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## Original Article

# Anti-tuberculosis activity of lipophilic isoniazid derivatives and their interactions with first-line anti-tuberculosis drugs

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## ABSTRACT

**Background:** Three lipophilic isoniazid (INH) derivatives, 1-isonicotinoyl-2-hexadecanoyl hydrazine (INH-C16), 1-isonicotinoyl-2-heptadecanoyl hydrazine (INH-C17) and 1-isonicotinoyl-2-octadecanoyl hydrazine (INH-C18) were chemically synthesized by attaching the INH to a 16, 17 and an 18-carbon hydrophobic moiety respectively. This paper reports the anti-TB activity of these derivatives and their interactions with INH, streptomycin (STR), rifampicin (RIF), and ethambutol (EMB).

**Methods:** The anti-TB activity of these derivatives and the first-line drugs was carried out by determining the minimum inhibitory concentration (MIC) against *Mycobacterium tuberculosis* H37Rv and clinical isolates using tetrazolium microplate assay (TEMA). The interaction study was performed using fixed-ratio method based on TEMAs on *M. tuberculosis* H37Rv.

**Results and discussion:** INH-C16, INH-C17 and INH-C18 were displayed good anti-TB activity against the strains tested. In combination, INH-C16 and INH-C18 showed additive/indifferent interaction with INH and EMB, and synergistic interaction with STR and RIF. INH-C17 showed synergism with RIF and additive/indifferent interaction with INH, STR and EMB.

**Conclusion:** INH-C16, INH-C17 and INH-C18 have the potential to be drugs lead worthy of further investigations.

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## 1. Introduction

*Mycobacterium tuberculosis* is a resilient human pathogen which causes tuberculosis (TB). The modern, standard short-course therapy for TB recommended by World Health Organization (WHO) is based on a combination of at least three first-line anti-TB drug regimen that relies on direct observation of patient compliance to ensure effective treatment.<sup>1</sup> Among the first-line anti-TB agents, isoniazid (INH) is the most prominent

drug. However, in the last decade, the number of INH resistant *M. tuberculosis* strains isolated from TB patients had been increasing at an alarming rate.<sup>1</sup> One of the intrinsic factors contributing to INH resistant in *M. tuberculosis* is the underlying architecture of the bacterial cell envelope.<sup>2,3</sup> The cell wall of *M. tuberculosis* is double-layered, comprising of an inner electron-dense layer of peptidoglycan and an outer electron-transparent layer containing mycolyl arabinogalactan complex and peptidoglycan.<sup>4</sup> In brief, the arabinogalactan

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chains covalently bond to cross-linked peptidoglycan via phosphoryl-*N*-acetylglucosaminosyl-rhamnosyl linkage units and then the arabinogalactan in turn is esterified to  $\alpha$ -alkyl,  $\beta$ -hydroxy mycolic acids.<sup>5,6</sup> Studies reported that the outer layer functions as an exclusion barrier towards hydrophilic drugs, especially INH.<sup>2,3</sup> Thus, the cell wall structure and INH penetration through the lipid domain provide opportunities for rational strategies for development of more effective and less toxic new anti-TB drugs which focused on drug lipophilicity.

Previous studies have shown that chemical modifications of INH by increasing its lipophilic property resulted in enhanced activity of INH against *M. tuberculosis*.<sup>2,7</sup> Encouraged by these studies, three lipophilic INH derivatives were synthesized and investigated for their *in vitro* anti-TB activities. We speculated that these new INH derivatives should easily penetrate the bacterial cell envelope to exert a better inhibitory activity on the growth of the bacteria. This study was also carried out to study the interactions between these INH derivatives with four most common first-line anti-TB drugs: INH, streptomycin (STR), rifampicin (RIF), and ethambutol (EMB). It is hoped that the findings of this study will point to a promising lead compound for future development of alternative therapeutic for INH resistant *M. tuberculosis* strains.

## 2. Materials and methods

### 2.1. Synthesis of 1-isonicotinoyl-2-hexadecanoyl hydrazine (INH-C16), 1-isonicotinoyl-2-heptadecanoyl hydrazine (INH-C17) and 1-isonicotinoyl-2-octadecanoyl hydrazine (INH-C18)

The INH-C16, INH-C17 and INH-C18 were synthesized following the procedure by Besra et al.<sup>8</sup> Dry dichloromethane and 4-dimethylaminopyridine (1.2 eq.) were added to hexadecanoyl chloride, heptadecanoyl chloride and octadecanoyl chloride for synthesis of INH-C16, INH-C17 and INH-C18 respectively, followed by INH (1.1 eq.). Each reaction mixture was stirred at ambient temperature overnight. It was then washed with 2% diluted hydrochloric acid and water. The organic layer obtained was dried over anhydrous magnesium sulphate. The solvent was removed under reduced pressure to afford the crude product, which was purified by column chromatography. Product confirmation was achieved by standard procedures involving IR, <sup>1</sup>H NMR, <sup>13</sup>C NMR, and mass spectroscopy. Fig. 1 displays the chemical structures of INH-C16, INH-C17 and INH-C18 as compared to INH.

