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Pilot Study of a Novel Anti-Tubercular Acylhydrazone Schiff Base Derivative

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Abstract

Aim: This study aimed to discover new Anti-Mycobacterium Tuberculosis (MTB) chemicals for treating drug-resistant MTB.

Method: A phenotypic screening model was construed using MTB H37Rv (ATCC27294) for searching anti-tuberculosis chemicals. Minimal inhibitory concentrations (MICs) of the positive compounds to sensitive and resistant MTB strains were detected to confirm antibacterial activities. The pharmacokinetic and metabolic properties and intracor-poral anti-MTB activity of the promising compound were determined in mice to assess drug suitability.

Results: HMPP, an acyl hydrazone Schiff base derivative of these compounds, was obtained based on this model. HMPP potently inhibited H37Rv (MIC, 0.72 μM) and a multiple drug-resistant MTB strain (MIC, 45 μM). The half maximal inhibitory concentrations (IC_{50}) for Vero and HepG2 were 457.06 μM and 720.86 μM, respectively. The IC_{50} value which inhibited the tail current of hERG channels was much >higher than 30 μM. Based on the mini-Ames experiment, HMPP did not induce>a 3-fold increase in reverse mutations. The elimination factor of HMPP was 7.75 L/h/kg, and the half-life ($t_{1/2}$) was 0.37 h and 1.82 h for Intravenous (iv) and Oral (po) administration in Sprague-Dawley rats. After one-hour incubation in murine plasma, HMPP hydrolyzed completely into M1 and M2 83.36%) HMPP reduced the load of MTB in mice lungs from 3.83×10^5 Colony Forming Units (CFU) to 3.32×10^3 CFU at a dose of 100 mg/kg.

Conclusion: These results indicate that HMPP is a promising antibacterial agent for treating mycobacterium tuberculosis.

Keywords: Mycobacterium tuberculosis; Phenotypic screening; Drug resistance; Antibacterial agent

Introduction

Tuberculosis (TB) remains the most serious infectious disease worldwide and predominantly occurs in developing countries. The annual death from TB caused by Multidrug-Resistant (MDR) and Extensively Drug-Resistant (XDR) Mycobacterium Tuberculosis (MTB) outnumbers Human Immunodeficiency Virus (HIV) [1]. Especially, 90% of active disease in MDR-TB occurs within the first two years and nearly all within three years, indicating the difficulties of MDR-TB treatment [2]. The traditional anti-TB drugs such as isoniazid, rifampicin, pyrazinamide, ethambutol, streptomycin, kanamycin, amikacin, capreomycin, levofloxacin, ofloxacin have been widely used to treat TB at the doses recommended by the World Health Organization (WHO) [3]. However, previous studies suggested that, MDR-TB did not respond to the two most powerful anti-TB drugs isoniazid and rifampicin. In 2017, there were 558,000 estimated cases of MDR-TB worldwide [4]. Furthermore, the process for developing new Anti-TB drugs with optimal efficacy and safety is still under experimentation. The one option to control the situation presently is to devise new methods that can help in deriving maximum benefits from traditionally available therapeutic agents [5,6]. Therefore, the discovery of novel chemicals with unique modes of action against drug-resistant TB is critical.

Automated chemical screening approaches, such as High-Throughput Screening (HTS) assays have been used to detect chemicals in a standardized manner [7]. HTS approaches also have been constructed to exploit anti-TB drugs, but limited success has been achieved [8-10]. For instance, HTS screening approach was used to identify inhibitors of the enzymatic activity of riboflavin synthase and find novel and effective antimicrobial compounds against brucellosis [11]. Recently, phenotypic screening approaches have been considered more relevant for drug repurposing, exceeding those discovered through the molecular target-based approaches [12,13]. In our library, the molecular HTS model was also constructed to search for inhibitors of isocitrate lyase and 4-diphosphocytidyl-2-C-methylerythritol synthetase. We showed that most of the inhibitors had no or weak bacteriostatic activity to MTB H37Rv. So, in the current study, a phenotypic screening approach via MTB H37Rv was adopted to screen new anti-TB agents through 150,000 synthetic compounds and a novel arylhydrazone Schiff base compound- 4-amino-5-Hydroxymethyl-2-Methyl-Pyrimidine Phosphate (HMPP) with strong antibacterial activity was identified. Further, the toxicity, pharmacokinetic properties, and anti-MTB capacities of HMPP *in vivo* were studied

comprehensively, and the results indicated it was a promising candidate.

