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Synthesis and Biological Evaluation of Isoniazid derivatives as potential Anti-Tuberculosis agents

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Abstract:

Due to emerging resistance of Mycobacterium Tuberculosis strains against all the currently used drugs, discovery of novel agents has become important. In the present study, isoniazid based derivatives were synthesized and characterized using various spectroscopic techniques. Antibacterial screening was done against gram positive and gram negative bacteria to evaluate selectivity of molecules, and the molecules were found to be selective for Mycobacterium tuberculosis cells.

Keywords: Antitubercular, novel, Isoniazid derivatives, antibacterial, selectivity

1. Introduction

Tuberculosis (TB) is a major global health problem, causing morbidity and mortality among millions of people each year [1,3]. According to World Health Organization (WHO), around one third of world population is infected with Mycobacterium tuberculosis. India accounts for more than 25% of TB incidence and deaths[2]. Mycobacterium tuberculosis is an obligate aerobic, slow growing, non-spore forming, non-motile and acid fast bacterium, with a unique cell wall structure, containing mycolic acid with high lipid content, which confers resistance to conventional antibiotics.

Current treatment[4,5] involves various first line drugs (Isoniazid, rifampicin, pyrazinamide, and ethambutol) and second-line drugs. The emergence of resistant strains[6-8] that are not affected by almost all currently used anti-tubercular drugs, has left very limited options for TB patients. Thus there is a need for immediate identification of new targets and to ligand scaffolds that can address the emerging resistance by acting through unique mechanism of action to prevent cross resistance.

Redesigning of privileged scaffolds represents one of the possible approaches for development of novel anti-infective agents. Isoniazid[9,10] is one of the front-line drugs of anti-TB therapy and is still one of the most effective drugs. Over the past few years, the use of isoniazid has decreased due to formation of toxic metabolites and development of resistant strains. Various derivatives of Isoniazid have been prepared with the aim of increasing the efficacy and safety. One such example LL-3858 an isoniazid –pyrrole hybrid, currently in clinical

trials. Previous lab work also shows isoniazid derivatives as good hits. Hence this was considered for the current work.

Isoniazid and its derivatives being *N*-containing heterocycles have gained prominence in medicinal chemistry due to their variety of biological activity such as anti-mycobacterial[11], anti-bacterial[12], anti-virus[13], anti-fungal[14], anti-tumor[15,16], anti-analgesic[17], anti-convulsant[18] activities. Amongst the various activities of its derivatives, the anti-TB activity is noteworthy. Isoniazid was selected as starting point and a preliminary set of molecules were synthesized as per the scheme shown in Fig **1**. The molecules were then screened against mycobacterium tuberculosis and were also tested against gram positive and negative bacteria to assess their selectivity. Some of the molecules were found to be selective for mycobacterium tuberculosis bacteria.

2. Experimental

All the chemicals were acquired from Sigma Aldrich, SD fine Chemicals and Spectrochem Ltd and were used without further purification. The purity and structures of the synthesized compounds was confirmed by melting point/boiling point, thin layer chromatography, infrared spectroscopy, nuclear magnetic resonance spectroscopy and mass spectrometry wherever possible. The melting points of the compounds synthesized were uncorrected and recorded by open glass capillary method on Oswald Precision Melting Point.

The general route of synthesis of the designed derivatives is shown in Fig **1**

Fig 1: Synthetic scheme

Step-1: General procedure for condensation of Hydantoin with substituted benzaldehydes

Hydantoin and benzaldehyde (2a-2i) were placed in a round bottom flask and dry piperidine was added. A reflux condenser protected by calcium chloride guard tube was fitted to the flask and the reaction mixture was slowly heated to 130 °C for 30 mins. After completion of the reaction, as indicated by TLC, the reaction mixture was cooled to 60 °C and 20 mL of water was added with stirring. The reaction mixture was cooled to room temperature, transferred to an Erlenmeyer flask and acidified by dropwise addition of conc. hydrochloric acid to pH 1. The reaction mixture was kept at room temperature for few hours, during which benzylidene hydantoin precipitated. The precipitate was collected on a Buchner funnel and washed thoroughly with cold water to obtain derivatives **3a-3i**, which were used for next step without further purification. Compounds **3a-3i** were synthesized as per the general procedure above and were characterized using melting points and FTIR which were complying with respective structures.

