by *X*-ray structures of both the minor and the major diastereomers of **5f** which contains a 6-membered ring, and the minor diastereomer of **5c** which contains a 7-membered ring (see Supporting Information section for the structures). For the assignment of the major diastereomer of the 7-membered ring-containing compound, we obtained an *X*-ray structure of the major isomer of an analogous quinoline derivative, compound **S1**, where bromo in the quinoline ring was replaced with the chloro group (Supporting Information). The conformations of the 5-membered ring-containing compounds (**5g-i**) were assigned on the basis of chemical shift patterns of major and minor isomers of 6- and 7-membered ring-containing analogs.

The syntheses of bisquinoline analogs of TMC207 (**IV** in Scheme 2a) were accomplished by treating appropriate Mannich bases with the anions of dialkoxybisquinolines (**V**) which were generated by treating the dichlorobisquinoline reagent (**10**) with various alkoxides. The procedure for the synthesis of **10** (Scheme 2) was inspired by a previous report that utilized Baylis-Hillman adducts to synthesize quinoline derivatives.[18] First, Baylis-Hillman adduct **7** was prepared by treating commercially available quinoline carboxaldehyde **6** with ethyl acrylate in presence of the tertiary amine, DABCO. Compound **7** was then acetylated to give **8** followed by nucleophilic substitution with aniline to yield compound **9**, which was subjected to three reactions in one pot—TFA-mediated Claisen rearrangement, K_2CO_3-mediated isomerization and $POCl_3$-mediated chlorination—to afford the bisquinoline reagent **10** in good yield. Treatment of **10** with various sodium alkoxides under refluxing conditions afforded bisalkoxyquinolines **11a-c**. Finally, deprotonation of compounds **11a-c** with LDA at -78 ^{o}C followed by treatment of the resulting carbanions with Mannich bases **12a-e**, gave nine *N,N*-dimethyl bisquinolines **13-21** (Scheme 2b).

To synthesize the macrocyclic bisquinoline TMC207 derivative **23** (Scheme 3), **11c** was cyclized via ring closure metathesis using Grubbs 2nd generation catalyst, 1,3-bis(2,4,6-trimethylphenyl)-2-imidazolidinylidene)dichloro(phenylmethylene),[19] to yield **22** (Scheme 3). LDA-mediated deprotonation of **22** followed by condensation of the resultant anion with the Mannich base **12b** gave the desired bisquinoline macrocycle, **23** (Scheme 3).

RESULTS AND DISCUSSION

Conformationally constrained analogs of TMC207 (compounds **5a-i**; Table 1) were evaluated as anti-tubercular agents by performing BACTEC assays[20] on the $H_{37}Rv$ strain of *M. tuberculosis* as described in the experimental section. Among these compounds, the lowest minimum inhibitory concentration (MIC) value was obtained for the minor isomer of compound **5g** (12.1 μM), a compound that has a methylene linker between the lateral side chain of the drug scaffold and an adjacent phenyl ring, resulting in the formation of a fused 5-membered ring. Introduction of a methyl substituent at the phenyl ring fused to the 5-membered ring of **5g** yielded compound **5i** which gave an MIC of 12.5 μM. Increasing the ring size to 6 (compounds **5d-f**) or 7 (compounds **5a-c**) resulted in a major loss in activity. The lower activity of our conformationally constrained compounds as compared to TMC207 (MIC value of 0.05 μM)[7] suggests that the lateral side chain of TMC207 requires mobility to allow the drug to bind effectively to its binding pocket. It is possible that the *N,N*-dimethyl amino moiety (which has been previously shown to be critical for the anti-tubercular activity of TMC207)[17] is unable to reach out and bind to the mycobacterial ATP synthase due to restricted mobility of the two carbon linker that connects it to the rest of the drug scaffold. This result uncovers an important aspect of the binding of the drug to its target that needs to be considered in future drug design efforts on this scaffold.

The bisquinoline compounds, on the other hand, displayed much higher anti-tubercular activity (Table 2) as compared to the conformationally constrained compounds discussed above. Indeed, among the 10 bisquinoline compounds tested (one of which contained a macrocyclic moiety), 6 compounds yielded MIC values < 2 μM (Table 2). In particular, compounds **13** (MIC: 0.39 μM), **19** (MIC: 0.39 μM), **20** (MIC: 0.66 μM) and **21** (MIC: 0.70 μM) were found to be especially potent. A common feature of these four potent compounds is the presence of the methoxy group on the 2 position of both the quinoline rings which seems to be important for their activity. Indeed, replacement of these methoxy groups with the more reactive allyloxy substituents (in compounds **15, 16** and **17**) caused a significant decrease in activity (Table 2). Further, introduction of a bromine substituent on the meta position of the lone phenyl substituent of **13** to yield the compound **14** caused a 4-fold reduction in activity. The bromo substitution is better tolerated in the para position as demonstrated by the MIC value of compound **20** (0.66 μM). Next, we tested the activity of a few of our promising compounds (**13**, **14**, **18** and **19**) on MDR strains of *M. tuberculosis* obtained from two clinical isolates. All four of these compounds were active against these isolates and yielded an MIC value of 3.12 μM (Table 2).

