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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 TEMA 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.¹ 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.¹ One of the intrinsic factors contributing to INH resistant in *M. tuberculosis* is the underlying architecture of the bacterial cell envelope.^{2,3} 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.⁴ 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 α -alkyl, β -hydroxy mycolic acids.^{5,6} Studies reported that the outer layer functions as an exclusion barrier towards hydrophilic drugs, especially INH.^{2,3} 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*.^{2,7} 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.⁸ 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, ¹H NMR, ¹³C 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.⁹ 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 μ 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% CO₂ 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.¹⁰ 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 μ L of sterile distilled water was added into the outer wells to prevent dehydration of broth during incubation. A volume of 100 μ 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 μ L of solutions from wells in column 10 were then discarded. Finally, 100 μ 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% CO₂ for 5 days. On day 5, 50 μ 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.

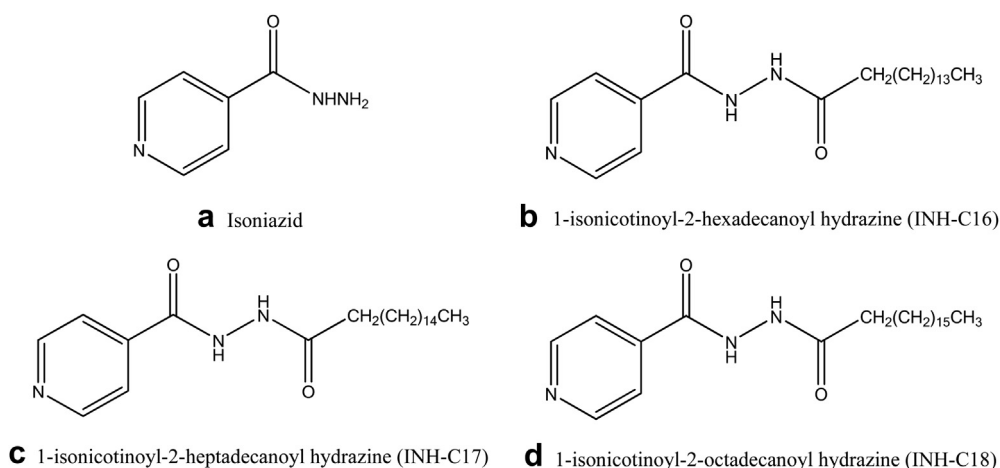


Fig. 1 – The chemical structure of (a) isoniazid and (b–d) its lipophilic derivatives. INH-C16, INH-C17 and INH-C18 were chemically synthesized by augmenting the amine side chain of INH with 16, 17 and 18-carbon acyl chain respectively.

2.5. Assessment of drug combination

Fractional Inhibitory Concentration (FIC) index was calculated to evaluate the drug interactions using the following formula¹¹:

FIC Index : MIC of drug in combination/MIC of drug alone

The sum of the FIC Index (\sum FIC) was calculated as follows¹¹:

$$\sum \text{FIC} : \text{MIC A (in combination)}/\text{MIC A (alone)} \\ + \text{MIC B (in combination)}/\text{MIC B (alone)}.$$

The interaction was expressed as synergistic if the value of \sum FIC \leq 0.5; additive/indifferent if $0.5 < \sum$ FIC \leq 4.0; and antagonistic if \sum FIC $>$ 4.0.

3. Results and discussion

The augmentation of the hydrophilic isoniazid (INH) into a lipophilic compound was achieved by increasing the molecular weight (g/mol) through the addition of hydrophobic hydrocarbon chain at the amine group of INH. The increase in the molecular mass will increase the lipophilicity/hydrophobicity of the compound. In order to further confirm this, the numerical measurement of hydrophobicity, Log $P_{\text{oct/wat}}$ was calculated using the software developed by Molinspiration Chemoin

formatics.¹² The Log $P_{\text{oct/wat}}$ value of 1-isonicotinoyl-2-hexadecanoyl hydrazine (INH-C16), 1-isonicotinoyl-2-heptadecanoyl hydrazine (INH-C17) and 1-isonicotinoyl-2-octadecanoyl hydrazine (INH-C18) is 6.423, 6.928 and 7.433 respectively compared to the INH value of -0.969 . It should be highlighted that Log $P_{\text{oct/wat}}$ of INH has a negative value due to its hydrophilic characteristic. Whereas, Log $P_{\text{oct/wat}}$ of INH-C16, INH-C17 and INH-C18 have positive values due to the presence of hydrophobic moiety which made them more hydrophobic.