Step-2 and 3: General procedure for synthesis of substituted Phenyl pyruvic acid and its condensation with Isoniazid

Benzylidene hydantoin (3a-3i) and a few chips of porcelain were placed in a three-necked round bottom flask, fitted with a reflux condenser and a gas-inlet tube. A slow stream of nitrogen gas was introduced, and 15 ml of 20% aqueous sodium hydroxide solution was added. The mixture was boiled at 170-180 °C for 3 hours under inert atmosphere. After completion of reaction, as indicated by TLC, the reaction mixture was cooled, and acidified with conc. hydrochloric acid to pH 1, without interrupting the stream of nitrogen gas. The mixture was extracted with diethyl ether, and the ether was evaporated on a boiling water bath to obtain the corresponding substituted phenylpyruvic acids (4a-4i), which were unstable, hence used for next step without further purification. Substituted Phenylpyruvic acids (4a-4i) and Isonicotinyl hydrazide (5) were dissolved in 10 mL methanol, and the solution was refluxed for 3 hours. After completion of the reaction, as indicated by TLC, the solvent was evaporated in vacuo, to obtain substituted schiff bases (6a-6i), which were used for next step without further purification.

Phenyl pyruvic acid (4a) and Schiff base (6a):

Benzylidene hydantoin 3a was treated according to **Step-2 & 3** of general procedure to give Schiff base 2-(2-isonicotinoylhydrazono)-3-phenylpropanoic acid. Phenylpyruvic acid (4a): Colour: orange; yield: 0.06 g (10%); R_f DCM:Methanol (19:1): 0.3; IR (KBr): 3466, 1672, 1489 cm^{-1}. Schiff base (6a): Colour: yellow; yield: 0.06 g (60%); R_f DCM:Methanol (9:1): 0.25; Melting point: more than 300 °C; IR (KBr): 3432, 1735, 1664, 1604, 1553, 1502 cm^{-1}.

4-Hydroxyphenylpyruvic acid (4b) and 4-hydroxy Schiff base (6b):

4-hydroxybenzylidene hydantoin 3b was treated according to **Step-2 & 3** of general procedure to give Schiff base 2-(2-isonicotinoylhydrazono)-3-(4-hydroxyphenyl)propanoic acid. 4-hydroxyphenylpyruvic acid (4b): Colour: yellowish-red; yield: 0.2 g (38%); R_f DCM:Methanol (19:1): 0.32; IR (KBr): 3449, 1724, 1672, 1604, 1510 cm^{-1}. 4-hydroxy Schiff base (6b): Colour: yellow; yield: 0.15 g (50%); R_f DCM: Methanol (9:1): 0.28; Melting point: more than 300 °C; IR (KBr): 3449, 3043, 1690, 1613, 1600, 1510 cm^{-1}.

4-Methoxyphenylpyruvic acid (4c) and 4-methoxy Schiff base (6c):

4-Methoxybenzylidene hydantoin was treated according to **Step-2 & 3** of general procedure to give Schiff base 2-(2-isonicotinoylhydrazono)-3-(4-methoxyphenyl) propanoic acid. 4-methoxyphenylpyruvic acid (4c): Colour: yellowish-red; yield: 0.021 g (10%); R_f DCM:Methanol (19:1): 0.32; IR (KBr): 3458, 2928, 2943, 1681, 1604, 1510 cm^{-1}. 4-methoxy Schiff base (6c): Colour: orange; yield: 0.01 g (30%); R_f DCM:Methanol (9:1): 0.3; Melting point: more than 300 °C ; IR (KBr): 3492, 1719, 1672, 1604, 1510 cm^{-1}.

4-Methylphenylpyruvic acid (4d) and 4-methyl Schiff base (6d)*:*

4-Methylbenzylidene hydantoin was treated according to **Step-2 & 3** of general procedure to give Schiff base 2-(2- isonicotinoylhydrazono)-3-(4-methylphenyl)propanoic acid. 4-methylphenylpyruvic acid (4d): Colour: buff; yield: 0.58 g (50%); R_f DCM:Methanol (19:1): 0.35; IR (KBr): 3479, 1681, 1613, 1514 cm^{-1}. 4-methyl Schiff base (6d): Colour: buff; yield: 0.6 g (60%); R_f DCM:Methanol (9:1): 0.3; Melting point: more than 300° C; IR (KBr): 3415, 1688, 1604, 1553, 1513 cm^{-1}.

