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5-Nitro-2-furoic acid hydrazones: Design, synthesis and in vitro antimycobacterial evaluation against log and starved phase cultures

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ABSTRACT

Various 5-nitro-2-furoic acid hydrazones were synthesized and evaluated for in vitro activities against log and starved phase culture of two mycobacterial species and *Mycobacterium tuberculosis* (MTB) isocitrate lyase (ICL) enzyme inhibition studies. Among twenty one compounds, 5-nitro-*N*′-[(5-nitro-2-furyl)methylidene]-2-furohydrazide (**4o**) was found to be the most active compound in vitro with MICs of 2.65 and 10.64 μM against log- and starved-phase culture of MTB. Compound **4o** also showed good enzyme inhibition of MTB ICL at 10 μM. The docking studies also confirmed the binding potential of the compounds at the ICL active site.

Mycobacterium tuberculosis (MTB) infects about 32% of the world's population. Every year, approximately 8 million of these infected people develop active tuberculosis (TB) and almost 2 million of these will die from the disease (WHO, 2005).[1] Despite 40 years of anti-TB chemotherapy, tuberculosis remains one of the leading infectious diseases worldwide. Among the main obstacles to the global control of the disease are the HIV epidemic that has dramatically increased the risk for developing active TB, the increasing emergence of multi-drug resistant TB (MDR-TB) and the recalcitrance of persistent infections to treatment with conventional anti-TB drugs.[2] The situation is exacerbated by the increasing emergence of extensively drug-resistant (XDR) TB.[3] Current chemotherapy for TB largely relies on drugs that inhibit bacterial metabolism with a heavy emphasis on inhibitors of the cell wall synthesis.[4] According to their mode of action, first and second line TB drugs can be grouped as cell wall inhibitors (isonizid (INH), ethambutol, ethionamide, cycloserine), nucleic acid synthesis inhibitors (rifampicin (RIF), quinolones), protein synthesis inhibitors (streptomycin, kanamycin) and inhibitors of membrane energy metabolism (pyrazinamide). Existing TB drugs are therefore only able to target actively growing bacteria through the inhibition of cell processes such as cell wall biogenesis and DNA replication. This implies that current TB chemotherapy is characterized by an efficient bactericidal activity but an extremely weak sterilizing activity, defined as the ability to kill the slowly growing or slowly metabolizing bacteria that persist after the growing bacteria have been killed by bactericidal drugs. Sterilizing activity also describes the ability to eliminate latent or 'dormant' bacteria that survive inside the host macrophages. This bias is hardly surprising as anti-TB drugs have traditionally been identified by their ability to suppress or kill replicating cultures of bacteria in vitro. The weak sterilizing property of available TB drugs is one of the major drawbacks for current TB chemotherapy.

Recently we reported some 5-nitrofuran-2-yl derivatives[5] and 2-[3-(4-bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phthalazinyl]-acetic acid hydrazones[6] with activity against both dormant and actively growing MTB. Tangallapally et al., reported some novel nitrofuranyl amides with potent anti-TB activity.[7] In this work we have designed (Fig. 1) and synthesized novel 5-nitrofuranyl-2-acid hydrazones and screened their activity against both growing and dormant mycobacterial species.

5-Nitrofuroic acid (**1**) is converted to its ethyl ester (**2**) by refluxing the acid **1** in absolute ethanol in presence of sulfuric acid. Ethyl ester (**2**) (1 mol) on treatment with hydrazine hydrate (2 mol) yielded acid hydrazide (**3**). This hydrazide which upon reacted with various carbonyl compounds in presence of glacial acetic acid at a pH 4–6 afforded the titled compounds **4a–u** (Scheme 1) in 52–89% yields.[6] The reaction utilizes the microwave irradiation in an unmodified domestic microwave oven at 80% intensity with 30 s/cycle for 3 min. The resultant solid was washed with dilute ethanol dried and recrystallized from ethanol–chloroform mixture. The purity of the synthesized compounds

