Novel Pthalazinyl Derivatives: Synthesis, Antimycobacterial Activities, and Inhibition of *Mycobacterium Tuberculosis* Isocitrate Lyase Enzyme

D. Sriram*, P. Yogeeswari, P. Senthilkumar, S. Dewakar, N. Rohit, B. Debjani, Pritesh Bhat, B. Veugopal, V.V.S. Pavan and H.M. Thimmappa

Medicinal Chemistry & Antimycobacterial Research Laboratory, Pharmacy Group, Birla Institute of Technology & Science - Pilani, Hyderabad Campus, Jawahar Nagar, Hyderabad – 500 078, Andhra Pradesh, India

Abstract: Novel 2-[3-(4-bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phthalazinyl]acetic acid hydrazones were synthesized from phthalic anhydride by a six step synthesis and evaluated for *in vitro*, *in vivo* activities against eight mycobacterial species and *Mycobacterium tuberculosis* (MTB) isocitrate lyase (ICL) enzyme inhibition studies. Among twenty six compounds *N*'1-[(4-nitrophenyl)methylene]-2-[3-(4-bromo-2-fluorobenzyl)-4-oxo-1,2,3,4-tetrahydro-1-phthalazinyl]ethanohydrazide (**7j**) was found to be the most active compound *in-vitro* with MIC's of 0.18 and <0.09 μM against log-phase cultures of MTB and multi-drug resistant MTB respectively. Compound **7j** inhibited all the eight mycobacterial species with MIC ranging from <0.09-12.25 μM and was not toxic to Vero cell lines till 122.5 μM. Seven compounds were tested against starved culture of MTB and they inhibited with MIC's ranging from 2.88-8.91 μM. Some compounds showed 45-61% inhibition against MTB ICL enzyme at 10 μM. In the *in vivo* animal model **7j** decreased the bacterial load in lung and spleen tissues with 1.87 and 3.03-log10 protections respectively at 25 mg/kg body weight dose.

Key words: Phthalazine, Antimycobacterial, *Mycobacterium tuberculosis*, Isocitrate lyase.

INTRODUCTION

Tuberculosis (TB) is a disease caused by an infection with the bacteria *Mycobacterium tuberculosis*. During the 19th century, up to 25% of deaths in Europe were caused by this disease. The death toll began to fall as living standards improved at the start of the 20th century, and from the 1940s, effective medicines were developed. However, there are now more people in the world with TB than there were in 1950, and 2 billion people, equal to one-third of the world's total population, are infected with TB bacilli. TB is a leading killer among HIV-infected people with weakened immune systems; about 200 000 people living with HIV/AIDS die from TB every year, most of them being in Africa. TB is a worldwide pandemic; although the highest rates per capita are in Africa (28% of all TB cases), half of all new cases are in 6 Asian countries (Bangladesh, China, India, Indonesia, Pakistan, the Philippines) [1]. Multi-drug resistant TB (MDR-TB) is a form of TB that does not respond to the standard treatments using first-line drugs; MDR-TB is present virtually in all countries recently surveyed by WHO and partners. 450, 000 new MDR-TB cases are estimated to occur every year; the highest rates of MDR-TB are in countries of the former Soviet Union and in China. Current short-course (6-month) combination therapy for TB is effective when administered reliably. However, TB control has long been hindered by the lengthy and complex treatment required by current drugs, and is further complicated by the disease's deadly interaction with HIV/AIDS and the rise of MDR-TB [2]. Current TB drugs are active against growing bacteria but are ineffective against non-growing bacteria. There are at least three types of non-growing bacteria that are phenotypically resistant to antibiotics: (*a*) the stationary phase bacteria, (*b*) residual survivors or persisters not killed during antibiotic exposure when a growing culture is treated with antibiotics, and (*c*) dormant bacteria. Current TB drugs are not good enough because they are mainly active against growing bacilli, and except for rifampicin (RIF) and pyrazinamide (PZA), they are not good at killing persisters. Although RIF and PZA are important sterilizing drugs that significantly reduce the number of bacilli in lesions and play an important role in shortening the therapy from 12–18 months to 6 months, there are still other persister populations that are not killed by RIF or PZA. The presence of persistent and dormant TB bacteria 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. These factors underscore the urgent public health need for new TB therapies. A new TB treatment should offer at least one of three improvements over the existing regimens: a). shorten the total duration of effective treatment and/or significantly reduce the total number of doses needed to be taken under DOTS supervision; b) improve the treatment of MDR-TB, which cannot be treated with isoniazid (INH) and RIF and/or c) provide more effective treatment of latent/dormant TB infection, which is essential for eliminating TB. Phthalazinyl derivatives were reported for vaso-relaxant activity [3], hypolipidemic [4], anti-HIV activity [5], and inhibition of poly(ADP-ribose) polymerase [6], PDE3/PDE4 [7], and thromboxane A2 synthetase [8]. In the course of screening to discover new antimycobacterial compounds [9-18], we identified 2-[3-(4-bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phthalazinyl] acetic acid hydrazones which inhibited *in-vitro Mycobacterium tuberculosis* H_{37} Rv (MTB) (log-phase and dormant

*Address correspondence to this author at the Medicinal Chemistry & Antimycobacterial Research Laboratory, Pharmacy Group, Birla Institute of Technology & Science - Pilani, Hyderabad Campus, Jawahar Nagar, Hyderabad – 500 078, Andhra Pradesh, India; Tel: +91-09010534101; E-mail: drdsriram@yahoo.com