4-Chlorophenylpyruvic acid (4e) and 4-chloro Schiff base (6e):

4-Chlorobenzylidene hydantoin was treated according to **Step-2 & 3** of general procedure to give Schiff base 2-(2- isonicotinoylhydrazono)-3-(4-chlorophenyl)propanoic acid. 4-chlorophenylpyruvic acid (4e): Colour: brown; yield: 0.9 g (64%); R_f DCM:Methanol (19:1): 0.35; IR (KBr): 3261, 1719, 1596, 1489 cm^{-1}. 4-chloro Schiff base (6e): Colour: brown; yield: 0.9 g (63%); R_f DCM:Methanol (9:1): 0.32; Melting point: 310 °C; IR (KBr): 3432, 1702, 1656, 1604,1549 cm^{-1}.

4-Fluorophenylpyruvic acid (4f) and 4-fluoro Schiff base (6f):

4-Fluorobenzylidene hydantoin was treated according to **Step-2 & 3** of general procedure to give Schiff base 2-(2- isonicotinoyl hydrazono)-3-(4-fluorophenyl)propanoic acid. 4-fluorophenylpyruvic acid (4f): Colour: buff; yield: 0.05 g (6%); R_f DCM:Methanol (19:1): 0.34; IR (KBr): 3475, 1702, 1604, 1506 cm^{-1}. 4-fluoro Schiff base (6f): Colour: yellow; yield: 0.05 g (63%); R_f DCM:Methanol (9:1): 0.32; Melting point: 345 °C; IR (KBr): 3304, 1688, 1604, 1553, 1500 cm^{-1}.

4-Trifluoromethylphenylpyruvic acid (4g) and 4-trifluoromethyl Schiff base (6g)*:*

4-Trifluoromethyl benzylidene hydantoin was treated according to **Step-2 & 3** of general procedure to give Schiff base 2-(2-isonicotinoylhydrazono)-3-(4- trifluoromethylphenyl)propanoic acid. 4-trifluoromethylphenylpyruvic acid (4g): Colour: green; yield: 0.86 g (68%); R_f DCM:Methanol (19:1): 0.35; IR (KBr): 3462, 1694, 1608, 1514 cm^{-1}. 4-trifluoromethyl Schiff base (6g): Colour: yellow; yield: 0.9 g (69%); R_f DCM:Methanol (9:1): 0.30; Melting point: more than 300 °C; IR (KBr): 3308, 1694, 1664, 1604, 1557 cm^{-1}.

4-Nitrophenylpyruvic acid (4h) and 4-nitro Schiff base (6h):

4-Nitrobenzylidene hydantoin was treated according to **Step-2 & 3** of general procedure to give Schiff base 2-(2- isonicotinoylhydrazono)-3-(4-nitrophenyl)propanoic acid (6h): Colour: brown; yield: 0.7 g (75%); R_f DCM:Methanol (9:1): 0.28; Melting point: more than 300 °C; IR (KBr): 3437, 1699, 1604, 1502, 1309 cm^{-1}.

3-Chlorophenylpyruvic acid (4i) and 3-chloro Schiff base (6i):

3-Chlorobenzylidene hydantoin was treated according to **Step-2 & 3** of general procedure to give Schiff base 2-(2- isonicotinoylhydrazono)-3-(3-chlorophenyl)propanoic acid. 3-chloro Schiff base (6i): Colour: yellow; yield: 0.4 g (55%); R_f DCM:Methanol (9:1): 0.32; Melting point: more than 300 °C; IR (KBr): 3313, 1694, 1660, 1625, 1513 cm^{-1}.

Step-4: General procedure for reduction of Schiff bases to form substituted2-(2-isonicotinoylhydrazinyl)-3-phenylpropanoic acid*:*

Schiff bases (6a-6i) were dissolved in methanol and Sodium borohydride ($NaBH_4$) was added. The mixture was stirred at room temperature for 2 hours. After completion of reaction, as indicated by TLC, solvent was evaporated in vacuo, and the product was washed with acetone, to obtain final products **7a-7i**.