Materials and Methods

Bacterial strains, medium, and culture conditions

MTB strain H37Rv (ATCC27294; ATCC, Manassas, VA, USA) and MDR strain (Beijing Tuberculosis and Thoracic Tumor Research Institute, Beijing, China) were cultured in Middlebrook 7H9 broth (supplemented with glycerol and polysorbate 80) in combination with Middlebrook OADC enrichment at 37°C [14]. Salmonella typhimurium histidine auxotrophs (TA98, TA100, TA1535, and TA1537) and Escherichia coli WP2 uvrA (pKM101) were purchased from Molecular Toxicology (Boone, NC, USA) and cultured in Oxoid nutrient broth No. 2 (NB2; Basingstoke, UK) at 37°C with a shake at approximately 150 rpm.

High Throughput Screening

MTB H37Rv was used to screen for Anti-TB agents in 96-well plates and the individual compound was added a final concentration of 20 μg/mL. Each plate had 4 negative controls containing Dimethyl Sulfoxide (DMSO) and 4 positive controls containing Isoniazid (INH) at 0.5 μg/mL. The ultrasonically-suspended, mid-log phase H37Rv were adjusted to approximately 5×10^5 CFU/mL with 7H9 broth (OD580 ≈ 0.001) and 100 μL suspensions were added to each well in the plates. The plates were incubated at 37°C for 10 d and the OD580 was detected via a Perkin Elmer Enspire 2300 Multilabel Reader (Perkin Elmer, MA, USA). Compounds which restrained the growth of MTB as potently as the positive controls were defined as hits for a further study.

Anti-TB activity of HMPP

The MICs of compounds that inhibited MTB were evaluated, as described by Darby and Nathan [15]. Briefly, the mid-log phase H37Rv and the MDR strain were adjusted to an OD580 of 0.001 with 7H9 broth, and then exposed to chemicals (HMPP or INH) in serial 2-fold dilutions from 64-0.125 μg/mL in 96-well plates in triplicate. After 10 d of incubation at 37°C, the final OD580 of the wells were measured and the MICs were defined at the concentrations when no growth of strains was detected. The acceptable MIC of INH on H37Rv and MDR strain was 0.125-0.25 μg/mL and>64 μg/mL, respectively [16].

Cytotoxicity assays

HepG2 cells (ATCCHB8065) and Vero green monkey kidney cells (ATCCC1008) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Waltham, MA, USA) supplemented with 10% Fetal Bovine Serum (FBS; Gibco) and 1% antibiotics (100 U/mL of penicillin and 100 mg/mL of streptomycin; Life Technologies, Carlsbad, CA, USA) and incubated in a humidified incubator (Heracell 150R; Thermo Electron Corp., Waltham, MA, USA) with 5% CO_2 at 37°C. The exponential cells were trypsinized, seeded in 96-well plates at a density of 1×10^4 cells/200 μL, and incubated for 24 h. The medium was removed and the cells were washed 3 times with Dulbecco's Phosphate-Buffered Saline (DPBS; Wako, Osaka, Japan). Medium containing various concentrations of HMPP (3-fold dilutions from 300-0.05 μg/mL) was added (final DMSO concentration=0.25%). After 2 days of incubation, cell viability was assessed using a 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) colorimetric assay. The IC_{50} values were calculated using the log (inhibitor) vs. normalized response-variable slope module of Graphpad prism 5.0 (GraphPad Software Inc., San Diego, CA, USA).