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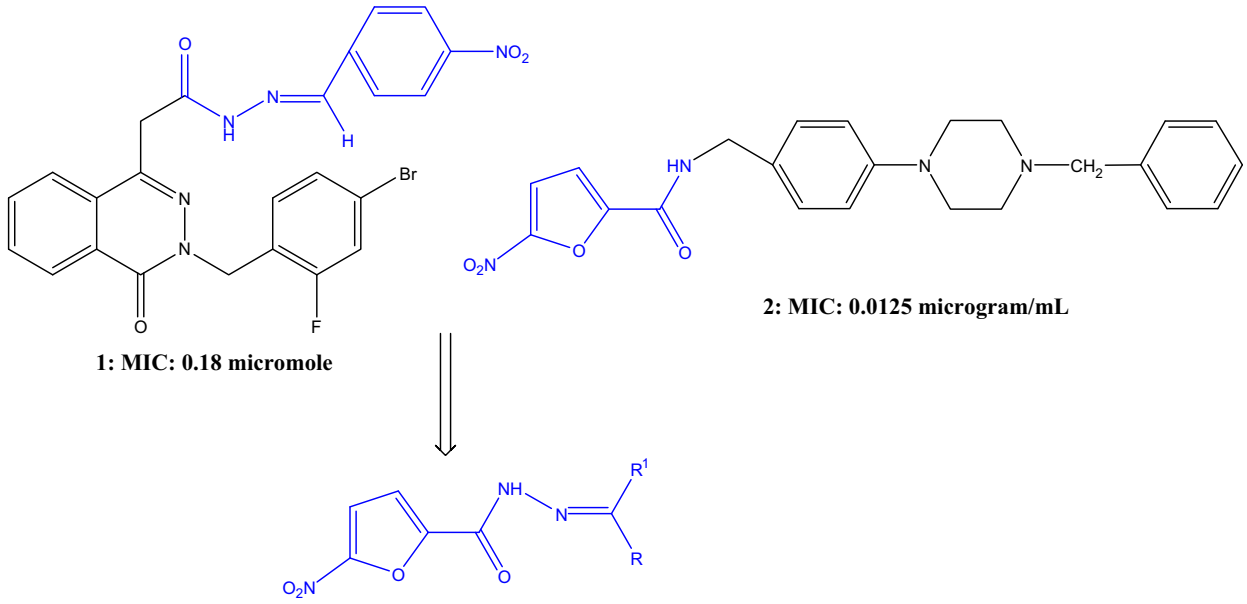
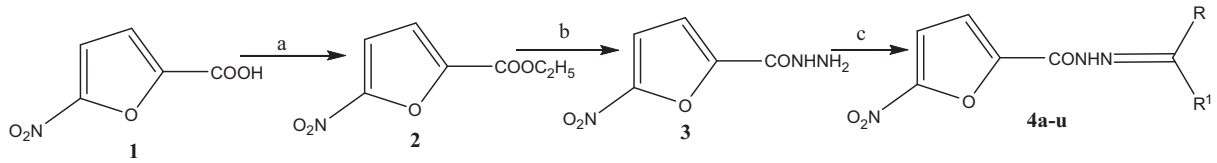


Figure 1. Development of 5-nitrofuranyl-2-acid hydrazones.



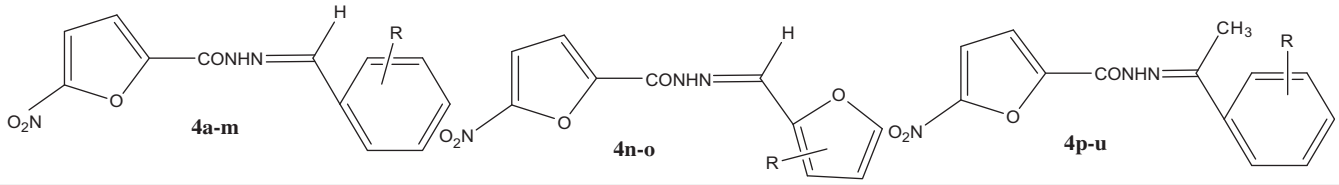
Scheme 1. Reagents: (a) H_2SO_4, C_2H_5OH; (b) $NH_2NH_2{\cdot}H_2O$; (c) CH_3COOH.

was monitored by thin layer chromatography (TLC) and elemental analyses and the structures were identified by spectral data.[8]