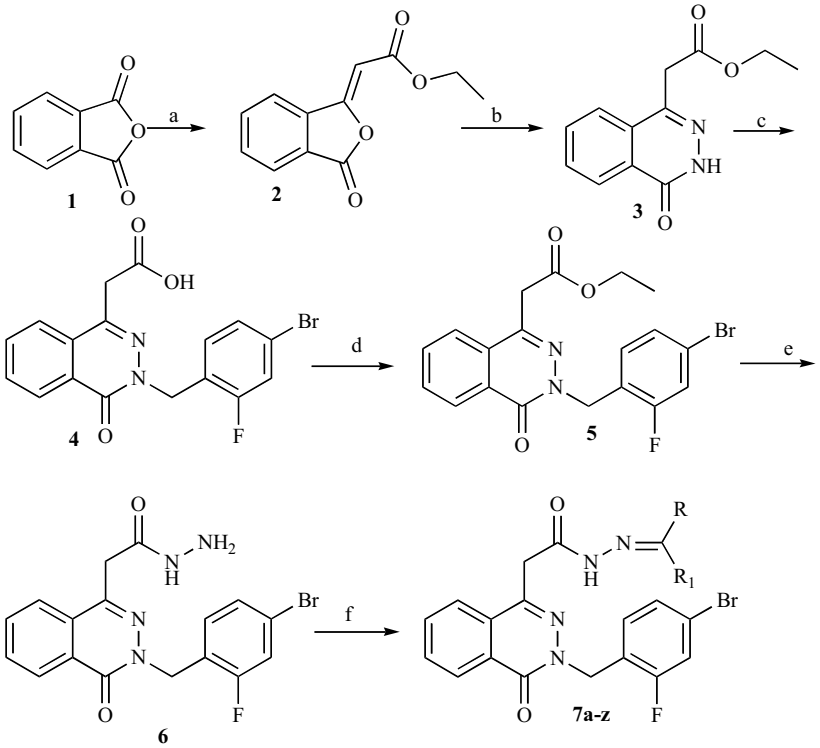
phase), multi-drug resistant *Mycobacterium tuberculosis* (MDR-TB) and various non-tuberculous mycobacteria (NTM). We present herein the results concerning the synthesis and the *in-vitro* and *in-vivo* antimycobacterial activities and MTB isocitrate lyase (ICL) enzyme inhibition studies of first representative compound of this family.

CHEMISTRY

2-[3-(4-Bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phthalazinyl]acetic acid hydrazones **7a-z** described in this study is shown in Table **1**, and a reaction sequence for the preparation is outlined in Scheme **1**. The first step, where phthalic anhydride (**1**) was reacted with ethyl 2-(1,1,1-triphenyl-λ^5-phosphanylidene)acetate, a phosphorus ylide to give ethyl 2-(3-oxo-1,3-dihydro-1-isobenzofuranyliden) acetate (**2**), an olefin. The reaction proceeds *via* a diionic compound called a betaine, as an intermediate, which forms oxaphosphetane which cleaves to form triphenylphosphine oxide and the corresponding olefin [19]. Ethyl 2-(4-oxo-3,4-dihydro-1-phthalazinyl)acetate (**3**) was prepared by the condensation of 1 mole equivalent of **2** and 0.8 mole equivalent of hydrazine hydrate using *p*-toluenesulphonic acid as a catalyst at room temperature for 8 min. There are a number for methods of synthesizing phthalazine nucleus [20] by refluxing phthalic anhydride and hydrazine hydrate and these methods are not very satisfactory due to drawbacks such as high temperature, long reaction time (6 hours), low yields (30-40%), effluent pollution and tedious workup procedure. In the present work using *p*-toluenesulphonic acid as a catalyst the reaction proceeded efficiently at room temperature with excellent yield (86%) and in a state of high purity. Compound **3** on N-alkylation with 4-bromo-1-bromomethyl-2-fluoro benzene in presence of sodium hydroxide yielded 2-[3-(4-bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phthalazinyl]acetic acid (**4**). The base treatment also hydrolyzed the ethyl ester into free acid. The ethyl ester (**5**) was prepared by refluxing the acid **4** in absolute ethanol in presence of sulphuric acid. Ethyl ester (**5**) (1 mol) on treatment with hydrazine hydrate (8 mol) yielded 2-[3-(4-bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phthalazinyl]ethanohydrazide (**6**). This hydrazide on reaction with various carbonyl compounds in presence of glacial acetic acid at a pH 4-6 yielded the titled compounds **7a-z**. The purity of the synthesized compounds was monitored by thin layer chromatography (TLC) and elemental analyses and the structures were identified by spectral data.

BIOLOGICAL ACTIVITY

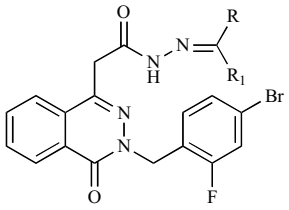
In the first phase the compounds were screened for their *in vitro* antimycobacterial activity against log-phase cultures of MTB, MDR-TB and NTM like *M. smegmatis* ATCC 14468, *M. microti* MTCC 1727, *M. vaccae* MTCC 997, *M. phlei* MTCC 1724, *M. fortuitum* MTCC 951, and *M. kansasii* MTCC 3058 by agar dilution method similar to that recommended by the National Committee for Clinical Laboratory Standards [21] for the determination of MIC in duplicate. The MDR-TB clinical isolate was obtained from



a. $Ph_3P{=}CHCOOC_2H_5$; b. $NH_2NH_2.H_2O$, PTSA; c. 4-Bromo-1-bromomethyl-2-fluorobenzene, NaOH; d. C_2H_5OH, H_2SO_4; e.$NH_2NH_2.H_2O$; f. Various aldehydes and ketones

Scheme.1. Synthetic protocol of the compounds.

Table 1. Antimycobacterial Activities and Cytotoxicities of Phthalazinyl Hydrazones