Solutions and voltage clamp recording from CHO-K1

CHO-K1 (AVIVA, London, England) was maintained in high-glucose DMEM (Gibco, Waltham, MA, USA) supplemented with 10% FBS, 100 U/mL of penicillin, and 100 mg/mL of streptomycin in an incubator with 5% CO_2 in a humidified atmosphere at 37°C. The experiments were performed as previously described with some modifications [17]. The adherent cells were perfused with an external solution (10 mM HEPES, 145 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose [pH was adjusted to 7.4 using 1 N NaOH], and an osmotic pressure of 290-300 mOsm at a rate of 1-2 mL/min. The solution also contained the positive control, amitriptyline (0.3 μM, 1 μM, 3 μM, 10 μM, or 30 μM), or HMPP (0.3 μM, 1 μM, 3 μM, 10 μM, or 30 μM). The glass microelectrode had a resistance of 2-5 MΩ and was filled with pipette solution (120 mM KCl, 31.25 mM KOH, 5.374 mM $CaCl_2$, 1.75 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES, 4 mM Na_2-ATP [pH was adjusted to 7.2 using 1 N KOH], and the osmotic pressure was adjusted to 280-290 mOsm) to record the tail current. The clamp voltage was maintained at -80 mV, the cells were depolarized (+60 mV) and repolarized (-50 mV), and the steady tail current was recorded.

Mini-Ames test

The mini-Ames assay was performed as recommended by the Economic Co-operation and Development (OECD) [18]. Overnight cultures of Salmonella typhimurium TA98, TA100, TA1535, and TA1537 and Escherichia coli WP2 uvrA (pKM101) were transferred to fresh Oxoid nutrient broth No. 2, and incubated until the OD_{600} was between 0.6 and 1.6. The bacteria were mixed with 2 mL of soft agar supplemented with 0.5% NaCl, 0.5 mM l-histidine/biotin, 0.5 mM l-tryptophan, 80 μL of chemical solution, 400 μL of S9 mix (prepared from the liver of Aroclor-induced rats, Moltox™; Boone, NC, USA) or Phosphate Buffered Saline (PBS; non-activated), and poured on 5 mL of minimum glucose agar medium (1.5% agar and 2% glucose in Vogel-Bonner medium E) in 6-well plates. Each treatment was performed in triplicate, except for untreated and solvent controls, which were tested in sextuplicates. After 60 h of incubation at 37°C, the colonies were counted. The HMPP concentrations used in this assay were 50, 20, 8, 3.2, 1.25, 0.5, 0.2, and 0.075 μg/μL. 2-Aminoanthracene (2-AA), 2-Nitrofluorene (2-NF), Sodium Azide (SA), acridine mutagen (ICR-191; Alfa Aesar, Ward Hill, MA, USA), and N-methyl-N-nitro-N-nitrosoguanidine (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) were used as positive controls.

Ethics and source of animals

The animal experiments were supervised and approved by the Institutional Animal Care and Use Committee of the Institute of Medicinal Biotechnology. Sprague-Dawley (SD) rats and Balb/c mice were purchased from the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences, and Peking Union Medical College.