In the first phase the compounds were screened for their in vitro antimycobacterial activity against log-phase cultures of MTB, and *Mycobacterium smegmatis* ATCC 14468 (MS) by agar dilution method similar to that recommended by the National Committee for Clinical Laboratory Standards[9] for the determination of MIC in duplicate. The minimum inhibitory concentration (MIC) is defined as the minimum concentration of compound required to give complete inhibition of bacterial growth. MICs of the synthesized compounds along with the standard drugs for comparison are reported (Table 1). In the initial screening against log-phase MTB, the newer compounds showed good activity with MICs ranging from 2.65 to 48.22 μM. Two compounds (**4k** and **4o**) showed excellent activity with MIC of <5 μM. When compared to INH (MIC: 0.72 μM) and RIF (MIC: 0.48 μM) all the compounds were less active. Compound 5-nitro-*N*′-[(5-nitro-2-furyl)methylidene]-2-furohydrazide (**4o**) was found to be the most active compound in vitro with MIC's of 2.65 μM against MTB. With respect to structure-MTB activity, substituents with electron-withdrawing groups like nitro and halogens on the benzaldehyde, actophenone and furanyl derived acid hydrazones (**4a–u**) enhanced the activity. Similarly substituents like hydroxyl, and methoxy groups reduce the activity. With respect to the carbimino terminal, the order of activity was found to be (sub)acetophenone > (sub)benzaldehyde. Among furanyl derivatives (**4n** and **4o**) introduction of nitro group at 5th position enhances the activity ~9 times. All the compounds were also screened for atypical mycobacteria MC_2 which infects lungs.[10] The synthesized compounds inhibited MC_2 with MICs ranging from 0.68 to 192.0 μM and 10 compounds were more potent than INH (MIC: 45.57 μM) and one compound was more potent than RIF (MIC: 1.89 μM). Compound **4o** was found to be 67 and 2.7 times more potent than INH and RIF, respectively, against log-phase culture of MC_2.

The compounds which showed good activity against log-phase culture of MTB and MC_2 were further screened against six-week-starved cells of MTB and MC_2 according to the literature procedure in duplicate and MICs were reported in Table 1.[11] Several in vitro model systems have been proposed to mimic the conditions found in the human chronic tuberculosis lesion (a granuloma), including oxygen starvation[12] nutrient deprivation[13] and rifampicin-induced persistence.[14] Development of a screen under carbon-starvation conditions is feasible and less challenging than for oxygen deprivation. Prolonged deprivation of nutrients results in a marked slowing of bacterial growth and concomitant phenotypic antibiotic resistance.[15] As bacteria can easily grow upon being returned to nutrient-rich media, this model allows easy quantification of antibiotic effectiveness. Against MTB, nine compounds were tested and they inhibited starved culture of MTB with MIC's ranging from 10.64 to 91.49 μM (Table 1). INH and RIF had poor activity against starved cells with MICs of >91.14 and >15.18 μM. All the nine tested compounds were more potent than INH and one compound was found to be more potent than RIF. The presence of persistent and dormant MTB is thought to be the cause for the lengthy TB chemotherapy, since the current TB drugs are not effective in eliminating persistent or dormant bacilli. Therefore, these drugs active against slowly growing or non-growing persistent bacilli are thought to be important to achieve a shortened therapy. In the case of starved MC_2 culture, the all the tested compounds inhibited with MICs ranging from 5.30 to 48.22 μM and were more potent than INH (MIC: >91.14 μM). Five compounds were found to be more potent than RIF (MIC: >15.18 μM). Compound *N*′-[1-(4-bromophenyl)ethylidene]-5-nitro-2-furohydrazide (**4r**) was found to be most active compound with MIC of 4.43 against starved MS.

Table 1
Physical constants and in vitro antimycobacterial and cytotoxicity of the compounds