### 2.2. Drug solution preparation

INH, STR, RIF, and EMB were obtained commercially from Sigma–Aldrich Chemical Company, United Kingdom. Stock solutions of INH, STR, and EMB were prepared by dissolving in distilled water to obtain a concentration of 1 mg/mL, 3.2 mg/mL, and 12.8 mg/mL respectively. RIF was dissolved in a small amount of dimethyl sulphoxide (DMSO) and then added with sterile distilled water to obtain a stock solution of 4 mg/mL. The derivatives, INH-C16, INH-C17 and INH-C18 were each dissolved in DMSO to obtain a stock solution of 1 mg/mL. These stock solutions were subsequently diluted with distilled

water on the day of experiment to attain the desired working concentrations and then filter-sterilized. For the interaction study, the configuration of drug combinations was based on a fixed-ratio method as described by Fivelman et al.<sup>9</sup> The concentrations of the drugs were prepared so that the MIC value for each drug alone would be at the fifth well of the two-fold serial dilution during the MIC determination assay as described in the following section. The dilutions of each of the two drugs were prepared in fixed-ratios of 0:10, 2:8, 4:6, 5:5, 6:4, 8:2 and 10:0 (in  $\mu$ g/mL). For instance, the seven combinations of INH and INH-C16 were prepared at concentrations of 0:1.25, 0.5:1.0, 1.0:0.75, 1.25:0.625, 1.5:0.5, 2.0:0.25, and 2.5:0 respectively with the first and last solutions being the drug tested individually.

### 2.3. Inoculum preparation

*M. tuberculosis*, strain H37Rv (ATCC 25618) and 7 *M. tuberculosis* clinical isolates (namely TB01, TB02, TB03, TB04, TB05, TB06, and TB07) were used in this study. For the purpose of standardization, a 10 day-old culture grown on Middlebrook 7H10 agar supplemented with 0.5% of glycerol and 10% OADC enrichment at 37 °C in 8% CO<sub>2</sub> was used throughout this study. The culture was then emulsified in 10 mL Middlebrook 7H9 broth supplemented with 0.2% glycerol and 10% ADC and grown for 3 days to reach log phase of growth. The turbidity of the log phase culture was adjusted to McFarland No. 1 standard solution and then further diluted to 1:25 in the Middlebrook 7H9 broth.

### 2.4. MIC value determination for each drug and in combination

The MIC values of the drugs were determined using the Tetrazolium Microplate assay (TEMA) as described by Caviedes et al.<sup>10</sup> The assay was performed in 96-well sterile microplates. Two different drugs either alone or in combination were tested in triplicate three times. Initially, a volume of 200  $\mu$ L of sterile distilled water was added into the outer wells to prevent dehydration of broth during incubation. A volume of 100  $\mu$ L of the enriched Middlebrook 7H9 broth was added into wells 3 until 11 in rows B to G. An equal volume of drug either alone or in combination was added in triplicate into wells in columns 2 and 3. The solutions were serially diluted with multichannel pipette from wells in columns 3 to 4 through to 10. The last 100  $\mu$ L of solutions from wells in column 10 were then discarded. Finally, 100  $\mu$ L of bacterial suspension was added into all the test wells. The wells in column 11 functioned as controls (without any drugs). The plates were sealed and incubated at 37 °C in 8% CO<sub>2</sub> for 5 days. On day 5, 50  $\mu$ L of Tetrazolium-Tween 80 mixture [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-tetrazolium bromide with a concentration of 1 mg/mL in absolute ethanol and 10% Tween 80 at 1:1] was added to well B11 and incubated for 24 h. If well B11 turned from yellow to purple, Tetrazolium-Tween 80 mixture was added to all wells and incubated for another 24 h. If well B11 remained yellow, incubation was continued and the tetrazolium-tween 80 mixture added to wells C11, D11, E11, F11, and G11 on day 7, 9, 11, 13, and 15 respectively. The MIC was defined as the lowest drug concentration that prevented a colour change of Tetrazolium dye from yellow to purple.

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