Despite the large structural change imposed on the TMC207 scaffold upon replacement of its phenyl group with quinoline to yield our bisquinoline compounds, these compounds demonstrated potent anti-tubercular activity (especially compounds **13** and **19**). The ability of the TMC207 scaffold to accommodate such radical structural changes while retaining its activity is fascinating as most drugs lose activity when subjected to comparatively minor changes.[21] One possible explanation for these results is that these bisquinoline compounds

employ a mechanism disparate from that of TMC207 (which acts by inhibiting the ATP synthase enzyme of *M. tuberculosis*)[7]. To test this hypothesis, we performed ATP synthase-inhibition assays on these compounds and found that all the 10 bisquinoline compounds were potent ATP synthase inhibitors (IC_{50} values ranging from 0.001 μM for compound **21** to 0.28 μM for **17**; Table 2) suggesting that the mode of antitubercular activity of these compounds was similar to that of TMC207.

Encouraged by the anti-tubercular activity of bisquinolines **13**, **14**, **18**, **19**, **20** and **21** (Table 2), we proceeded to perform cytotoxicity experiments. Cytotoxicity of the compounds (with MIC ≤ 3.12 μM) was evaluated towards VERO cells and mouse bone marrow-derived macrophages as described in the experimental section. In these studies, a compound was considered as potentially toxic if its IC_{50} (concentration causing 50% loss in cell viability) was ≤ 10 times its MIC for *M. tuberculosis* $H_{37}R_v$.[22] We found that none of these compounds were toxic (Table 2). This result underlies the great potential of bisquinolines as potential antitubercular agents.

After demonstrating the anti-tubercular properties of these compounds and obtaining encouraging cytotoxicity data, we studied the efficacy of our most promising compounds, **13** and **19**, in mouse bone marrow macrophage model of tuberculosis. Upon testing the compounds at 4 times their MIC values, we found that compound **13** demonstrated a 90% reduction in the intracellular colony forming units (CFUs) of *M. tuberculosis*, whereas **19** demonstrated a 91% reduction in CFUs. By comparison, the standard drugs, isoniazid and rifampicin produced 98% reduction in the CFUs. These results are extremely promising as the macrophage model not only mimics the growth environment of natural infection, but also serves as a model for hypoxia-induced latent TB infection since the tissue concentration of oxygen is considerably lower than that in ambient air. Encouragingly, similar results were obtained with human bone marrow macrophage model of tuberculosis (data not shown).

To test the therapeutic efficacy of the compound **13** *in vivo*, outbred Swiss mice were infected intravenously with the $H_{37}R_v$ strain of *M. tuberculosis* and the compound was then orally administered. Results of mouse experiment (Table 3) show that administration of compound **13** reduced the bacterial load in the lungs by 118-fold at 50 mg/kg dose and 808 -fold at 100 mg/kg dose. The reduction in viable bacilli counts (CFU) was statistically significant at both test doses as compared to untreated control, e.g. P = 0.0012 and 0.0001, respectively, for 50 and 100 mg/kg doses. All the mice survived up to day 29 in the group fed with 50 mg/kg dose whereas one mouse died on day 9 in the group given the compound at 100 mg/kg.

These results are extremely exciting for the clinical prospects of **13** because when infected with a high number of *M. tuberculosis* CFUs by the intravenous route, the mouse harbors a bacillary population that is similar in number and in metabolic state to that present in the lung cavity of human TB. Thus, the mouse model is able to reproduce bacteriologic conditions close to those present in the natural human disease and provide information on compound activity that can be extrapolated to humans. Moreover, differences in the immune status among

outbred Swiss mice also exist in humans, and so a drug or regimen that is active against the mycobacteria in Swiss mice is likely to be active in humans.

The potent anti-tubercular activities of bisquinoline analogs of TMC207 described above motivated us to explore the molecular basis of the anti-tubercular activity of this class of compounds. The mode of action of TMC207 is known to involve inhibition of the ATP synthase enzyme.[7] Since our compounds also acted as inhibitors of this enzyme (Table 2), we decided to investigate the mode of binding of our compounds to this enzyme. Towards this goal, we built a homology model of this enzyme as described previously[23] and performed docking studies of the binding of selected compounds (**14**, **19**, and **23**) and TMC207 to the active site of the enzyme by using the Glide software.[24] The active site is located near the conserved acidic residue Glu-61 and basic residue Arg186 located in a- and c-subunits respectively, where the process of proton translocation occurs. Earlier reports have highlighted this residue (Glu-61) plays crucial role in the proton translocation with the side chain of Arg-186 (a-subunit), and hence is decisive for the initiation of the rotary movement of the subunit-c, and hence, in the ATP synthesis.[11,25-26] As shown in Figure 2, the conformational preference and stability were governed by both electrostatic and hydrophobic interactions. The common moiety, *N*,*N*-dimethylaminopropanol, present in the synthesized compounds and also in TMC207, exhibited favorable bidentate H-bond interaction with the carboxylate side chain of Glu-61(K) residue located in the central membrane-spanning part of subunit-c.