Comp. No.	R	R_1	IC_{50}[a] (μM)	Minimum Inhibitory Concentration (μM)							
				MTB[b]	MDR TB[c]	MS[d]	MM[e]	MV[f]	MP[g]	MF[h]	MK[i]
7a	H	Phenyl	NT[j]	6.73	NT	53.73	53.73	53.73	53.73	1.68	3.35
7b	H	4-Fluorophenyl	>129.3	0.81	0.81	3.23	51.73	3.23	6.48	3.23	6.48
7c	H	3-Fluorophenyl	>129.3	1.61	0.81	25.87	51.73	25.87	25.87	3.23	12.93
7d	H	2-Trifluoromethylphenyl	117.2	0.36	0.36	1.46	11.72	1.46	1.46	1.46	2.93
7e	H	2,6-Dichlorophenyl	>117.0	1.46	2.92	2.92	11.70	1.46	0.73	2.92	5.86
7f	H	3-Bromophenyl	>114.9	1.43	0.72	5.75	45.94	11.49	5.75	2.87	5.75
7g	H	4-Bromophenyl	>114.9	0.72	0.72	11.49	91.88	22.97	22.97	11.49	1.43
7h	H	2-Nitrophenyl	>122.5	0.76	0.18	3.06	97.99	3.06	6.13	3.06	1.53
7i	H	3-Nitrophenyl	>122.5	0.37	0.37	1.53	24.49	6.13	3.06	3.06	0.77
7j	H	4-Nitrophenyl	>122.5	0.18	<0.09	1.53	12.25	3.06	3.06	6.13	0.77
7k	H	5-Nitrofuran-2-yl	NT	12.49	NT	49.98	24.99	12.49	49.98	3.12	24.99
7l	H	2-Hydroxyphenyl	NT	6.50	NT	25.97	12.99	12.99	12.99	1.62	6.50
7m	H	4-Methoxyphenyl	NT	6.32	NT	25.24	3.15	25.24	25.24	12.62	25.24
7n	H	4-Hydroxy-3-methoxyphenyl	>122.2	3.05	3.05	24.45	12.22	12.22	24.45	3.05	12.22
7o	H	2-Methylphenyl	NT	13.04	NT	26.08	6.53	26.08	13.04	3.25	26.08
7p	H	4-Methylphenyl	NT	6.53	NT	1.63	6.53	1.63	3.25	3.25	6.53
7q	H	4-Dimethylaminophenyl	122.9	3.07	6.16	49.18	49.18	49.18	49.18	12.29	49.18
7r	H	4-Benzyloxyphenyl	NT	21.88	NT	43.75	21.88	21.88	21.88	1.37	21.88
7s	CH_3	Phenyl	NT	13.04	NT	26.08	52.16	52.16	26.08	13.04	26.08
7t	CH_3	4-Fluorophenyl	125.7	1.57	1.57	1.57	25.14	3.14	0.78	1.57	3.14
7u	CH_3	4-Bromophenyl	111.9	2.79	2.79	2.79	11.19	22.39	11.19	1.39	5.61
7v	CH_3	2-Hydroxyphenyl	NT	6.32	NT	25.24	6.32	12.62	12.62	3.15	6.32
7w	CH_3	4-Hydroxyphenyl	NT	6.32	NT	12.62	1.57	25.24	6.32	1.57	3.15
7x	CH_3	4-Methylphenyl	>126.7	3.16	0.79	12.67	25.34	6.34	12.67	3.16	12.67
7y	Ph	Phenyl	115.4	0.35	0.72	1.44	11.54	5.78	23.09	1.44	5.78
7z	Ph	4-Bromophenyl	100.8	2.52	1.26	10.08	20.15	10.08	2.52	0.63	5.05
Cipro	-	-	-	4.71	37.68	2.35	2.35	4.71	4.71	4.71	9.45
Rif	-	-	-	0.23	3.79	1.89	30.38	3.80	30.38	1.89	7.59
INH	-	-	-	0.66	45.57	45.57	22.82	182.3	91.15	22.82	182.3

[a] Cytotoxicity in mammalian vero cell lines; [b] *M. tuberculosis*; [c] Multidrug-resistant *M. tuberculosis*; [d] *M. smegmatis*; [e] *M. microti*; [f] *M. vaccae*; [g] *M. phlei*; [h] *M. fortuitum*; [i] *M. kansasii*; [j] Not tested.

Tuberculosis Research Center, Chennai, India, and was resis-tant to INH, RIF, ethambutol and quinolones. The minimum inhibitory concentration (MIC) is defined as the minimum concentration of compound required to give complete inhibition of bacterial growth. MIC's of the synthesized compounds along with the standard drugs for comparison are reported (Table **1**). In the initial screening against MTB, the newer compounds showed good activity with MIC's ranging from 0.18-21.88 μM. Seven compounds (**7b, 7d, 7g-j** and **7y**) showed excellent activity with MIC of <1 μM. When compared to INH (MIC: 0.66 μM), four compounds (**7d, 7i, 7j,** and **7y**) were found to be more active and compound **7j** was found to be more active than RIF (MIC: 0.23 μM). Sixteen compounds were more potent than ciprofloxacin. Compound *N*'1-[(4-nitrophenyl)methylene]-2-[3-(4-bromo-2-fluorobenzyl)-4-oxo-1,2,3,4-tetrahydro-1-phthalazinyl]etha-nohydrazide (**7j**) was found to be the most active compound *in vitro* with MIC's of 0.18 μM against MTB. With respect to structure-MTB activity, among the benzaldehyde and actophenone derived acid hydrazones (**7a-x**), substituents with electron withdrawing groups like nitro and halogens enhanced the activity. With respect to the carbimino terminal, the order of activity was found to be (sub)benzaldehyde > (sub)benzophenone > (sub)acetopheno ne. Replacement of phenyl ring of benzaldehyde with heteroaryl furanyl ring (**7k**) drastically reduced the activity. Similarly introduction of bulky benzyloxy group at 4^{th} postion of phenyl ring of benzaldehyde also reduced the activity several fold (**7r**: MIC of 21.88 μM). Among the nitro substituted at the benzaldehyde derivatives (**7h-j**) the order of activity was 4-NO_2> 3-NO_2> 2-NO_2. Introduction of electron donating groups like methyl, hydroxyl, methoxy and dimethylamino substituents at the benzaldehyde moiety reduced the activity. Most importantly against MDR-TB, when compared to INH (MIC 45.57 μM) and ciprofloxacin (MIC 37.68 μM), all the sixteen compounds that screened were more active with MIC's in the range of <0.09-6.16 μM. Some compounds (**7h, 7j,** and **7x**) endowed greater activity towards the MDR-TB than MTB. Compound **7j** was found to be the most active compound and was 42, 418 and 506 times more potent than RIF, ciprofloxacin and INH respectively. All the compounds were also screened for atypical mycobacteria (AM), AM infection [22] an illness caused by a type of myco-bacterium other than TB which cause a wide variety of infections such as abscesses, septic arthritis, and osteomy-elitis (bone infection). They can also infect the lungs, lymph nodes, gastrointestinal tract, skin, and soft tissues. The rate of AM infections is rare, but it is increasing as the AIDS population grows. Populations at risk include individuals who have lung disease and weakened immune systems. The synthesized compounds inhibited *M. smegmatis* (MS) with MIC's ranging from 1.44-53.73 μM and twenty three compounds were more potent than INH (MIC: 45.57 μM); MS infects lungs [23]. With regard to activity against *M. micriotii* (which causes sepsis tuberculosa acutissima in immuno-competant persons [24]) the compounds showed activity with MIC's ranging from 1.57-97.99 μM and fourteen compounds were more potent than INH (MIC: 22.82 μM). *M. vaccae,* which causes cutaneous and pulmonary infections [25] was inhibited by the synthesized compounds with MIC's ranging from 1.63-53.73 μM and all compounds were more potent than INH (MIC: 182.3 μM). All the compounds also inhibited *M. phlei* (MP) which causes abscesses [26] with MIC's ranging from 0.73- 53.73 μM and were more potent than INH (MIC: 91.15 μM). Against *M. fortuitum* (which causes infection in immuno-competant persons [27]) the compounds showed excellent activity with MIC's ranging from 0.63-13.04 μM and all compounds were more potent than INH (MIC: 22.82 μM). The compounds were also screened against *M. kansasi* which causes central nervous system infection and cutaneous lymphadenitis [28], was inhibited with MIC's ranging from 0.71-49.18 μM and all compounds were more potent than INH (MIC: 182.3 μM). Compound **7j** inhibited all the eight mycobacterium species with MIC ranging from <0.09-12.25 μM and was more potent than INH.