Pharmacokinetics experiment

Six males SD rats (6-8 weeks old; 180-200 g) were housed in stainless steel cages in a ventilated animal room at 21 ± 2°C with a relative humidity of 60 ± 10% and a 12-h light/dark cycle. Distilled water and sterilized food were available ad libitum. HMPP diluted in 5% N-methyl pyrrolidine, 40% polyethylene glycol 400, and 55% 20% hydroxypropyl-beta-cyclodextrin water to 1 mg/mL was administered by Intravenous (iv) injection at a dose of 1 mg/kg, and diluted in 0.5% CMC-Na to 20 mg/mL and administered by oral gavage at a dose of 10 mg/kg. Approximately 0.2 mL of blood was collected from cardiac puncture before drug administration and after 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, and 24 h of administration. Four microliters of K3EDTA (0.5 M) was used for anti-coagulation. Serum was separated by centrifugation at 8000 rpm [g values are preferred] for 6 min and stored at -80°C. The calibration measurement of HMPP was used to assess the veracity of the analyzing conditions. The stock solutions (200 μg/mL) of HMPP in methanol were diluted with blank SD rat plasma to 1000, 500, 100, 50, 10, 5, and 1 ng/mL; 50 μL of the solutions were added to 1.5 mL of Eppendorf (EP) tubes containing 250 μL of celecoxib (10 μg/mL in methanol for use as an Internal Standard [IS]). The mixtures were centrifuged at 8000 rpm for 6 min. Ultra-Performance Liquid Chromatography/Tandem Mass Spectrometry (UPLC-MS/MS) was performed using an Acquity UPLC (Waters, Milford, MA, USA) coupled with an API 5500 (Applied Biosystems, Foster City, CA, USA) to analyze the samples. An Acquity UPLC BEH C18 1.7 μm column (50 mm × 2.10 mm; Waters) was eluted with a 0.1% formic acid aqueous solution-0.1% formic acid acetonitrile solution (80:20 to 20:80 in 1 min of elution time) mobile phase at a flow rate of 500 μL/min. Chemicals were monitored using the positive multiple reaction monitoring (MRM) mode at a transition of m/z 400.0-300.0. Raw data from the mass spectrometry was processed using Analyst 1.6.2 software (Applied Biosystems, Foster City, CA, USA) and pharmacokinetic parameters were computed using WinNonlin 5.2 non-compartment model software (Pharsight, Palo Alto, CA, USA).

Metabolism of HMPP in plasma

HMPP (10 μM) and the positive control (enalapril, 10 μM) were incubated in SD rat plasma at 37°C for 60 min. Samples were precipitated with acetonitrile (containing 0.1% formic acid [1:3]) and centrifuged again. The supernatants were dried with N_2 gas and the residues were reconstituted with 200 μL of 10% acetonitrile/0.1% formic acid. UPLC-MS/MS was conducted as described above to analyze the metabolites, which were monitored at a transition of 400.0-60.0.

In vivo anti-TB activity test

The anti-TB activity test was conducted as described previously with some modifications [19]. The log phase H37Rv bacteria were centrifuged, washed with distilled pyrogen-free water, diluted to 3.4×10^8 CFU/mL, and stored at -80°C. The frozen bacteria, diluted with 0.9% NaCl solution (containing 0.01% Tween 80) to 5×10^6 CFU/mL, were used to infect 26 male Balb/c mice (6-8 weeks old; 18-20 g) through an Inhalation Exposure System 099C A4212 (Glas-col, Terre Haute, IN, USA). On day 3, three mice were sacrificed to verify the success of infection and on day 10, 3 mice were sacrificed to determine the pre-treatment bacterial load. Fifteen days after infection, the remaining 20 mice were randomly divided into 4 treatment groups. Negative control mice were administered CMC-Na solvent, while positive controls received INH at dosage of 25 mg/kg [20]. HMPP was administered at 25 mg/kg or 100 mg/kg. The mice were dosed for 5 consecutive days each week. All mice were weighed and sacrificed 3 d after the final dose to minimize carryover from the lung homogenates to the plating medium. The lung tissues were homogenized and diluted in Hanks' Balanced Salt Solution (HBSS)-Tween and the number of bacteria were determined using plating homogenized organs on 7H10 agar to quantify the CFU.

Results

Screening of HMPP

In this study, we screened through 150,000 chemicals using the pathogenic MTB H37Rv expecting to acquire inhibitors with diversity structures. Chemcals at concentration of 20 μg/mL had antibacterial activities comparable to the potency of INH (0.5 μg/mL) were defined as hits.

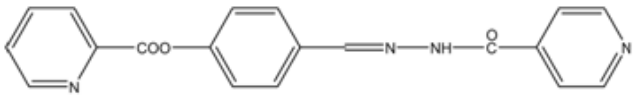

Figure 1: The structure of HMPP.