The bis-(2-methoxyquinoline) moiety present in compounds **14** and **19** attained an orientation almost perpendicular to each other and was extended toward the a-subunit exhibiting hydrophobic interactions and the 2-methoxy groups remained anti-parallel to each other. One of the two quinoline substructures was accommodated in the pocket corresponding to the 6-bromo-2-methoxyquinoline substructure of TMC207 and exhibited hydrophobic contacts with the two hydrophobic residues Ala-62(K) and Phe-65(K) located in the c-subunit, while another quinoline moiety was extended toward another interface site between a- and c-subunits where the phenyl moiety of TMC207 binds. In this pocket, quinoline exhibited stronger hydrophobic interactions with residues Tyr-64(L), Leu-68(L), Met-71(L) and Ala-193 than the phenyl of TMC207 and hence may be considered as one of the potential factors for their better ATPase inhibitory activity than TMC207 (Figure 2).

The compound **23** (ATPase IC_{50} = 0.01 μM) which comprises 1,6-bis(quinolin-2-yloxy)hex-3-ene substructure occupied similar site as that of compounds **14** (ATPase IC_{50} = 0.05 μM) and **19** (ATPase IC_{50} = 0.03 μM), but the cyclization enabled the quinoline nitrogen to favorably orient toward the same side, and thus exhibited electrostatic interaction with the residue Asn-190 (a-subunit) (Figure 2C). This may be one of the reasons behind the 5-fold better ATPase inhibitory activity of the cyclic analogue **23** than the acyclic analogue **14**.

Another important interaction explaining the observed ATPase inhibitory activity of the series of compounds was the hydrophobic interaction between the aromatic groups attached to the chiral carbon

(attachment point of the hydroxyl group) and the located hydrophobic (aromatic/aliphatic) residues Phe-65(L), Met-17(K) and Ile-16(L). The aromatic groups with greater bulk and hydrophobicity exhibited stronger hydrophobic contacts with these amino acid residues (Figure 2). The compounds **14** (ATPase IC_{50} = 0.05μM) and **19** (ATPase IC_{50} = 0.03μM) comprising 3-bromophenyl and 2-naphthyl groups respectively exhibited stronger hydrophobic contacts with residues Phe-65(L), Met-17(K) and Ile-16(L), and hence led to better ATPase inhibition than the compound **13** (ATPase IC_{50} = 0.07μM) containing phenyl ring at the corresponding position. This observation predicts that incorporation of bulky hydrophobic group such as naphthalene at this position will generate potent inhibitors of ATPase.

CONCLUSION

We have synthesized a series of TMC207 derivatives and evaluated their antitubercular activity by performing *in vitro* bacterial assays, *ex vivo* mouse bone marrow macrophage assays, and subjecting them to *in vivo* mouse models of the disease. We discover that introduction of steric constraint by linking a phenyl substituent to the lateral chain of TMC207 causes an appreciable loss in the antitubercular activity suggesting an important role of the mobility of the lateral chain in the activity of TMC207. Another series of compounds wherein a phenyl substituent of TMC207 is replaced by a quinoline moiety to give bisquinolines, demonstrate potent antitubercular activity. Encouragingly, toxicity studies demonstrate that these compounds are non-toxic to mammalian cells. Furthermore, by performing ATP synthase assays we demonstrate that these compounds potently inhibit the mycobacterial ATP synthase enzyme, suggesting that the mechanism of antitubercular activity of these compounds is similar to that of TMC207. Finally, to gain molecular insights into binding of our compounds to their target we performed docking studies which reveal an important role of both electrostatic as well as hydrophobic interactions in the stabilization of these ligands within the active site of the ATP synthase enzyme, leading to potent enzyme inhibition.

EXPERIMENTAL METHODS

General. All reactions were done using flame-dried glassware under nitrogen atmosphere. *N*,*N*-diisopropylethylamine (DIPA) was distilled over calcium hydride (CaH_2) and tetrahydrofuran (THF) was distilled from sodium/benzophenone ketyl immediately before use, while other solvents and reagents were used as received from the supplier. Reactions were monitored by thin layer chromatography (TLC) using 0.25 mm Merck pre-coated (Merch 60 F254) silica gel plates and using UV light and $KMnO_4$ as visualizing agent. Purification was performed by flash chromatography using silica gel (230-400 mesh). NMR spectra were recorded on a Bruker Advance-300 spectrometer. Chemical shifts are reported as parts per million (δ) relative to TMS as internal standard. Mass spectra were recorded on LCQ Advantage MAX (ESI) and JOEL JMS-600H (EI/HRMS) mass spectrometers. IR spectra were recorded on a Perkin Elmer FT-IR RXI spectrometer. The work plan for all

the animal experiments reported in this study was approved by the Institute's Ethical Committee (No. IAEC/2102/27/Renewed 07(22/13) dated 7.3.2013) and all were performed in compliance with the relevant laws and institutional guidelines.

General procedure for the synthesis of Mannich bases 2a-i

To a stirred suspension of Eschenmoser's salt[27] (1.0 mmol) in anhydrous CH_3CN (5 ml) were added cyclic ketones **1a-i** (1.0 mmol). The reaction mixtures were refluxed at 120 ^oC for 10–30 min and after completion of the reaction (TLC monitoring), the reaction mixtures were concentrated in vacuo to yield pale yellow hygroscopic solids which upon washing with anhydrous ether gave analytically pure hydrochloride salts of the desired Mannich bases **2a-i**.