The compounds which showed good activity against log-phase culture of MTB and MS were further screened against 6-week-starved cells of MTB and MS according to the literature procedure [29]. Several *in vitro* model systems have been proposed to mimic the conditions found in the human chronic tuberculosis lesion (a granuloma), including oxygen starvation [30] nutrient deprivation [31] and rifampicin-induced persistence [32]. 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 [33]. As bacteria can easily grow upon being returned to nutrient-rich media, this model allows easy quantification of antibiotic effectiveness. Against MTB, seven compounds were tested and they inhibited starved culture of MTB with MIC's ranging from 2.88-8.91 μM (Table **2**). INH had poor activity against starved cells with MIC of 729.1 μM. As previously observed [29] RIF retained activity, although it is considerably less active against non-growing than against log-phase cells. All the seven tested compounds were more potent than INH and RIF (MIC: 15.2 μM). 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 MS culture, the tested compounds inhibited with MIC's ranging from 11.68-32.13 μM and were more potent than INH (MIC: >729.1 μM). Compound *N*'1-[(2-trifluoromethylphenyl)methylene]-2-[3-(4-bromo-2-fluorobenzyl)-4-oxo-1,2,3,4-tetrahydro-1-phthalazinyl]ethanohydrazide (**7d**) was found to be most active compound with MIC's of 2.88 and 11.68 μM against starved MTB and MS.

Mycobacterial persistence refers to the ability of tubercle bacillus to survive in the face of chemotherapy and/or immunity [34]. The nature of the persistent bacteria is unclear but might consist of stationary phase bacteria, post-chemotherapy residual survivors and/or dormant bacteria that do not form colonies upon plating [35]. The presence of such persistent bacteria is considered to be the major reason for lengthy therapy [36]. A lot of research activity is currently aimed at understanding the biology of persistence of the tubercle bacillus and developing new drugs that target the

Table:2 Inhibitory Activities of Selected Compounds Against Log-Phase and 6-Week-Starved Mycobacterial Cultures.

No	MIC in µM Against MTB[a]		No	MIC in µM Against MS[b]	
	Log-Phase Cells	6-Wk-Starved Cells		Log-Phase Cells	6-Wk-Starved Cells
7b	0.81	8.91	7d	1.46	11.68
7d	0.36	2.88	7i	1.53	19.89
7g	0.72	7.92	7j	1.53	32.13
7h	0.76	6.08	7p	1.63	14.67
7i	0.37	4.81	7t	1.57	17.27
7j	0.18	3.24	7y	1.26	17.64
7y	0.35	6.30			
INH	0.66	729.10	INH	45.57	>729.1
Rifampin	0.23	15.20	Rifampin	1.89	22.6

[a]*M. tuberculosis;* [b]*M. smegmatis*

persister bacteria [37]. Gene products involved in mycobacterial persistence, such as isocitrate lyase (ICL) [38], PcaA (methyl transferase involved in the modification of mycolic acid) [39], RelA (ppGpp synthase) [40], and DosR (controlling a 48-gene regulation involved in mycobacterial survival under hypoxic conditions) [41], have been identified and could be good targets for the development of drugs that target persistent bacilli. As these synthesized compounds showed activity against dormant mycobacterium, we decided to explore the possible mechanism by screening some compounds against ICL enzyme of MTB. 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 [38]. 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 [42] as MTB ICL inhibitors; however, none has been developed as a drug for MTB. Isocitrate lyase activity was determined at 37^0C by measuring the formation of glyoxylate-phenylhydrazone in the presence of phenylhydrazine and isocitrate lyase at 324nm based on the method described [43]. 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 **3**). All the seven compounds inhibited MTB ICL with percentage inhibition ranging from 45.1-61.6 at10 µM. Four compounds (**7d, 7h-j**) showed more than 50% inhibition and all these compounds were found to be more potent than standard 3-NP at the dose level tested. Compound *N*'1-[(3-nitrophenyl)methylene]-2-[3-(4-bromo-2-fluorobenzyl)-4-oxo-1,2,3,4-tetrahydro-1-phthalazinyl]ethanohydrazide (**7i**) was found to be most active compound in the enzyme inhibition studies. This is the first report that screening of newer synthetic compounds, which have an inhibition to MTB ICL. Further investigation could provide lead compounds for drug development against persistent tuberculosis.

Some compounds were further examined for toxicity (IC_{50}) in a mammalian Vero cell line at concentrations of 62.5 µg/mL (Table **1**). 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 [44]. The compounds with benzophenone derivatives were found to be toxic, which is followed by acetophenone substituted derivatives and benzaldehyde substituted derivatives show no or less toxicity. These results are important as these compounds with their decreased cytoliability, are much better attractive in the development of a compounds for the treatment of TB. This is primarily due to the fact that the eradication of TB requires a lengthy course of treatment, and the need for an agent with a high margin of safety becomes a primary concern. Compound **7j** showed selectivity index (IC_{50}/ MIC) of more than 680 and 1361 against log-phase MTB and MDR-TB infections. For the persistent culture of MTB the selectivity index of compound **7d** is 40.