Synthesis of 2-(2-isonicotinoylhydrazinyl)-3-phenylpropanoic acid (7a)

Schiff base **6a** was reduced in presence of sodium borohydride (0.008 g, 0.175 mmol) according to **Step-4** of general procedure to give 2-(2-isonicotinoylhydrazinyl)-3-phenylpropanoic acid (7a). *Spectral and physical data*: Colour: yellow; Yield: 0.06 g (100%); R_f DCM:Methanol (9:1): 0.11; Melting point: more than 300 °C; IR (KBr): 3227, 2920, 1741, 1672, 1600, 1514 cm^{-1}.

Synthesis of 3-(4-hydroxyphenyl)-2-(2-isonicotinoylhydrazinyl)propanoic acid (7b): 4-Hydroxy Schiff base was reduced in presence of sodium borohydride (0.021 g, 0.55 mmol) according to **Step-4**of general procedure to give 3-(4-hydroxyphenyl)-2-(2- isonicotinoylhydrazinyl)propanoic acid (7b). *Spectral and physical data*: Colour: brown; Yield: 0.15 g (100%); R_f DCM:Methanol (9:1): 0.1; Melting point: more than 300° C; IR (KBr): 3411, 2924, 1688, 1608, 1510 cm^{-1}.

Synthesis of 3-(4-methoxyphenyl)-2-(2-isonicotinoylhydrazinyl)propanoic acid (7c): 4-Methoxy Schiff base was reduced in presence of sodium borohydride (0.002 g, 0.047 mmol) according to **Step-4** of general procedure to give 3-(4-methoxyphenyl)-2-(2- isonicotinoylhydrazinyl)propanoic acid (7c). *Spectral and physical data*: Colour: yellow; Yield: 0.01 g (100%); R_f DCM:Methanol (9:1): 0.11; Melting point: more than 300°C; IR (KBr): 3402, 3027, 1942, 1688, 1625, 1544 cm^{-1}.

Synthesis of 3-(4-methylphenyl)-2-(2-isonicotinoylhydrazinyl)propanoic acid (7d): 4-Methyl Schiff base was reduced in presence of sodium borohydride (0.114 g, 3.03 mmol) according to **Step-4** of general procedure to give 3-(4-methylphenyl)-2-(2- isonicotinoylhydrazinyl)propanoic acid (7d). *Spectral and physical data*: Colour: buff; Yield: 0.6 g (100%); R_f DCM:Methanol (9:1): 0.12; Melting point: 320° C; IR (KBr): 3415, 3026, 1664, 1630, 1540 cm^{-1}.

Synthesis of 3-(4-chlorophenyl)-2-(2-isonicotinoylhydrazinyl)propanoicacid (7e):

4-Chloro Schiff base was reduced in presence of sodium borohydride (0.16 g, 4.2 mmol) according to **Step-4** of general procedure to give 3-(4-chlorophenyl)-2-(2- isonicotinoylhydrazinyl)propanoic acid (7e). *Spectral and physical data*: Colour: brown; Yield: 0.9 g (100%); R_f DCM:Methanol (9:1): 0.14; Melting point: more than 300° C; IR (KBr): 3419, 3039, 1681, 1596, 1538 cm^{-1}.

Synthesis of 3-(4-fluorophenyl)-2-(2-isonicotinoylhydrazinyl)propanoicacid (7f):

4-Fluoro Schiff base was reduced in presence of sodium borohydride (0.009 g, 0.25mmol) according to **Step-4** of general procedure to give 3-(4-fluorophenyl)-2-(2- isonicotinoylhydrazinyl)propanoic acid (7f). *Spectral and physical data*: Colour: yellow; Yield: 0.05 g (100%); R_f DCM:Methanol (9:1): 0.12; Melting point: more than 300 °C; IR (KBr): 3407, 3026, 1672, 1625, 1544 cm^{-1}.