General procedure for the synthesis of conformationally-constrained diarylquinolines (5a-i).

*Preparation of HCl free Mannich base **2a-i***: Crude hydrochloride salt of Mannich adducts **2a-i** were dissolved in water (10 mL) and extracted with diethyl ether (2 × 15 mL). The aqueous layer was cooled with ice-water mixture and then basified to pH ~ 8 with cold 5% aqueous $NaHCO_3$ solution followed by extraction with CH_2Cl_2 (2 × 25 mL). The combined organic layers were dried (Na_2SO_4) and concentrated in vacuo to give Mannich bases as pale liquids (80-90%) and were dried under vacuum for 1h before being used for the reaction.

To a cooled (-30^{o}C) solution of *N,N*-diisopropyl amine (1.8 mmol) in THF (10 ml) was added *n*BuLi (1.6 M in hexane, 1.6 mmol) drop wise under nitrogen atmosphere and stirred for 30 min. The mixture was then cooled to -70 °C and was added drop wise a solution of quinoline derivative **3** (1.0 mmol) in THF (5 mL). Resultant anion of **3** gave a deep red color. After 1h, a solution of freshly prepared free Mannich base (**2a-i**; 1.2 mmol) in THF (5 mL) was added drop wise to the reaction mixture and stirring was continued for additional 2h. The reaction was quenched with H_2O/THF (1:1) at -70 °C, allowed to warm to room temperature, extracted with EtOAc (3 × 15 mL). The combined organic extracts were washed with brine (2 × 10 mL), dried (Na_2SO_4) and concentrated *in vacuo* to give yellow foam as mixture of two diastereomers of **5a-i** which were purified by flash column chromatography over silica gel column using EtOAc/hexane as eluent.

Synthesis of Mannich adduct (12a-e) as HCl salts. Compounds **12a**[28] (yield 93%), **12b**[28] (yield 89%), **12c**[29] (yield 91%), **12d**[30] (yield 78%), **12e**[31] (yield 86%) were synthesized following procedure as described above for compounds **2a-i** by treating Eschenmoser's salt with acetophenone, 3-bromoacetophenone, 4-bromoacetophenone, 2-naphthylacetophenone, and 1-naphthylacetophenone respectively.

Synthesis of bisquinolines (13-21): Following procedure as described for diarylquinolines **5a-i**, bisquinolines **13-21** were synthesized by treating **11a-c** with Mannich bases **12a-e** (HCl salt free). In this case, the anions of **11a-c** gave a deep blue colour.

Biological evaluation.

In vitro screening of compounds against the $H_{37}R_v$ strain of M. tuberculosis. The compounds were dissolved in dimethyl sulfoxide (DMSO) to make stocks (5 mg/ml). Serial dilutions from stocks were also made in DMSO. To 1.9 ml MB 7H10 agar medium (in tubes, temp. 45-50° C, with OADC supplement), 0.1 ml of compound or DMSO (negative control) or isoniazid (positive control) was added. The contents were mixed and allowed to solidify as slants. Three-week old culture of *M. tuberculosis* $H_{37}R_v$ was harvested from L-J medium and its suspension (1 mg/ml, equivalent to 10^7/mL bacilli approximately) was made in normal saline containing 0.05% Tween-80. This suspension (10 µl ~ 10^5 bacilli) was inoculated into each tube and incubated at 37ºC for 4 weeks. The lowest concentration of a compound up to which there was no visible growth of bacilli was observed was considered its minimal inhibitory concentration (MIC).

Testing compounds against MDR strain of M. tuberculosis. Testing of compounds against MDR strains of *M. tuberculosis* were performed on two clinical isolates procured from the Department of Biotechnology (Govt. of India) Mycobacterial Repository located at National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Agra, India.

Bactec Assay (in vitro). Stock solution of the test compounds prepared in DMSO at 1 mg/mL was sterilized by passage through 0.22 µm filters. Fifty microliters were added to 4 mL radiometric 7H12 Broth (BACTEC 12B; Becton Dickinson Diagnostic Instrument System US) to achieve final concentrations. Controls received 50lL DMSO. Isoniazid and rifampin (Sigma Chemical Co. St. Louis, MO) were included as positive drug control. In BACTEC method, 104 to 105 CFU/mL of *M. tuberculosis* $H_{37}Rv$ was inoculated in 4 mL fresh BACTEC 12B broth containing the test compounds. An additional control was inoculated with 1:100 dilution of the inoculum to represent 1% of the bacterial population [102 to 103 CFU/mL]. The vials were incubated at 37^0C and GI (Growth Index) readings were recorded daily until the GI in 1:100 controls had reached 30. The concentration of the drug producing final GI reading lower than those in 1:100 control was considered to have inhibited more than 90% of the bacteria and was defined as the MIC.