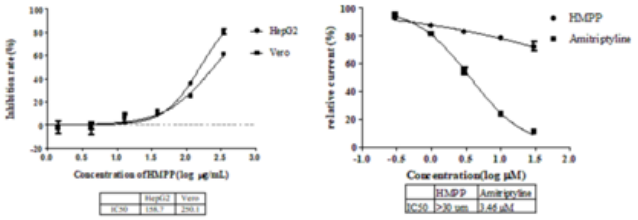

Figure 2: Cytotoxicity of HMPP. A: MTT assay was used to measure the inhibition of HepG2 or Vero cells exposed to 300-0.05 μg/mL HMPP. The OD of the unexposed cells was taken as 100% survival; B: Whole-cell voltage-clamp recordings from CHO-K1 cells expressing hERG channels. The IC_{50} for HMPP calculated in this study was not accurate because the concentration of HMPP used in the study ranged from 0.3-30 μM, which was not wide enough to calculate the IC_{50}. All experiments were conducted in triplicate and the data are reported as the mean ± standard deviation.

MICs of these hits (1108 chemicals) to MTB were detected to eliminate the false-positive chemicals and confirm their bacteriostatic activities. Finally, 516 compounds were shown to have affirmative anti-tuberculous activity (the positive rate=0.34%). Eighty-five of these compounds had the strongest antibacterial activity, which was equivalent to a MIC<5 μg/mL. Notably, among the 85 compounds, HMPP inhibited the sensitive H37Rv with a MIC of 0.25 μg/mL (equivalent to 0.72 μM. the MDR MTB at the level of 6.19 μg/mL (approximately 45 μM), So HMPP was chosen for further studies. As shown in Figure 1, the structure of HMPP suggested a molecular weight of 346.11 kD. In addition, drug likeness was predicted using the Drug Likeness prediction module of Molsoft (http://www.molsoft.com/). It was suggested that HMPP had 6 hydrogen bond acceptors and only one hydrogen bond donor, and the logP was

2.60. There was no stereo center in HMPP, and the drug-likeness score was 0.74.

Toxicity of HMPP

The potential toxicity of HMPP was first assessed by detecting the capacity to affect the survival of HepG2 and Vero cells. As shown in Figure 2A, the IC_{50} of HMPP for HepG2 and Vero cells was 158.7 μg/mL (458.5 μM) and 250.1 μg/mL (722.4 μM), respectively. Compared with the antibacterial activity *in vitro*, the Safety-Index (SI) of HMPP was up to 634.8. Furthermore, the IC_{50} value of HMPP to inhibit the tail current of hERG channels was much higher than 30 μM (Figure 2B). The positive compound, amitriptyline, had an IC_{50} of 3.46 μM, suggesting that HMPP minimally inhibited hERG channels.

	TA98		TA100		TA1535		TA1537		WP2 uvrA (pKM101)	
	-	+S9	-	+S9	-	+S9	-	+S9	-	+S9
75 μg/mL	0.97	1.11	0.96	0.95	0.6	1.69	0.43	0.95	0.92	0.93
200 μg/mL	1.03	1.7	1.17	1.03	0.8	1.08	1.43	0.74	0.9	0.97
500 μg/mL	1.23	0.98	1.07	0.85	0.9	1.69	1	1.16	0.94	0.93
1250 μg/mL	1.13	0.98	1.04	1.1	1	1.85	0.71	0.74	1.05	1.08
3200 μg/mL	1.08	0.94	0.84	0.89	1	0.62	1.43	1.68	1.07	0.89
8000 μg/mL	0.77	1.15	0.93	0.93	0.5	0.92	1	1.16	1.02	0.86
20000 μg/mL	1.18	1.11	0.82	0.81	1.6	1.08	0.71	0.95	0.82	0.79
50000 μg/mL	1.13	0.89	0.97	0.74	0.9	0.92	1	0.63	0.79	0.81