Table. 3. Inhibitory Activities of Selected Compounds and 3-NP Against MTB ICL

No	% Inhibition (µM)
3-NP	63.2 (100)
7b	45.1 (10)
7d	55.0 (10)
7g	48.6 (10)
7h	51.6 (10)
7i	61.6 (10)
7j	54.8 (10)
7y	49.2 (10)

Prior to *in vivo* antimycobacterial screening the maximum tolerated dose (MTD) was performed for the com-

pound **7j** using C57BL/6 female mice by administration of a one-time dose/animal of an escalating dose of drug (100, 300, 500 and 1000 mg/Kg). The nine mice (3 mice/dose) in each study were observed for a total of 1 week. Surviving mice were sacrificed and organs examined for signs of overt toxicity. Compound **7j** showed no effect or adverse reactions/toxicity at the maximum dose. Subsequently, compound **7j** was tested for efficacy against MTB at a dose of 25mg/Kg (Table **4**) in six-week-old female CD-1 mice. In this model [45], the mice were infected intravenously through caudal vein approximately 10^7 viable *M. tuberculosis* ATCC 35801. Drug treatment began after inoculation of the animal with microorganism for 10 days by intra peritoneal route. After 35 days post infection the spleens and right lungs were aseptically removed and ground in a tissue homogenizer, the number of viable organisms was determined by serial 10-fold dilutions and subsequent inoculation onto 7H10 agar plates. Cultures were incubated at 37°C in ambient air for 4 weeks prior to counting. Bacterial counts were measured, and compared with the counts from negative (untreated) controls (Mean culture forming units (CFU) in lung: 7.99 ± 0.16 and in spleen: 9.02 ± 0.21). Compound **7j** decreased the bacterial load in lung and spleen tissues with 1.87 and 3.03-log10 protections respectively and was considered to be promising in reducing bacterial count in lung and spleen tissues. When compared to isoniazid at the same dose level **7j** was found to be less active in the *in vivo* study. The reason for this less *in vivo* activity might be due to the instability of the compounds, as it gets hydrolyzed in to the less active intermediate 2-[3-(4-bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1- phthalazinyl]ethanohydrazide (**6**) which showed *in vitro* MIC of 12.5 μg/ml against MTB.

CONCLUSION

Screening of the antimycobacterial activity of these novel series, identified 2-[3-(4-bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phthalazinyl]acetic acid hydrazones as a new lead endowed with high activity towards MTB, MDR-TB, NTM, dormant MTB and ICL of MTB. The present study reveals the importance of these compounds effective for the treatment of TB, MDR-TB, persistent TB and NTM infections. In conclusion, it has been shown that the potency, selectivity, and low cytotoxicity of these compounds make them valid leads for synthesizing new compounds that possess better activity. Further structure-activity and mechanistic studies should prove fruitful.

EXPERIMENTAL SECTION

Melting points were taken on an electrothermal melting point apparatus (Buchi BM530) in open capillary tubes and are uncorrected ^{1}H-NMR spectra were scanned on a JEOL Fx 400MHz NMR spectrometer using $CDCl_3$, DMSO-d_6 as solvent. Chemical shifts are expressed in δ (ppm) relative to tertamethylsilane. Elemental analyses (C, H, and N) were performed on Perkin Elmer model 240C analyzer and the data were within ± 0.4% of the theoretical values.

Synthesis of ethyl 2-(3-oxo-1,3-dihydro-1-isobenzofurany liden)acetate (2)

A solution of phthalic anhydride (1.0 equiv.) and ethyl 2-(1,1,1-triphenyl-λ^5-phosphanylidene)acetate (1.1 equiv.) in 300 ml of dichloromethane (DCM) was refluxed for 3 hr. DCM was removed by vacuum at 40-50 °C. 2x150 ml of hexane was added to the resulting sticky solid, stirred for 10 min and the un-reacted 2-(1,1,1-triphenyl-λ^5-phosphanylidene)acetate was removed by filtration. The organic solvent was removed under vacuum and the resulting crude semisolid was taken to next step without further purification. Yield: 84%. ^{1}H-NMR $CDCl_3$; δ (ppm): 1.1 (t, 3H), 4.2 (q, 2H), 6.0 (s, 1H), 7.6 (t, 1H), 7.7 (t, 1H), 7.8 (d, 1H), 8.9 (d, 1H).

Synthesis of ethyl 2-(4-oxo-3,4-dihydro-1-phthalazinyl) acetate (3)

A mixture of **2** (1.0 equiv.), hydrazine hydrate (0.8 equiv) and PTSA (1.0 equiv.) was ground by pestle and mortar at room temperature for 8 min. On completion, as indicated by TLC, the reaction mixture was treated with water. The resultant product was filtered, washed with water and recrystallized from DMF to give **3** in high yields (86%).^{1}H-NMR $CDCl_3$; δ (ppm): 1.1 (t, 3H), 3.9 (s, 2H), 4.1 (q, 2H), 7.6 (t, 1H), 7.7 (t, 1H), 7.8 (t, 1H), 8.3-8.4 (d, 1H), 10.0 (s, 1H).

Synthesis of 2-[3-(4-bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phthalazinyl]acetic acid (4)

A mixture of 3 (1.0 equiv.), NaOH (5.0 equiv.), and THF was stirred for 30 min at 40-50 °C. 4-bromo-1-bromomethyl-2-fluoro benzene (1.1 equiv.) was added to the reaction mixture and stirred for 2 hr at 50-60 °C. Water was added to the reaction mixture and stirred at room temperature for 1 hr. pH was adjusted to 2-3 using cold acetic acid. THF was removed and the aqueous phase was extracted with ethyl acetate (2x50 ml), washed with brine, dried over sodium sulphate and evaporated. The solid was crystallized with methanol to give **4** with 54 % yield. ^{1}H-NMR (DMSO-d_6); δ (ppm): 3.98 (s, 2H), 5.3 (s, 2H), 7.17 (t, 1H), 7.35 (dd, 1H, J_1= 8.0, J_2= 1.6), 7.55 (dd, 1H, J_1= 8.0, J_2= 1.6), 7.87 (t, 1H), 7.9 (t, 1H), 7.95 (t, 1H0, 8.29 (d, 1H).