Entry	R	Yield (%)	Mp (°C)	CC_{50}[a]	MIC in log phase[a]		MIC in starved phase MTB[a]	MIC in acetate for MC_2[a]
					MTB	MC_2		
4a	H	89	197	NT	48.22	192	NT	48.22
4b	4-NO_2	88	237	NT	20.55	82.23	NT	20.55
4c	4-Cl	84	214	NT	21.28	42.56	NT	10.65
4d	4-Br	89	180	NT	18.48	73.94	NT	36.97
4e	4-F	86	224	NT	22.54	90.19	NT	45.09
4f	4-OH	86	>250	NT	>45.42	>45.42	NT	45.42
4g	4-CH_3	84	198	>228.74	11.45	45.74	91.49	22.87
4h	4-OCH_3	60	120	NT	21.60	43.20	NT	21.60
4i	3-Br	77	156	NT	18.48	36.97	NT	36.97
4j	2-NO_2	89	174	205.57	10.29	10.29	41.11	10.29
4k	2-CF_3	89	170	>191.01	4.76	4.76	19.10	9.56
4l	4-OH,3-OCH_3	88	228	NT	40.97	81.94	NT	20.48
4m	2,6-$(Cl)_2$	87	220	NT	19.04	76.18	NT	38.09
4n	H	76	182	NT	25.08	50.16	NT	25.08
4o	5-NO_2	87	227	212.52	2.65	0.68	10.64	5.30
4p	H	87	176	>228.74	11.45	45.74	45.74	45.74
4q	4-NO_2	71	218	>196.52	9.84	19.65	19.65	19.65
4r	4-Br	52	170	>177.50	8.88	17.75	17.75	4.43
4s	4-F	66	152	214.64	5.35	21.46	21.46	42.92
4t	4-CH_3	76	168	>217.57	10.89	43.51	43.51	43.51
4u	2-OH	85	194	NT	>43.21	>43.21	NT	>43.21
Isoniazid				>455.73	0.72	45.57	>91.14	>91.14
Rifampicin				>75.94	0.48	1.89	>15.18	>15.18

[a] CC_{50} and MIC in (μM), NT indicates not tested.

As these synthesized compounds showed activity against dormant mycobacterium, we decided to explore the possible mechanism by screening some compounds against MTB ICL enzyme. ICL is an important enzyme in the glyoxylate cycle during carbohydrate starvation in MTB it catalyzes the cleavage of isocitrate to glyoxylate and succinate, allowing the organisms to survive on acetate or fatty acids.[16] The glyoxylate cycle is not present in higher animals, and due to its necessity for survival for the persistent phase of the infection, ICL is considered an ideal drug target for persistent MTB. Several small-molecule inhibitors have been described[17] as MTB ICL inhibitors; however, none has been developed as a drug for MTB. Isocitrate lyase activity was determined at 37 °C by measuring the formation of glyoxylate-phenylhydrazone in the presence of phenylhydrazine and isocitrate lyase at 324 nm based on the method described.[18] The compounds were screened with a single concentration of 10 μM and percentage inhibition of the screened compounds along with the standard MTB ICL inhibitor 3-nitropropionic acid (3-NP) (at 100 μM) for comparison are reported (Table 2). All the nine compounds inhibited MTB ICL with percentage inhibition ranging from 19.82% to 86.8% at 10 μM. Two compounds (**4o, 4r**) showed more than 50% inhibition and all these compounds were found to be more potent than standard 3-NP at the dose level tested. Further investigation could provide lead compounds for drug development against persistent tuberculosis.

Some compounds were further examined for cytotoxicity (CC_{50}) in a mammalian Vero cell line at single concentration of 62.5 μg/mL. After 72 h of exposure, viability was assessed on the basis of cellular conversion of MTT into a formazan product using the Promega Cell Titer 96 non-radioactive cell proliferation assay.[19] Most of the compounds were not cytotoxic to Vero cells.

The crystal structure of MTB ICL bound with inhibitor, 3-nitropropionate was taken from the Protein Data Bank (PDB entry 1F8I Cys191 mutated to Ser191) and bromopyruvate (PDB entry 1F8M) were used for docking. Docking was carried out using GOLD,[20] which uses the genetic algorithm (GA). For each of the 100 independent GA runs, a maximum number of 100,000 GA operations were performed, whereby all variables for the GA were set to their default values. Default cutoff values of 2.9 Å (dH-X) for hydrogen bonds and 6.0 Å for van der Waals were employed. When the top three solutions attained rmsd values within 1.5 Å, GA docking was terminated. The GOLD docking run showed very strong electrostatic and hydrophobic pattern of interaction for the ligand (Fig. 2), very well comparable to interaction pattern observed with both inhibitors bromopyruate and 3-nitropropionic acid crystallized with MTB ICL, indicating a high affinity to the enzyme. The following interactions were observed for ligand docked to mutated protein (Cys 191 to Ser 191, PDB entry 1F8I), nitro group of nitrofuroic acid moiety forms multicentred trifurcated H-bonding with Arg 228 and Tyr 89 (ARG228:NH12···NFA:O11, 2.27 Å; ARG228:NH11···NFA:O11,

Table 2
Inhibitory activities of selected compounds and 3-nitropropionic acid against MTB ICL