Table 1: Mutagenic index triggered by various concentrations of HMPP *a, *b; *a the mutagenic indexes were calculated using M (revertants per well of HMPP)/M (revertants per well of vehicle). (n=3), *b the positive controls were as follows: for TA98, 100 μg/mL 2-Nitrofluorene (MI=51.38); for TA98(+S9) (MI=26.17), TA100(+S9) (MI=5.62), A1535(+S9) (MI=8.31), and TA1537(+S9) (MI=6.11), 20 μg/mL 2-aminoanthracene; for TA100 (MI=7.06) and TA1535 (MI=35.30), 10 μg/mL sodium azide; for TA1537 (MI=35.57), 10 μg/mL acridine mutagen ICR-191; for WP2 uvrA (pKM101) (MI=6.76), 10 μg/mL N-methyl-N-nitro-N-nitrosoguanidine; and for WP2 uvrA (pKM101) (+S9) (MI=4.80), 100 μg/mL 2-aminoanthracene (n=3).

The mutagenicity of HMPP was evaluated based on mini-Ames experiments. As shown in Table 1, the mutation index (MI) of HMPP to Salmonella typhimurium (TA98, TA100, TA1535, and TA1537) and Escherichia coli WP2 uvrA (pKM101) was lower than 2.0 whether S9 mix was added or not. There was no apparent cytotoxity or dose-dependent effect observed at any doses with or without S9 mix in any test strains. All positive compounds induced>a 4-fold mutation in the mean number of revertant colonies in the presence or absence of S9 mix, which confirmed the reliability of the results.

Pharmacokinetics of HMPP

The plasma concentration-time courses of HMPP after single IV and po administration to rats are illustrated in (Figure 3).

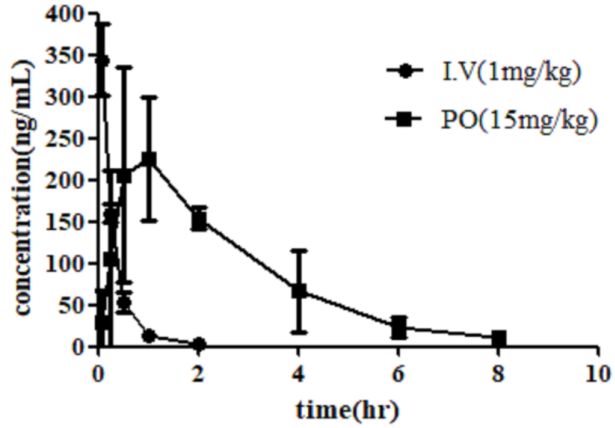

Figure 3: Concentration-time curves of HMPP in SD rat plasma after a single iv (1 mg/kg) and po administration (15 mg/kg).

The plasma concentration-time curve showed that HMPP was disposed quickly in the circulation following iv administration of 1 mg/kg HMPP and deceased within 8 h after po administration of 15 mg/kg HMPP. The pharmacokinetic parameters including half-life ($t_{1/2}$), the time to reach peak concentration (T_{max}), peak concentration (C_{max}), Area under the Concentration-Time Curve (AUC), the apparent volume of distribution during the terminal phase (Vz), Clearance (CLz), Mean Residue Time (MRT) were listed in (Table 2). It was suggested that HMPP exhibited an elimination $t_{1/2}$ of 0.37 ± 0.06 h (IV) and 1.82 ± 0.44 h (po). The C_{maxs} obtained from iv (344.24 ng/L ± 42.43) and po (249.85 ng/L ± 34.98) was lower than the MIC level *in vitro*. The volume of distribution was 4.10 ± 0.88 L/kg, and the clearance was 7.75 ± 0.69 L/h/kg for iv administration. Comparison to iv AUC with oral AUC of HMPP in plasma over time resulted in an oral bioavailability of 27.99 ± 1.93%.