Synthesis of 3-(4-trifluoromethylphenyl)-2-(2-isonicotinoylhydrazinyl)propanoic acid (7g):

4-Trifluoromethyl Schiff base was reduced in presence of sodium borohydride (0.15 g, 3.8 mmol) according to **Step-4** of general procedure to give 3-(4-trifluoromethylphenyl)-2-(2-isonicotinoylhydrazinyl)propanoic acid (7g). *Spectral and physical data*: Colour: green; Yield: 0.9 g (100%); R_f DCM:Methanol (9:1): 0.13; Melting point: more than 300 °C; IR (KBr): 3240, 2924, 1737, 1672, 1600, 1514 cm^{-1}.

Synthesis of 3-(4-nitrophenyl)-2-(2-isonicotinoylhydrazinyl)propanoic acid (7h):

4-Nitro Schiff base was reduced in presence of sodium borohydride (0.12 g, 3.2 mmol) according to **Step-4** of general procedure to give 3-(4-nitrophenyl)-2-(2- isonicotinoylhydrazinyl)propanoic acid (7h). *Spectral and physical data*: Colour: brown; Yield: 0.7 g (100%); R_f DCM:Methanol (9:1): 0.11; Melting point: more than 300 °C; IR (KBr): 3419, 3039, 1681, 1596, 1536, 1322 cm^{-1}.

Synthesis of 3-(3-chloro phenyl)-2-(2-isonicotinoyl hydrazinyl)propanoicacid (7i):

3-Chloro Schiff base which was reduced in presence of sodium borohydride (0.07 g, 1.88 mmol) according to **Step-4** of general procedure to give 3-(3-chlorophenyl)-2-(2-isonicotinoylhydrazinyl)propanoic acid (7i). *Spectral and physical data*: Colour: yellow; Yield: 0.4g (100%); R_f DCM:Methanol (9:1): 0.15; Melting point: more than 300° C; IR (KBr): 3411, 3035, 1677, 1596 cm^{-1}.

3. Results and discussion:

3.1. Chemistry

The different isoniazid derivatives have been obtained by the method described in general procedure. Appropriately, substituted phenyl pyruvic acids were reacted with Isonicotinyl hydrazide resulting in several Schiff bases. These Schiff bases were reduced using sodium borohydride to give **7a-7i** in good to excellent yields. All the compounds were identified by spectral data. In general, the NMR spectrum of representative compound **7h** (^{1}H NMR and D_2O exchange spectra) shows integration of 8 protons in the aromatic region 7.9 ppm to 8.4 ppm. The carboxylic acid (COOH) proton and NH proton (adjacent to C=O) are observed as superimposed at 13 ppm. The CH proton and NH proton (adjacent to CH) are observed at at 1.1 ppm. The CH_2 proton is observed at 3.5 ppm. The mass spectrum of representative compound **7h** shows M+1 peak (base peak) at 330.9 (positive ionization mode). The other peaks observed are 354.1 (sodium adduct), 314.1 (loss of OH, α-fission).

3.2. Biological evaluation

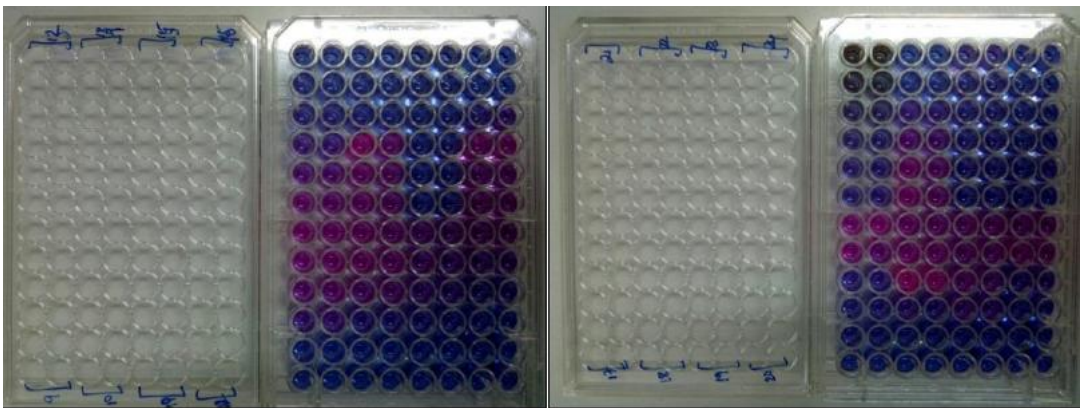
The Minimum Inhibitory Concentration of synthesized molecules was determined in duplicates by

microbroth dilution method i.e. Resazurin microtitre assay method (REMA) [19-21] a microbroth dilution technique. It is seen that molecules with Carboxylic acid group (COOH) (7a-7i) are showing good to moderate activity as shown in Table 1. It is also seen that molecules with H-bonding substituent (7b) show good activity, while even though molecule with nitro (-NO_2) substituent and Schiff base show good activity, these substituents are generally reported to be toxic. Hence, 7b (-OH Substituent) was found to be the best molecule in the series. It is also seen that substitution at 3-position (3-Cl) decreases activity as compared to substitution at 4-position (4-Cl).