In vitro toxicity evaluation. In vitro cytotoxicity of active compounds was measured using VERO cells and mouse bone-marrow derived macrophages. VERO C-1008 cell line was procured from Laboratory Animals Division (LAD) of CDRI. For obtaining the bone marrow derived macrophages, a Swiss mouse (bred in LAD) was euthanized by exposure to CO_2 and femurs dissected out. The bones were trimmed at each end and marrow flushed out with Dulbecco's Minimal Essential Medium containing 10% fetal bovine serum (DMEM-FBS) and antibiotics. The medium was also supplemented with 15% (v/v) L929 fibroblast conditioned medium and non-essential amino acids. The cell suspension (VERO/macrophage) was plated in 96-well tissue culture plates (20,000 cells/200µl/well) and incubated overnight (37 ºC, 5% CO_2) to allow their adherence. Compounds at

different concentrations were added to the wells. A known toxic compound, staurosporine, was used as a positive control and DMSO was used as negative control. After 24 h of incubation, 20 µl of MTS solution (tetrazolium compound, Owen's reagent) was added to each well and incubated for further 2 h (37 ºC, 5% CO_2). O.D. was read at 490 nm using an ELSA plate reader.

Assay for mouse bone marrow macrophage model of TB. The effect of compounds on the survival and multiplication of *M. tuberculosis* $H_{37}R_v$ within mouse bone-marrow derived as well as human blood monocyte derived macrophages was evaluated by counting of colony-forming units (CFU). Mouse bone-marrow derived macrophages were prepared as mentioned above. Five-day old cultures of adherent macrophages (10^6 cells/mL/well, in 24-well plates) were infected for 3 h with 1 ml/well suspension containing $5x10^6$ *M. tuberculosis* $H_{37}R_v$ in antibiotic-free medium. Later, the wells were washed to remove extracellular bacteria and replenished with fresh 1 ml antibiotic-free medium containing 4x MIC of standard drugs or test compounds. In order to determine the number of bacilli phagocytosed during the 3 h infection period (0 day count), one well was lysed with 0.1% saponin (15 min) and lysate (50 µl of 1:100 dilution) was plated onto MB 7H11 agar medium containing 10% (final concentration) OADC enrichment (in petri dishes) for colony counting. Other wells, after further 4 days of incubation, were gently washed and cells were lysed (with 0.1% saponin). The lysates (50 µl of 1:200 dilutions) were plated on MB 7H11 agar plates and CFUs were counted after 4 weeks of incubation at 37˚C.[32]

In vivo assays in mouse model of tuberculosis. Experiments were performed in out bred Female Swiss mice infected with $H_{37}R_v$ strain of *M. tuberculosis*, which maintains virulence through regular passages in the mouse. Efficacy of the compound was monitored by survival/mortality rate (MST, Mean Survival Time), % survivors on day 30[33] and the enumeration of the viable bacilli Counts (CFUs) in lungs. Mice (obtained from CDRI-LAD) were infected with *M. tuberculosis* $H_{37}R_v$ (10^7 CFU/ mouse, *i.v.*) and divided into groups of 5 animals each. The experimental group received daily oral dose of test compound, 100 mg/kg or 50mg/kg body weight, 6 days/week, for 28 days) dissolved in Sterile Distilled Water (DW). The drug-treated control groups received isoniazid (25 mg/kg) or ethambutol (100 mg/kg) for 28 days. The untreated control group received only DW. Three mice from each group were sacrificed on day 29 for determination of viable bacilli in the lungs. Serial dilutions of lung homogenates were plated onto MB7H11 agar medium (containing 10% OADC enrichment) and colonies (CFU) were counted after 3-4 weeks of incubation at 37 °C.

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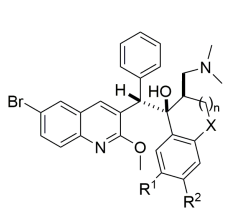
Abbreviations used: SAR: Structure-activity relationships; TB: Tuberculosis; MBMDM: Mouse bone marrow derived macrophages; DABCO: 1,4-diazabicyclo[2.2.2]octane; DMSO: Dimethyl sulfoxide; NMR: Nuclear

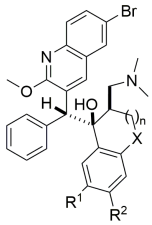
Magnetic Resonance, HRMS: High resolution mass spectrometry; MIC: Minimum inhibitory concentration; CFU: Colony-forming unit; DIPA: *N*,*N*-diazopropylamine; r.t.: room temperature; *i.v.*: intravenous.

Supporting Information Available: The crystal structures of compounds **5f**, **5c** and **S1** are depicted in the supporting information section. This material is available free of charge via the Internet at http://pubs.acs.org.

Table 1: Anti-TB activity of conformationally-constrained analogs of TMC207. Experiments were performed on the $H_{37}Rv$ strain of *M. tuberculosis*.



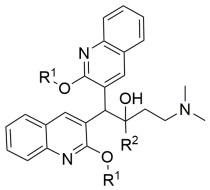
5-(±)-minor



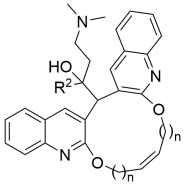
5-(±)-major

Compound	R^1	R^2	n	X	BACTEC MIC µM (minor isomer)	BACTEC MIC µM (major isomer)
5a	H	H	2	CH_2	not isolated	45.8
5b	H	H	2	O	not isolated	22.8
5c	Cl	H	2	O	>50	>50
5d	H	H	1	CH_2	>50	>50
5e	Br	H	1	CH_2	>50	>50
5f	H	H	1	O	>50	>50
5g	H	H	0	CH_2	12.1	48.3
5h	H	Br	0	CH_2	>50	>50
5i	Me	H	0	CH_2	12.5	>50

Table 2: Evaluation of anti-TB and ATP synthase-inhibitory activity of bisquinoline compounds.