Synthesis of ethyl 2-[3-(4-bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phthalazinyl]acetic acid (5)

Compound **4** (0.01mol) and ethanol (20 mL) refluxed for 8 hr at a temperature of 70-75°C in presence of few drops of

Table 4. ***In Vivo*** **Activity Data of 7j and INH Against** ***M. Tuberculosis*** **ATCC 35801 in Mice at 25 mg/kg**

Compound	Lungs (log CFU ± SEM)	Spleen (log CFU ± SEM)
Control	7.99 ± 0.16	9.02 ± 0.21
Isoniazid	5.86 ± 0.23	4.71 ± 0.10
7j	6.12± 0.21	5.99± 0.12

concentrated sulfuric acid as a catalyst. The reaction progress was monitored by TLC using a mixture of ethyl acetate: hexane (1:1) as the mobile phase. After the reaction, ethanol was distilled off from the reaction mixture and the residue was collected and DCM was added. To this solution a water wash was given to remove the H_2SO_4 present. Then the DCM layer was collected and to this sodium sulphate was added to remove traces of water. Then the DCM was distilled of to get the residue of the product. On cooling white crystals of **5** was observed with 59% yield and M.P. of 112^{0}C. ^{1}H-NMR (DMSO-d_6); δ (ppm): 1.1 (t, 3H), 3.98 (s, 2H), 4.12 (q, 2H), 5.3 (s, 2H), 7.17 (t, 1H), 7.35 (dd, 1H, J_1= 8.0, J_2= 1.6), 7.55 (dd, 1H, J_1= 8.0, J_2= 1.6), 7.87 (t, 1H), 7.9 (t, 1H), 7.95 (t, 1H0, 8.29 (d, 1H).

Synthesis of 2-[3-(4-bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1- phthalazinyl]ethanohydrazide (6)

To methanolic solution of (**2**) (1 equiv.) was added hydrazine-hydrate (7.5 equiv.) and refluxed for 12 hr at 50-60^{0}C. The reaction progress was monitored by TLC using a Ethyl acetate as the mobile phase. After the completion of reaction, the reaction mixture was then cooled, diluted with water and allowed to stand overnight. The solid precipitated was washed with water, dried and recrystallised twice from methanol to give **6** with 75% yield and m.p. of 232^{0}C. ^{1}H-NMR (DMSO-d_6); δ (ppm): 3.96 (s, 2H), 5.32 (s, 2H), 7.18 (t, 1H), 7.35 (dd, 1H, J_1= 8.0, J_2= 1.6), 7.55 (dd, 1H, J_1= 8.0, J_2= 1.6), 7.87 (t, 1H), 7.9 (t, 1H), 7.95 (t, 1H0, 8.3 (d, 1H), 9.42 (s 2H), 12.1 (s, 1H).

General procedure for the synthesis of 2-[3-(4-bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phthalazinyl]acetic acid hydrazones (7a-z):

A solution of **6** (1.0 equiv.), equimolar amount of appropriate aldehyde or ketone, and acetic acid (to maintain pH 4-6) in ethanol was refluxed for 5-6 hr at 70^{0}C. . After completion of the reaction, the precipitate obtained was filtered off, washed with water and cleaned twice with boiling ethanol to give **7a-z** with 40-96% yield.

2-[3-(4-Bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phtha-lazinyl]-*N*'-[phenylmethylidene]acetohydrazide (7a)

Yield: 65 %; m.p.: 237 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 4.01 (s, 2H), 5.32 (s, 2H), 7.2-8.2 (m, 13H), 12.01 (s, 1H); ^{13}C NMR (DMSO-d_6) δ (ppm): 186.8, 161.7, 158.7, 143.1, 141.3, 133.7, 132.3, 131.2, 130.7, 129.8, 129.2, 128.8, 127.6, 127.1, 122.7, 120.5, 38.9; Calculated for $C_{24}H_{18}BrFN_4O_2$: C, 58.43; H, 3.68; N, 11.36; found: C, 58.48; H, 3.66; N, 11.33.

2-[3-(4-Bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phtha-lazinyl]-*N*'-[(4-fluorophenyl)methylidene]acetohydrazide (7b)

Yield: 70 %; m.p.: 241 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 3.98 (s, 2H), 5.33 (s, 2H), 7.4-8.19 (m, 12H), 12.0 (s, 1H); ^{13}C NMR (DMSO-d_6) δ (ppm): 186.8, 165.7, 161.7, 158.7, 143.1, 141.3, 132.3, 131.2, 130.7, 129.8, 129.3, 127.6, 127.1, 122.7, 120.5, 115.7, 38.9; Calculated for $C_{24}H_{17}BrF_2N_4O_2$: C, 56.38; H, 3.35; N, 10.96; found: C, 56.40; H, 3.33; N, 10.89.

2-[3-(4-Bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phtha-lazinyl]-*N*'-[(3-fluorophenyl)methylidene]acetohydrazide (7c)

Yield: 70 %; m.p.: >250 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 3.99 (s, 2H), 5.33 (s, 2H), 7.4-8.18 (m, 12H), 12.0 (s, 1H); ^{13}C NMR (DMSO-d_6) δ (ppm): 186.8, 163.4, 161.7, 158.7, 143.1, 141.3, 135.5, 132.3, 131.2, 130.7, 129.8, 129.2, 127.6, 127.1, 124.7, 122.7, 120.5, 117.9, 114.2, 38.9; Calculated for $C_{24}H_{17}BrF_2N_4O_2$: C, 56.38; H, 3.35; N, 10.96; found: C, 56.34; H, 3.37; N, 10.95.

2-[3-(4-Bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phtha-lazinyl]-*N*'-[(2-trifluoromethylphenyl)methylidene]aceto-hydrazide (7d)

Yield: 78%; m.p.: 205 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 4.01 (s, 2H), 5.28 (s, 2H), 7.27-8.19 (m, 12H), 12.0 (s, 1H); ^{13}C NMR (DMSO-d_6) δ (ppm): 186.8, 161.7, 158.7, 143.1, 141.3, 132.3, 131.3, 130.7, 129.8, 129.4, 128.1, 127.6, 127.1, 125.5, 122.7, 120.5, 38.9; Calculated for $C_{25}H_{17}BrF_4N_4O_2$: C, 53.49; H, 3.05; N, 9.98; found: C, 53.51; H, 3.08; N, 10.02.