Compd	% Inhibition (μM)
4g	19.82 (10)
4j	46.80 (10)
4k	30.12 (10)
4o	86.8 (10)
4p	34.86 (10)
4q	48.90 (10)
4r	73.12 (10)
4s	28.13 (10)
4t	32.54 (10)
3-NP	63.2 (100)

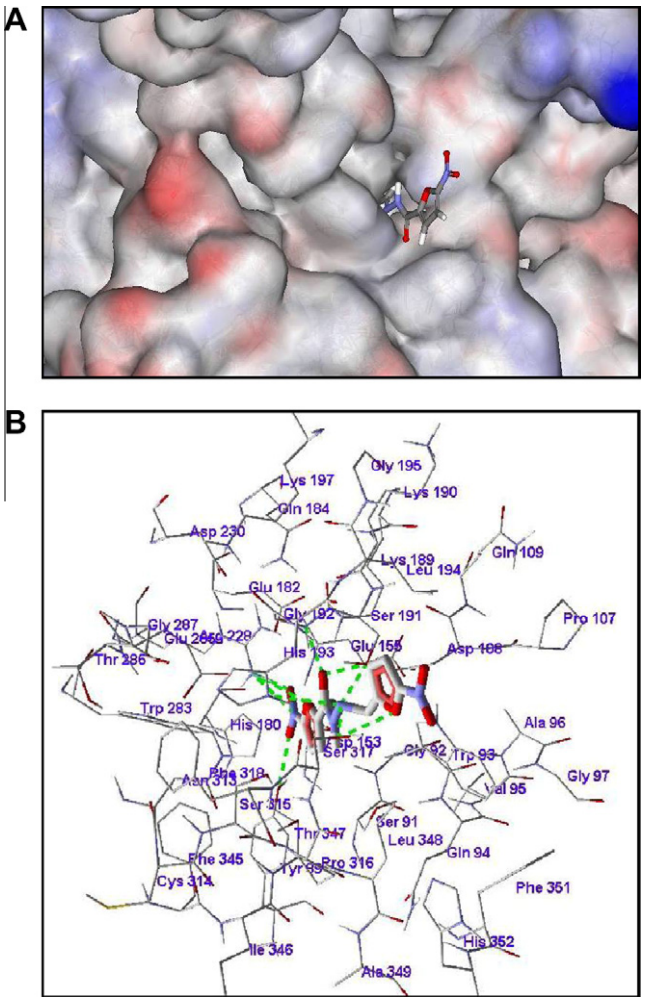


Figure 2. (A) Ligand docked inside the active site pocket of the MTB ICL Cys191 muteated to Ser191. (B) Binding mode of ligand with the MTB ICL (Cys191 mutated to Ser191) only the residues in the active site were shown (residues within a distance of less than 11.0 Å are shown), hydrogen bonds are displayed by dotted green lines; Mg^{2+} is shown as black sphere.

2.53 Å; TYR89:OH···NFA:O10, 2.39 Å). The oxygen of nitrofuroic acid group forms H-bond with Arg228 (ARG228:NH12···NFA:O5, 1.99 Å). The carboxyl group of nitrofuroic acid forms multicentred H-bond with Ser191, Gly 192, and His193 (SER191:HO···NFA:O12, 1.68 Å; GLY192:HN···NFA:O12, 1.42 Å; HIS193:HN···NFA:O12, 2.87 Å). The hydrazinehydrazide group (N–NH) forms a bifurcated H-bond with Ser 191 and His 193 (SER191:HO···NFA:N14, 2.00 Å; HIS193:HD1···NFA:N14, 2.38 Å) The oxygen of nitrofuraldehyde group forms H-bond with Ser 317 (SER317:HO···NFA:O20). All most all the amino acid residues involved in the interaction with the ligands were common compared to the interaction observed with the enzyme crystallized with 3-nitropropionic acid except for residues Tyr 89, Ser 191 which are involved in H-bond and Leu 194, Trp 283 which forms hydrophobic interactions with the ligand.

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8. Spectroscopic data for representative compound **4o** is given below. *5-Nitro-N′-[(5-nitro-2-furyl)methylidene]-2-furohydrazide*: ^{1}H NMR (300 MHz, $CDCl_3$) δ_H 7,2 (s, 1H), 7.43 (s, 1H), 7.32 (dd, 1H), 7.46 (dd, 1H), 8.01 (dd, 1H), 8,52 (dd, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ_C 111.3, 111.4, 116.1, 116.2, 134.6, 150.9, 151.8, 152.0, 153.9, 166.3.
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