13-21



23

Compd.	R^1 or n	R^2	ATP synthase IC_{50} (μM)#	Anti-tubercular activity against $H_{37}Rv$ strain MIC (μM)	Anti-tubercular activity against MDR *M. tb* isolates MIC (μM)		Cell Toxicity Assay	
					Isolate 1	Isolate 2	VERO cells	Mouse Macrophages
13	Me	Phenyl	0.07	0.39	3.12	3.12	>100 μM	>100 μM
14	Me	3-bromophenyl	0.05	1.56	3.12	3.12	>100 μM	>100 μM
15	CH_2-CH=CH_2	Phenyl	0.04	>50	nd	nd	nd	nd
16	CH_2-CH=CH_2	3-bromophenyl	0.03	>50	nd	nd	nd	nd
17	CH_2-CH=CH_2	4-bromophenyl	0.28	>50	nd	nd	nd	nd
18	CH_2-CH_2-CH =CH_2	4-bromophenyl	0.08	1.56	3.12	3.12	>100 μM	>100 μM
19	Me	2-naphthyl	0.03	0.39	3.12	3.12	>100 μM	>100 μM
20	Me	4-bromophenyl	0.01	0.66	nd	nd	>100 μM	>100 μM
21	Me	1-naphthyl	0.001	0.70	nd	nd	>100 μM	>100 μM
23	2	3-bromophenyl	0.01	nd	nd	nd	nd	nd

#IC_{50} in ATP-synthase assay were calculated using nonlinear regression/log inhibitor *vs* response using Graph Pad Prism software; nd, not determined; Ø mouse bone marrow-derived macrophages; @ The VERO cell assay (VCA) has been widely used to assess the cytotoxicity of verotoxin-producing *E. coli* strains.

Table 3. Therapeutic efficacy of compound **13** in mice infected with *M. tuberculosis* H37Rv.

Groups	CFUs (per gram of lungs)	Fold reduction in CFU	MST (Mean Survival Time)
Control Mean ± SD	$1.18 \times 10^9 \pm 0.32$	-	27.80
Compound **13** [50 mg/kg] Mean ± SD	$1.00 \times 10^7 \pm 0.60$ (P = 0.0012)*	118.00	29.00
Compound **13**[100 mg/kg] Mean ± SD	$1.46 \times 10^6 \pm 0.12$ (P = 0.0001)**	808.22	25.00
Isoniazid [25 mg/kg]	$4.59 \times 10^6 \pm 0.114$ (P= 0.0002)**	257.08	29.00
Ethambutol [100 mg/kg]	$4.38 \times 10^7 \pm 0.30$ (P= 0.0027)*	26.94	29.00

Doses administered were mg/kg body weight. CFUs represent average bacterial burden in the lungs of 3 mice each. Significance of variance (P) and fold reduction in CFUs were calculated with respect to untreated control mice.*Statistically very significant; **Extremely significant.

Figure 1: Structures of compounds evaluated in this study a) TMC207; b) Conformationally-constrained analogs of TMC207 (X = CH_2 or O); c) Bisquinoline analogs of TMC207 (R = alkyl/aryl group); d) Bisquinoline macrocycles (R = alkyl/aryl group)

