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Synthesis and antitubercular activity of 1,2,4-trisubstitued piperazines

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ABSTRACT

Parallel solid phase synthesis offers a unique opportunity for the synthesis and screening of large numbers of compounds and significantly enhances the prospect of finding new leads. We report the synthesis and antitubercular activity of chiral 1,2,4-trisubstituted piperazines derived from resin bound acylated dipeptides against Mycobacterium tuberculosis strain H37Rv.

Tuberculosis (TB) is the biggest comeback infectious disease in history and kills an estimated 3 million people worldwide each year.[1–4] Although many believe TB to be a scourge of the past, the disease continues to strike people throughout the world at an alarming rate. Tuberculosis (TB) is more prevalent in the world today than at any other time in human history. *Mycobacterium tuberculosis* (*Mtb*), the pathogen responsible for TB, uses diverse strategies to survive in a variety of host lesions and to evade immune surveillance. Almost 2 billion people are infected with *Mtb*, the TB bacterium, and each year, 8 million of them develop active TB.[1–4] Tuberculosis is particularly dangerous to those with weakened immune systems and is the primary cause of death among people infected with HIV.[5]

Herein we present the synthesis and the screening results against *Mtb* of a collection of 1,2,4-trisubstituted piperazine compounds. These compounds were prepared following the strategy outlined in Scheme 1.

Starting from resin-bound amino acid and trityl (Trt) protection of the amino group, a selective *N*-alkylation of the amide linked to the solid support was performed using lithium *t*-butoxide in THF, followed by addition of methyl iodide in dimethyl sulfoxide (DMSO). Following removal of the Trt protecting group with 2% TFA in DCM and neutralization, the second Fmoc amino acid was added using traditional solid-phase peptide chemistry. The Fmoc group was deprotected in the presence of piperidine in DMF and the resulting dipeptide was treated with a variety of carboxylic acids to generate the corresponding acylated dipeptides. The reduction of the amide groups of the resin-bound *N*-acylated dipeptide using borane in THF at 65 °C yielded a single tertiary and two secondary amines.[6–10] We previously reported that the borane reduction of amide bonds was free of racemization by comparing the relative absorbances of different pairs of diastereomers that do not coelute.[6–10] The same observations were later reported by other groups using different reduction workup procedures.[11] The treatment of the resin-bound polyamine with oxalyldiimidazole in anhydrous DMF yielded the corresponding trisubstituted diketopiperazine. The oxamide moiety was treated with borane in THF at 65 °C and the desired 1,2,4-trisubstituted piperazine compounds were obtained following HF cleavage of the solid support.

All the individual piperazine compounds were purified and first evaluated in primary screen for activity against *M.* tuberculosis (Fig. 1). Four select compounds with desirable activity profiles were then characterized in detail using a panel of antimicrobial assays presented below.

Primary Screen for minimum inhibitory concentration (MIC): Stock solutions of compounds were prepared in DMSO at 10 mM and used within 7 day for antimicrobial assays. *Mycobacterium tuberculosis* (*Mtb*) H37Rv was cultured in 7H9-OADC-Tween80 liquid media (4.7 g/L 7H9 base broth, 0.05% Tween-80, 10% OADC supplement) under aerobic conditions.[12]

Determination of minimum inhibitory concentration: The minimum inhibitory concentration (MIC) of each compound was

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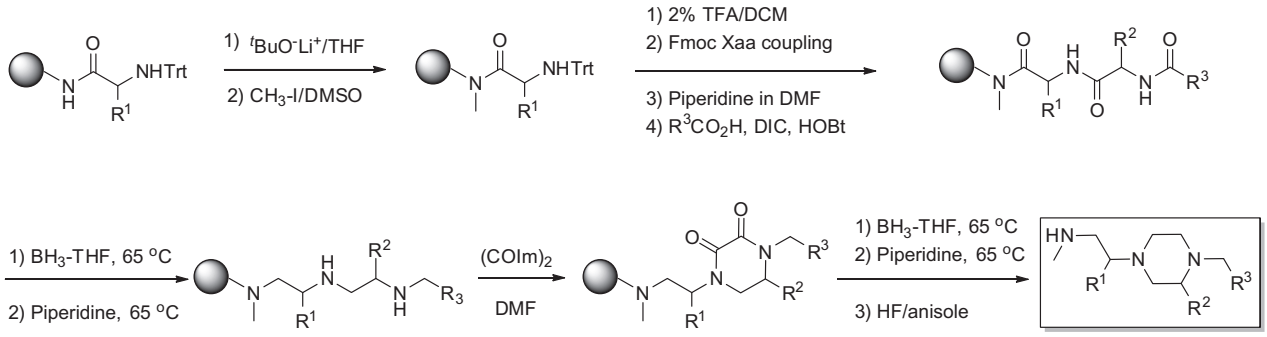

Scheme 1. Synthesis of 1,2,4-trisubstituted piperazines.

determined as the concentration required to prevent growth as measured by optical density (OD) of the culture. Compounds were dispensed into 96-well plates as either 10-pt (compounds **9**–**24**) or 20-pt (compounds **1**–**8**) two-fold serial dilutions into 7H9-Tw-OADC medium starting at the highest concentration of 200 mM, with a final DMSO concentration of 2%. Each plate included a 10-pt dilution series of control drug (rifampicin), a background control (medium + DMSO only, no bacteria), a no growth control (100 mM Rif), and a maximum growth control (DMSO only). Wells were inoculated with *Mtb* and incubated for 5 days at 37° C. Growth was measured by OD_{590} using a BioTek™ Synergy 4 plate reader. To calculate MIC values, dose response curves were plotted as % growth and fitted to the Gompertz model using GraphPad Prism 5. The MIC is the minimum concentration that completely inhibited growth, which corresponds to the inflection point of the fitted curve to the lower asymptote (zero growth baseline).