Parameters	IV(1 mg/kg)	PO(15 mg/kg)
t1/2 (h)	0.37 ± 0.06	1.82 ± 0.44
T_{max} (h)	0.083	1.167 ± 0.764
C_{max} (ng/L)	344.24 ± 42.43	249.85 ± 34.98
AUC (0-t) (ng/L.h)	128.06 ± 11.16	695.12 ± 34.98
AUC (0-∞) (ng/L.h)	129.61 ± 11.00	725.64 ± 50.08
Vz (L/kg)	4.10 ± 0.88	-
CLz (L/h/kg)	7.75 ± 0.69	-
MRT (0-∞) (h)	0.3 ± 0.02	2.62 ± 0.73
F (%)	-	27.99 ± 1.93

Table 2: The kinetic parameter of HMPP*a,b,c; *a Data expressed as means ± standard deviation (N=3); b Oral bioavailability: F (%)=[AUC(0-∞) PO/dose po)/(AUC(0-∞)iv/dose iv)] × 100%; c $t_{1/2}$, half-life; T_{max}, the time to reach peak concentration; C_{max}, peak concentration; AUC, area under the concentration-time curve; Vz, the apparent volume of distribution during the terminal phase; CLz, clearance; MRT, mean residue time.

Metabolic stability of HMPP

Metabolic profiling of HMPP was conducted in SD rat plasma after incubation for 1 h. Under the same conditions, the hydrolysis of enalapril (376.45) formed a fragment of 348.39, confirming the reliability of the experiment. The metabolites of HMPP were detected and further characterized by LC-MSn (n=1-2). The structures of the putative metabolites were elucidated by comparative analysis of fragments between the parent and individual metabolites. The results indicated that HMPP was metabolized into pieces via hydrolysis and oxidation. Structures of the putative metabolites and the transformation modes were shown in Figure 4. HMPP was quickly and completely hydrolyzed into M1 and M2, and then 83.36% of M2 was oxidized into M3 and M4. The degradation ratio of M2 was inferred from the ratio of the UV peak areas.

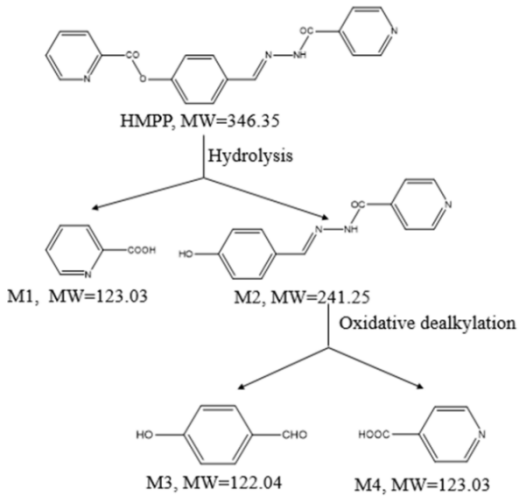

Figure 4: Proposed metabolic pathways of HMPP in rat plasma, HMPP was completely hydrolyzed in plasma forming M1 and M2, and 83.36% of M2 was oxidized to form M3 and M4.

In vivo anti-TB activity of HMPP

The bacteria load of MTB in the lungs 3 d after infection was approximately 60 CFUs, which confirmed the success of the acute MTB infection mice model. The load of MTB reached 1.39×10^4 CFU 10 d later. After administered with HMPP or INH 15 times, there was no significant change in weight compared with the negative groups (data not shown). However, the bacteria load in the lungs declined significantly ($p<0.01$; Figure 5). The log10 CFU of the positive group was a 173.19-fold reduction from 5.58 ± 0.166 (equivalent to 3.83×10^5 CFU) to 3.34 ± 0.874 (equivalent to 2.20×10^3 CFU). While bacteria load of mice administered with HMPP reduced to 4.041 ± 0.379 (25 mg/kg) and 3.521 ± 0.543 (100 mg/kg), demonstrating dose-dependent antibacterial activity *in vivo*.