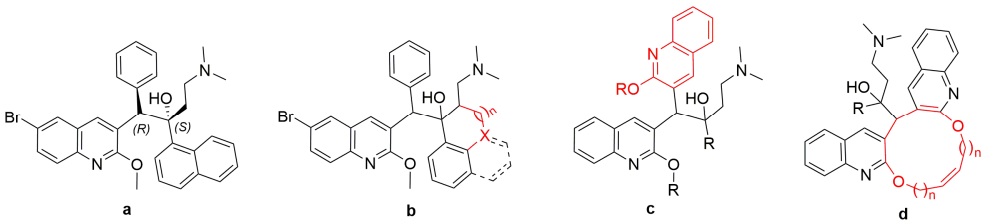
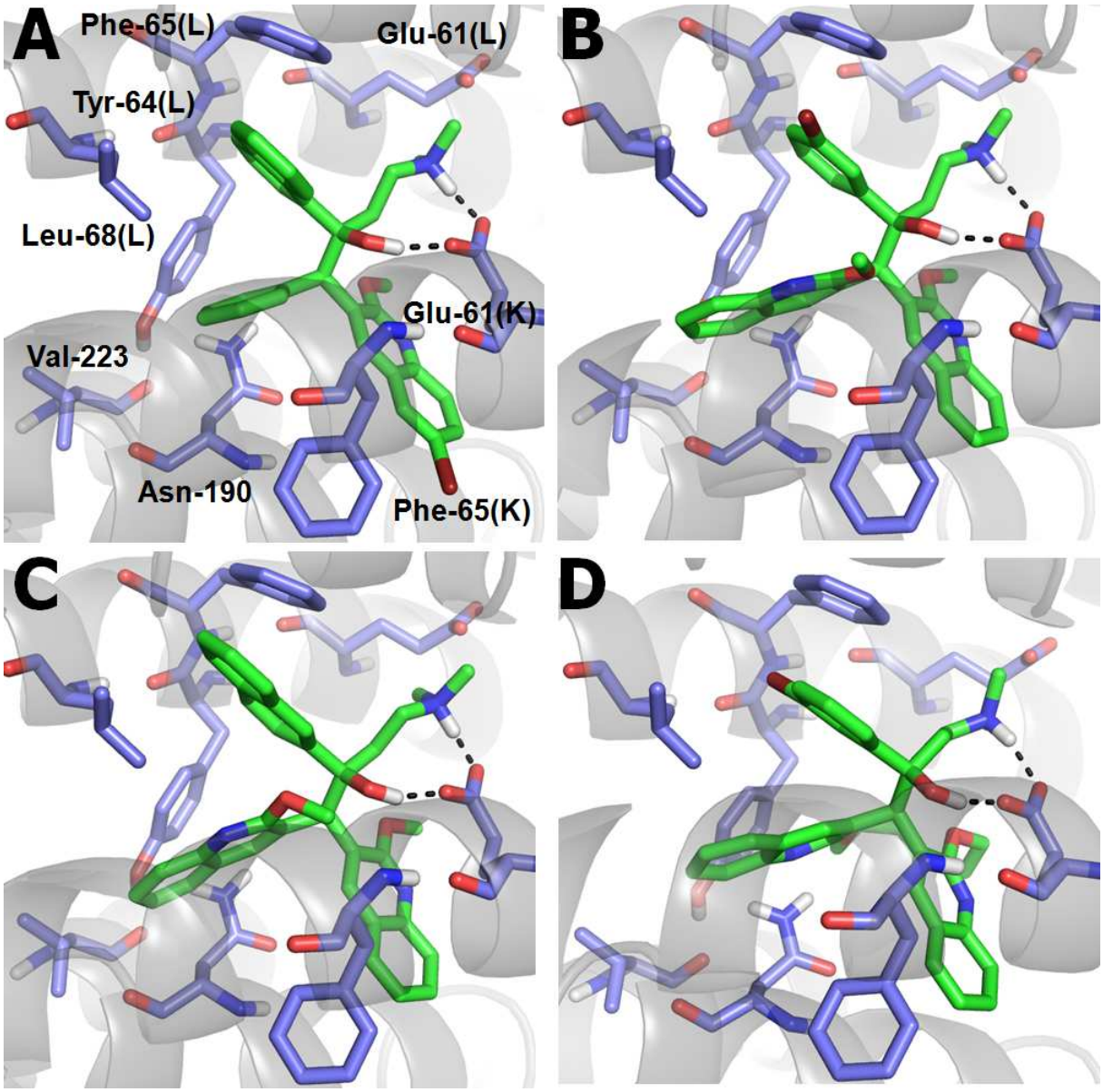
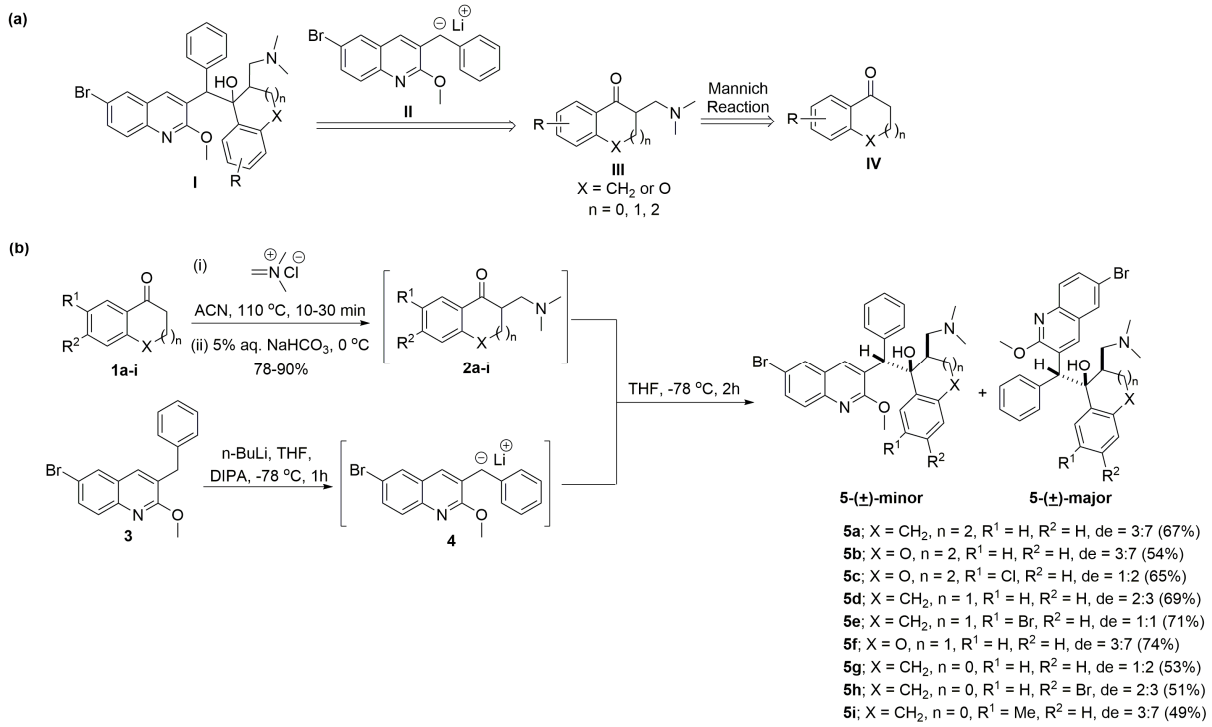