Determination of Antimicrobial Activity against Non-replicating Hypoxic Mtb: The antibacterial activity of compounds against *Mtb* grown in hypoxic conditions was tested, to mimic the phenotypically drug tolerant state of bacilli in vivo. Using the low oxygen recovery assay (LORA) developed by S.H. Cho et al.[13] *Mtb* expressing bacterial luciferase *luxABCDE* (*Mtb:lux*) [12] are preadapted under hypoxic conditions followed by exposure to test compounds for 10 days under hypoxia. A 5 day period of aerobic outgrowth allows for discrimination between killed and unaffected wells based on luminescence signal. Serial dilutions of compounds were prepared as described above for MIC assays. Rifampicin and metronidazole were included as positive controls for aerobic (Rif) and anaerobic (Met) killing.

Minimum bactericidal activity: The bactericidal activity of compounds against *Mtb* grown to log phase in aerobic 7H9-Tw-OADC broth was determined at four different compound concentrations: 10× MIC, 5× MIC, 1× MIC and 0.25× MIC. Treatments duration was 21 days, with bacterial viability at day 0, 7, 14, and 21 determined by enumeration of colony forming units (CFU) of serial dilutions plates on 7H10 agar plates. The MBC was defined as the minimum drug concentration needed to achieve a 2-log kill in 21 days. For compounds with >1-log kill, the time-and/or concentration dependence was determined based on the kill kinetics.

Activity against intracellular Mtb: Murine J774 macrophage cell-line were infected with *Mtb:lux* at an MOI of 1:1 in 96-well plates. After 18 h, infected monolayers were washed to remove extracellular bacteria before addition of compounds. Infected macrophages were treated for 4 days with 1× and 10× MIC. At day 4, bacteria were released from macrophages by lysis with 0.1% SDS, inoculated into 7H9-Tw-OADC media, and incubated aerobically for 5 days. Viable bacteria present were quantitated by luminescence. All assays were conducted in triplicate and included a positive control (4 mM isoniazid) and a negative vehicle control (2% DMSO). The intracellular activity was expressed as a log reduction of *Mtb* based on the formula [Log RLU Day 4 compound]—[Log RLU Day 4 DMSO].

Activity against drug resistant Mtb: The activity of compounds against five drug resistant *Mtb* strains—2 isoniazid resistant (INH-R1 and INH-R2), two rifampicin resistant strains (RIF-R1 and RIF-R2) and a fluoroquinolone resistant strain (FQ-R1)—was assessed by determination of MIC based on an OD readout as described above.

Results of antitubercular screening of compounds: The primary MIC assay results (MIC, IC_{50}, and IC_{90}) for all compounds indicating their ability to inhibit the growth of replicating *Mtb* grown in aerobic conditions are shown in Figure 1. Four of the more potent compounds (compounds **2**, **3**, **4**, and **10**) were further analyzed for bactericidal mode of action, antimicrobial activity against anaerobic dormant bacilli and intracellular *Mtb*, and ability to kill strains resistance to front-line and second-line TB drugs. Results are summarized in Table 1. All four compounds showed moderate potency against replicating *Mtb* (Table 1—Aerobic MIC). Compounds **2** and **4** had MBC < MIC, indicating a bactericidal mode of action, whereas **3** and **10** had MBC > MIC consistent with a bacteriostatic activity. Compounds **2**, **4**, and **10** but not **4** retained appreciable activity against nonreplicating anaerobic *Mtb* which are notably drug tolerant (Table 1—LORA MIC). Interestingly, **2** exhibited enhanced activity in the LORA assay compared to the aerobic assay, a desirable feature for targeted activity against dormant bacilli within granuloma lesions. All four compounds led to >2 logs of killing of intracellular *Mtb*, indicating their ability to access the pathogen within the macrophage host cell. Finally, strains resistant to the front-line drugs isoniazid (INH) and rifampicin (Rif) and second-line fluoroquinolone (FQ) were susceptible to all four piperazine compounds, indicating a novel target and mechanism of action.

Encouraged by the interesting antitubercular activity of the individual piperazines, a positional scanning library (PSL) of 1,2,4-trisubstituted piperazine containing 33,640 compounds (29 R^1 × 29 R^2 × 40 R^3) will be screened for antitubercular activity.

The use of PSL will enable the most active groups at each position of the bis-diazacyclic libraries to be determined directly from the initial screening data. Screening the three sets of mixtures for each library, totaling 98 mixtures (29 R^1 + 29 R^2 + 40 R^3) in the same assay will yield information about the most important functional groups at each fixed position in each library. After selecting the most active mixtures from screening of the piperazine library, active functional groups will be selected for R^1, R^2 and R^3 for the synthesis of individual compounds making all possible combinations of defined R^1, R^2 andR^3. This will enable a better analysis of the SAR for the potential design and identification of compounds with great potential for success in the clinic. The deconvolution of the PS piperazine library and screening results will be reported elsewhere.