2-[3-(4-Bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phtha-lazinyl]-*N*'-[(2,6-dichlorophenyl)methylidene]acetohydra-zide (7e)

Yield: 49 %; m.p.: >250 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 4.011 (s, 2H), 5.33 (s, 2H), 7.15-8.67 (m, 11H), 12.0 (s, 1H); ^{13}C NMR (DMSO-d_6) δ (ppm): 186.8, 161.7, 158.7, 143.1, 141.3, 138.9, 135.8, 132.1, 131.8, 131.2, 130.7, 129.8, 129.2, 127.6, 127.1, 122.7, 120.5, 38.9; Calculated for $C_{24}H_{16}BrCl_2FN_4O_2$: C, 51.27; H, 2.87; N, 9.97; found: C, 51.31; H, 2.86; N, 9.94.

2-[3-(4-Bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phtha-lazinyl]-*N*'-[(3-bromophenyl)methylidene]acetohydrazide (7f)

Yield: 90 %; m.p.: >250 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 3.98 (s, 2H), 5.30 (s, 2H), 7.13-8.18 (m, 12H), 12.0 (s, 1H); ^{13}C NMR (DMSO-d_6) δ (ppm): 186.8, 161.7, 158.7, 143.1, 141.3, 136.2, 134.0, 132.9, 132.3, 131.2, 130.7, 129.8, 129.2, 128.1, 127.6, 127.1, 123.2, 122.7, 120.5, 38.9; Calculated for $C_{24}H_{17}Br_2FN_4O_2$: C, 50.37; H, 2.99; N, 9.79; found: C, 50.40; H, 2.96; N, 9.77.

2-[3-(4-Bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phtha-lazinyl]-*N*'-[(4-bromophenyl)methylidene]acetohydrazide (7g)

Yield: 40%; m.p.: 212 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 3.98 (s, 2H), 5.32 (s, 2H), 7.34-8.18 (m, 12H), 12.0 (s, 1H); ^{13}C NMR (DMSO-d_6) δ (ppm): 186.8, 161.7, 158.7, 143.1, 141.3, 132.9, 132.3, 131.8, 131.2, 130.7, 129.8, 129.2, 127.6, 127.1, 125.3, 122.7, 120.5, 38.9; Calculated for $C_{24}H_{17}Br_2FN_4O_2$: C, 50.37; H, 2.99; N, 9.79; found: C, 50.36; H, 3.02; N, 9.78.

2-[3-(4-Bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phtha-lazinyl]-*N*'-[(2-nitrophenyl)methylidene]acetohydrazide (7h)

Yield: 52 %; m.p.: 221 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 3.98 (s, 2H), 5.33 (s, 2H), 7.27-8.38 (m, 12H), 12.0 (s, 1H); ^{13}C NMR (DMSO-d_6) δ (ppm): 186.8, 161.7, 158.7, 149.3, 143.1, 141.3, 135.5, 132.3, 131.8, 131.2, 130.7, 130.1, 129.8, 129.2, 127.6, 127.1, 126.4, 122.7, 121.2, 120.5, 38.9; Calculated for $C_{24}H_{17}BrFN_5O_4$: C, 53.55; H, 3.18; N, 13.01; found: C, 53.52; H, 3.14; N, 12.98.

2-[3-(4-Bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phtha-lazinyl]-*N′*-[(3-nitrophenyl)methylidene]acetohydrazide (7i)

Yield: 59 %; m.p.: >250 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 4.01 (s, 2H), 5.31 (s, 2H), 7.28-8.39 (m, 12H), 12.0 (s, 1H); ^{13}C NMR (DMSO-d_6) δ (ppm): 186.8, 161.7, 158.7, 148.5, 143.1, 141.3, 135.6, 134.7, 132.3, 131.2, 130.7, 129.8, 129.2, 127.6, 127.1, 124.2, 123.4, 122.7, 120.5, 38.9; Calculated for $C_{24}H_{17}BrFN_5O_4$: C, 53.55; H, 3.18; N, 13.01; found: C, 53.51; H, 3.21; N, 13.04.

2-[3-(4-Bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phtha-lazinyl]-*N′*-[(4-nitrophenyl)methylidene]acetohydrazide (7j)

Yield: 52 %; m.p.: >250 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 3.99 (s, 2H), 5.33 (s, 2H), 7.27-8.38 (m, 12H), 12.0 (s, 1H); ^{13}C NMR (DMSO-d_6) δ (ppm): 186.8, 161.7, 158.7, 150.7, 143.1, 141.3, 139.9, 132.3, 131.2, 130.7, 130.1, 129.8, 129.2, 127.6, 127.1, 122.7, 121.1, 120.5, 38.9; Calculated for $C_{24}H_{17}BrFN_5O_4$: C, 53.55; H, 3.18; N, 13.01; found: C, 53.53; H, 3.16; N, 13.03.

2-[3-(4-Bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phtha-lazinyl]-*N′*-[(5-nitro-2-furyl)methylidene]acetohydrazide (7k)

Yield: 60 %; m.p.: >250 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 3.98 (s, 2H), 5.33 (s, 2H), 7.28-8.19 (m, 10H), 12.0 (s, 1H); ^{13}C NMR (DMSO-d_6) δ (ppm): 186.8, 161.7, 158.7, 156.6, 153.0, 141.3, 134.8, 132.3, 131.2, 130.7, 129.8, 129.2, 127.6, 127.1, 122.7, 120.5, 112.1, 38.9; Calculated for $C_{22}H_{15}BrFN_5O_5$: C, 50.02; H, 2.86; N, 13.26; found: C, 49.97; H, 2.83; N, 13.27.

2-[3-(4-Bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phtha-lazinyl]-*N′*-[(2-hydroxyphenyl)methylidene]acetohydra-zide (7l)

Yield: 78 %; m.p.: >250 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 3.98 (s, 2H), 5.33 (s, 2H), 7.19-8.19 (m, 12H), 11.04 (s, 1H), 12.0 (s, 1H); ^{13}C NMR (DMSO-d_6) δ (ppm): 186.8, 161.7, 160.9, 158.7, 143.1, 141.3, 132.3, 131.2, 130.7, 129.8, 129.2, 127.6, 127.1, 122.7, 121.3, 120.5, 119.0, 116.4, 38.9; Calculated for $C_{24}H_{18}BrFN_4O_3$: C, 56.60; H, 3.56; N, 11.00; found: C, 56.57; H, 3.58; N, 10.98.