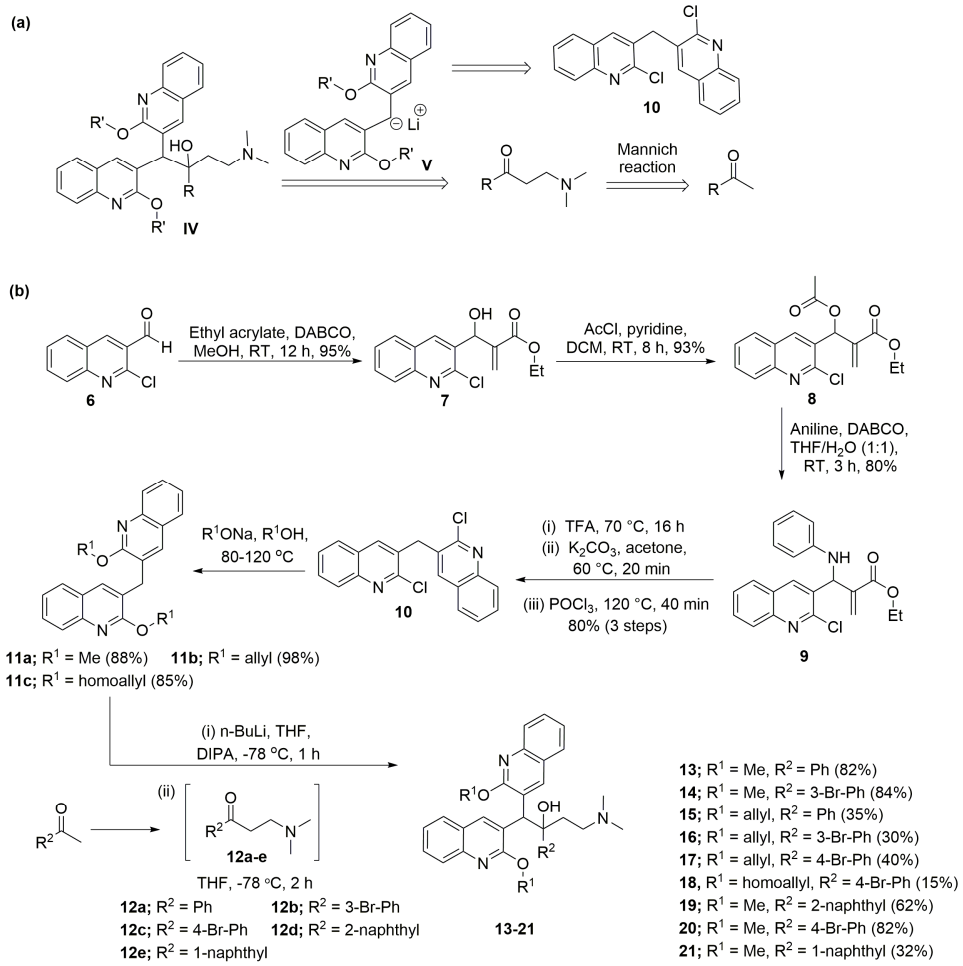
Figure 2: Binding modes of the compounds (**A**) TMC207; (**B**) **14**; (**C**) **19**; (**D**) **23** into the active site of homology modeled ATPase. The black dashed lines indicate H-bonds. The "(K)" and "(L)" mentioned along with some of the labeled residues indicate the amino acid chains of c-subunit in which they are located. The protein is shown in ribbon-cartoon form. The images are taken using PyMol graphics.



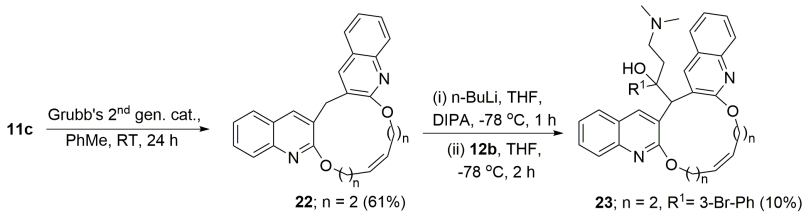
Scheme 1. Synthesis of conformationally constrained analogs of TMC207. a) Retrosynthetic analysis b) Synthetic scheme.



Scheme 2. Synthesis of bisquinolines. a) Retrosynthetic analysis b) Synthetic scheme.



Scheme 3. Synthesis of a bisquinoline macrocycle.



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