The individual MICs of INH-C16, INH-C17, INH-C18, INH, streptomycin (STR), rifampicin (RIF), and ethambutol (EMB) are tabulated in Table 1. The results showed that INH-C16, INH-C17 and INH-C18 lowered the MIC value of their parent compound INH against *M. tuberculosis* H37Rv, thus surpassing the activity of INH by 2-fold. Among the clinical isolates tested, INH-C16 showed lower MIC than INH only in an isolate and INH-C17 and INH-C18 in 2 out of 7 isolates. Hence, it is very apparent that there could be other factors other than hydrophobicity properties which influence the uptake and distribution of an anti-TB drug in *M. tuberculosis*. Such factors could be the structural properties of the compounds and the complex microenvironment within the cell as well as cell wall permeability differences between the strains.

In an earlier report, Rastogi et al⁷ suggested that penetration of molecules through the periplasmic space is influenced by the size of the molecules and the addition of side chains would increase the miscibility of the drug in the lipids of the outer layer

Table 1 – MIC ($\mu\text{g/mL}$) values of INH-C16, INH-C17, INH-C18, INH, STR, RIF, and EMB.

Compound	Molecular weight (g/mol)	Log $P_{\text{oct/wat}}$	<i>M. tuberculosis</i> H37Rv	TB 01	TB 02	TB 03	TB 04	TB 05	TB 06	TB 07
INH-C16	375.55	6.423	0.0391	0.0781	0.0781	0.0391	0.0781	0.156	0.313	0.0098
INH-C17	389.57	6.928	0.0391	0.0781	0.0098	0.156	0.0195	0.0781	0.156	0.0098
INH-C18	403.60	7.433	0.0391	0.625	0.0098	0.313	0.0391	0.0781	0.0391	0.0098
INH	137.14	-0.969	0.0781	0.0195	0.0195	0.0195	0.0195	0.0781	0.0098	0.0195
STR	581.57	–	1.0	0.125	0.0625	0.125	0.5	0.25	0.0625	0.0625
RIF	822.94	–	0.125	0.0156	0.0039	0.0156	0.0156	0.0078	0.0039	0.008
EMB	204.31	–	2.0	1.0	1.0	1.0	0.5	0.125	2.0	2.0

Table 2 – Interactions between first-line anti-tuberculosis drugs and the derivatives against *M. tuberculosis* H37Rv.

Derivative and ratio	INH		STR		RIF		EMB	
	Σ FIC	Interaction	Σ FIC	Interaction	Σ FIC	Interaction	Σ FIC	Interaction
INH-C16								
2:8	1.0	A/I	0.499	Syn	0.25	Syn	1.001	A/I
4:6	0.999	A/I	0.499	Syn	0.251	Syn	0.998	A/I
5:5	0.999	A/I	0.498	Syn	0.25	Syn	0.999	A/I
6:4	1.0	A/I	0.499	Syn	0.251	Syn	2.001	A/I
8:2	0.501	A/I	0.5	Syn	0.5	Syn	1.999	A/I
INH-C17								
2:8	1.0	A/I	1.001	A/I	0.25	Syn	1.999	A/I
4:6	0.999	A/I	0.999	A/I	0.499	Syn	1.999	A/I
5:5	0.999	A/I	0.999	A/I	0.499	Syn	2.0	A/I
6:4	0.507	A/I	0.999	A/I	0.5	Syn	0.999	A/I
8:2	0.501	A/I	1.0	A/I	0.5	Syn	1.0	A/I
INH-C18								
2:8	1.0	A/I	0.499	Syn	0.499	Syn	1.998	A/I
4:6	0.999	A/I	0.499	Syn	0.251	Syn	1.999	A/I
5:5	0.999	A/I	0.498	Syn	0.25	Syn	2.0	A/I
6:4	1.0	A/I	0.499	Syn	0.25	Syn	0.999	A/I
8:2	0.501	A/I	0.5	Syn	0.251	Syn	0.999	A/I