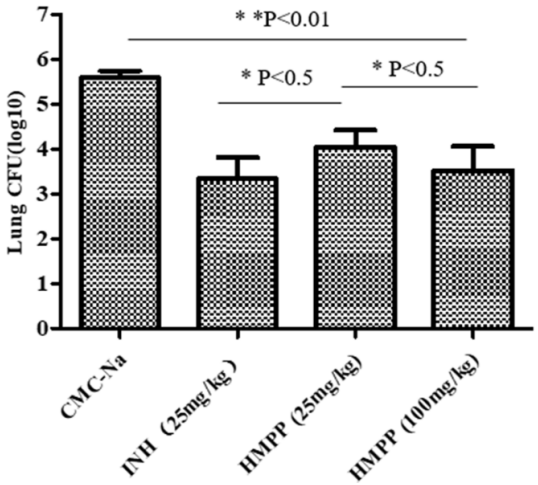

Figure 5: Bacteriostatic activity of HMPP in Balb/c mice. HMPP was administered at concentrations of 25 and 100 mg/kg for 15 d and INH administered at level of 25 mg/kg was regarded as a positive control. **Statistically significant difference from the negative control ($p<0.01$; n=3); *statistically significant difference between mice administered INH and PBIM ($p<0.5$; n=3).

Discussion

The spread of drug-resistant TB has inspired researchers to exploit Anti-TB agents via biochemical assays [21-26]. However, limited success has been achieved, though it facilitates the subsequent structure-relation-ship research on compounds for their unambiguous antimicrobial mechanisms. In contrast, the stereotyped approach to screen for inhibitors at the whole-cell level is still fruitful [27]. Therefore, we believe that it would be efficient to seek lead compounds through cell-based phenotypic screening models. Previous studies suggested that phenotypic screening is valuable, notably in recapitulating relevant biological conditions and can reveal desirable or potentially therapeutic effects linked to a disease [28,29]. Accordingly, we screened through our chemical library using H37Rv for novel compounds, and 85 chemicals with different structures were obtained with potent Anti-TB activities.

It had proven that Schiff bases are important in the development of coordination chemistry due to their ease of synthesis and structural tenability, and their ability to form a wide variety of complexes of chemical, biological and industrial importance [30,31]. As an acyl hydrazone Schiff base derivative, HMPP had 6 hydrogen bond acceptors, only one hydrogen bond donor, 2.60 logP, no stereo center and the drug-likeness score was shown to be 0.74. All of the predicted properties of HMPP adhered to the rule of five [32]. The mutagenicity of HMPP evaluated by mini-Ames experiments indicated that the mean number of his+- and trp+- revertant colonies observed for the solvent control for all the tester strains used in this study was comparable to data obtained from a previous study [33]. In addition, there was no apparent cytotoxity or dose-dependent effect observed at any dose level with or without S9 mix in any test strain.

INH, a first-line anti-TB drug, could be converted to an active intermediate showing antimycobacterial action in MTB. It was shown that INH binded to NAD and further inhibited inhA (2-trans-enoyl-

acyl carrier protein reductase) of MTB, thus blocking the synthesis of mycolic acid, a major lipid of the mycobacterial cell wall [34,35]. In this study, among the 85 compounds, HMPP was shown to have equivalent exoteric antibacterial activity to INH. Moreover, HMPP moderately inhibited the MDR MTB with a MIC of 45 μM, while INH did not inhibit this strain, even at a concentration of 300 μM. Even though INH was contained in the structure of HMPP, INH was not the intermediate or terminal metabolite of HMPP. Therefore, the antibacterial mechanisms of HMPP might be different from INH. To resolve this issue, a conditional mutant MTB should be constructed to detect its sensitivity to HMPP when inhA is expressed at different levels, which is deemed to be the target of INH [36,37]. The fast-complete hydrolysis of HMPP in plasma suggested that HMPP is probably the prodrug of M2 that inhibits MTB *in vivo*, which was demonstrated by the potent bacteriostatic activity of M2 *in vitro* (data not shown). The degradation of HMPP do not produce acethydrazide, which is associated with severe hepatotoxicity in the metabolism of INH *in vivo* [38], indicating that clinical application of HMPP should not cause liver damage in the host.

Conclusion

In conclusion, results of the current study demonstrate that HMPP is a promising candidate to withstand MDR-MTB. Nevertheless, the antimicrobial activity of HMPP to more clinical drug-resistant MTB strains should be evaluated to confirm its validity, and further studies should be undertaken to expound its bacteriostatic mechanisms.

Disclosure of Potential Conflicts of Interest

The authors have declared that no competing interests exist.

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