Also, to assess the activity of compounds against various microbial strains, all the compounds were evaluated against an array of microorganisms including *S.aureus* (Gram positive bacterium) and *E.coli* (Gram negative bacterium) using 96 well plate micro-titre assay. All the compounds were inactive upto 125 µg/ml against both *S.aureus* and *E.coli*, as shown in Table 2 as InhA is present only in *Mycobacterium tuberculosis* for synthesis of mycolic acid. Thus all the compounds were found to be specific for *Mycobacterium tuberculosis*.

Table 1: Anti tubercular activity

S.No.	Molecule	*Mtb* activity
1	7a	219.29 µM
2	7b	12.97 µM
3	7c	198.41 µM
4	7d	104.51 µM
5	7e	195.61 µM
6	7f	412.54 µM
7	7g	88.52 µM
8	7h	≤2.95 µM
9	7i	391.23 µM
10	Isoniazid	≤7.12 µM
11	Streptomycin	≤1.67 µM



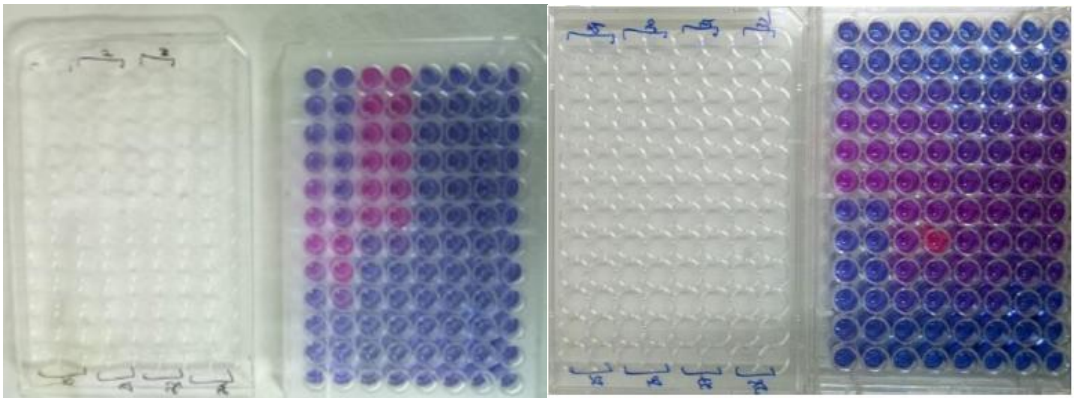


Table 2: Anti-bacterial activity

S.No.	Molecule	*S.aureus* (µg/ml)	*E.coli* (µg/ml)
1	7a	>125	>125
2	7b	>125	>125
3	7c	>125	>125
4	7d	>125	>125
5	7e	>125	>125
6	7f	>125	>125
7	7g	>125	>125
8	7h	>125	>125
9	7i	>125	>125
10	Streptomycin	<7.81	<7.81
11	Kanamycn	<7.81	<7.81

4. Conclusion:

In the present work Isoniazid derivatives with substituted phenylpyruvic acid were designed taking into consideration the previously reported Isoniazid derivatives. These molecules were successfully synthesized and characterized using spectroscopic techniques. In conclusion, the preliminary anti mycobacterial activities study of 9 compounds based on the coupling of isoniazid with different substituted phenyl pyruvic acids described here suggest that they may be selectively targeted to *Mycobacterium tuberculosis* growth. The compounds exhibited activity against *Mycobacterium tuberculosis* in the range ≤2.95 µM to 400 µM when compared with first line drugs such as isoniazid (INH) and could be a good starting point for further studies as well as find new lead compounds.

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