A/I: additive/indifferent; Syn: synergistic.

of mycobacterial cell wall. They also suggested that the side chain added to INH would be metabolized so that the active form of INH liberates inside the bacteria. In a subsequent related study, Rastogi and Goh² also floated the idea that a palmitic acid chain that was attached to the amphipathic INH derivative was possibly utilized as an energy source and liberates the parent INH molecule inside the bacteria, thus, exerts its natural anti-mycobacterial activity. In a similar study, David et al¹³ reported that the highly hydrophobic low-polar drugs are the most active anti-mycobacterial drugs because they could easily dissolved in the lipids of the outer cell wall layer and interact with surface amphiphils. On the basis of these considerations, it is assumed that the lipophilic derivatives were penetrated through the lipophilic periplasmic space of the mycobacterial cell wall and metabolized in such a way that the active INH molecule is released inside the bacteria. Thus, it can be reckoned that the mechanism of action of the INH derivatives on *M. tuberculosis* could be similar to that of their parent INH, which is via the inhibition of mycolic acid synthesis.

With regards to the drug interaction studies, we have used fixed-ratio method because it is easier to conduct and fewer calculations are needed. The Σ FICs of INH-C16, INH-C17 and INH-C18 in combination with first-line drugs are shown in Table 2.

The combinations of INH-C16, INH-C17 and INH-C18 with both INH and EMB showed additive/indifferent interaction at all the combination ratios. Additive/indifferent or no synergistic interaction could be due to the indifferent mechanisms of action of the drugs which is based on the idea that the combined drugs were not interacting, causing only one metabolic pathway to become the growth limiting factor of an organism at a time.¹¹ For instance, Rastogi et al¹⁴ reported that INH in combination with EMB did not show any synergistic activity against *M. tuberculosis* H37Rv because both drugs target the cell wall. INH inhibits the mycolic acid synthesis in the cell wall, whereas EMB inhibits cell wall arabinogalactan

synthesis.¹⁵ Therefore, the additive/indifferent between the derivatives and INH and EMB respectively probably due to the similar target (the cell wall) of these drugs which neither enhance nor hinder their anti-TB activity when combined.

On the other hand, INH-C16 and INH-C18 in combinations with STR and RIF indicated synergism. One of the reasons for synergistic interaction could be due to the contradictory mechanisms of action of the individual drugs.¹⁴ The mechanism of action of STR is via the inhibition of protein synthesis and RIF interferes with RNA synthesis.¹⁵ In the case of INH-C16 and INH-C18, if their target is mycolic acid synthesis, synergism with STR and RIF is expected as the mechanisms of action of these drugs are also totally different. Hence, in combinations, these drugs exert increasing killing effects on the bacterial cells.

INH-C17 showed synergism with RIF but additive/indifferent interaction with STR. This could be due the structure of INH-C17 which might be hindered by the cell wall in the presence of STR. However, author could not obtain a better explanation for such phenomenon.

Moreover, not all *in vitro* drug interactions could be acknowledged meticulously for predicting efficiency of these drugs in combination in clinical practices against TB as these interactions can only provide information about synergistic, additive/indifferent, or antagonistic actions of the drugs in inhibiting the bacterial growth. Therefore, this *in vitro* study should be further assessed with *in vivo* studies for clinical significance against TB.

4. Conclusion

The lipophilic derivatives, INH-C16, INH-C17 and INH-C18 showed a better anti-TB activity against *M. tuberculosis* H37Rv and interacted positively with the first-line drugs. Therefore, they have the potential to be drug leads worthy of further investigations as anti-TB drugs.

Conflicts of interest

All authors have none to declare.

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