2-[3-(4-Bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phtha-lazinyl]-*N′*-[(4-methoxyphenyl)methylidene]acetohydra-zide (7m)

Yield: 41 %; m.p.: >250 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 3.84 (s, 3H), 3.98 (s, 2H), 5.33 (s, 2H), 6.7-8.19 (m, 12H), 12.0 (s, 1H); ^{13}C NMR (DMSO-d_6) δ (ppm): 186.8, 163.2, 161.7, 158.7, 143.1, 141.3, 132.3, 131.2, 130.7, 130.1, 129.8, 129.2, 127.6, 127.1, 126.1, 122.7, 120.5, 114.5, 56.8, 38.9; Calculated for $C_{25}H_{20}BrFN_4O_3$: C, 57.37; H, 3.85; N, 10.71; found: C, 57.32; H, 3.88; N, 10.70.

2-[3-(4-Bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phtha-lazinyl]-*N′*-[(4-hydroxy-3-methoxyphenyl)methylidene] acetohydrazide (7n)

Yield: 52%; m.p.: >250 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 3.8 (s, 3H), 3.98 (s, 2H), 4.9 (s, 1H), 5.33 (s, 2H), 6.8-8.19 (m, 11H), 12.0 (s, 1H); ^{13}C NMR (DMSO-d_6) δ (ppm): 186.8, 161.7, 158.7, 152.4, 148.2, 143.1, 141.3, 132.3, 131.2, 130.7, 129.8, 129.2, 127.5, 127.1, 122.7, 120.5, 117.6, 114.9, 58.6, 38.9; Calculated for $C_{25}H_{20}BrFN_4O_4$: C, 55.67; H, 3.74; N, 10.39; found: C, 55.70; H, 3.75; N, 10.34.

2-[3-(4-Bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phtha-lazinyl]-*N′*-[(2-methylphenyl)methylidene]acetohydrazide (7o)

Yield: 63%; m.p.: 232 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 2.78 (s, 3H), 3.98 (s, 2H), 5.33 (s, 2H), 6.90-8.18 (m, 12H), 12.0 (s, 1H); ^{13}C NMR (DMSO-d_6) δ (ppm): 186.8, 161.7, 158.7, 143.1, 141.3, 139.2, 132.3, 131.2, 130.7, 129.8, 129.2, 127.6, 127.1, 126.8, 125.8, 122.7, 120.5, 38.9, 18.6; Calculated for $C_{25}H_{20}BrFN_4O_2$: C, 59.18; H, 3.97; N, 11.04; found: C, 59.21; H, 4.01; N, 11.00.

2-[3-(4-Bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phtha-lazinyl]-*N′*-[(4-methylphenyl)methylidene]acetohydrazide (7p)

Y ield: 63%; m.p.: >250 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 2.79 (s, 3H), 4.01 (s, 2H), 5.32 (s, 2H), 6.94-8.18 (m, 12H), 12.0 (s, 1H); ^{13}C NMR (DMSO-d_6) δ (ppm): 186.8, 161.7, 158.7, 143.1, 141.3, 140.9, 132.3, 131.2, 130.7, 129.8, 129.2, 127.6, 127.1, 122.7, 120.5, 38.9, 24.5; Calculated for $C_{25}H_{20}BrFN_4O_2$: C, 59.18; H, 3.97; N, 11.04; found: C, 59.17; H, 3.99; N, 11.07.

2-[3-(4-Bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phtha-lazinyl]-*N′*-[(4-dimethylaminophenyl)methylidene]aceto-hydrazide (7q)

Yield: 67%; m.p.: >250 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 2.44 (s, 6H), 3.98 (s, 2H), 5.33 (s, 2H), 6.36-8.19 (m, 12H), 12.0 (s, 1H); ^{13}C NMR (DMSO-d_6) δ (ppm): 186.8, 161.7, 158.7, 151.9, 143.1, 141.3, 132.3, 131.2, 130.7, 130.1, 129.8, 129.2, 127.6, 127.1, 123.5, 122.7, 120.5, 114.5, 40.6, 38.9; Calculated for $C_{26}H_{23}BrFN_5O_2$: C, 58.22; H, 4.32; N, 13.06; found: C, 58.24; H, 4.30; N, 13.11.

2-[3-(4-Bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phtha-lazinyl]-*N′*-[(4-benzyloxyphenyl)methylidene]acetohydra-zide (7r)

Yield: 96%; m.p.: 193 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 3.98 (s, 2H), 4.98 (s, 2H), 5.33 (s, 2H), 6.60-8.18 (m, 17H), 12.0 (s, 1H); ^{13}C NMR (DMSO-d_6) δ (ppm): 186.8, 161.7, 158.7, 143.1, 141.3, 132.3, 131.2, 130.7, 130.1, 129.8, 129.2, 127.6, 127.1, 126.5, 122.7, 120.5, 114.5, 71.2, 38.9; Calculated for $C_{31}H_{24}BrFN_4O_3$: C, 62.11; H, 4.04; N, 9.35; found: C, 62.09; H, 4.07; N, 9.33.

2-[3-(4-Bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phtha-lazinyl]-*N*'-[1-phenylethylidene]acetohydrazide (7s)

Yield: 94%; m.p.: 217 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 2.14 (s, 3H), 3.96 (s, 2H), 5.33 (s, 2H), 7.18-8.18 (m, 13H), 12.0 (s, 1H); ^{13}C NMR (DMSO-d_6) δ (ppm): 186.8, 170.6, 161.7, 158.7, 141.3, 134.2, 132.3, 131.2, 130.7, 129.8, 129.2, 128.6, 127.6, 127.1, 122.7, 120.5, 38.9, 15.2; Calculated for $C_{25}H_{20}BrFN_4O_2$: C, 59.18; H, 3.97; N, 11.04; found: C, 59.21; H, 3.99; N, 10.99.

2-[3-(4-Bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phtha-lazinyl]-*N*'-[1-(4-fluorophenyl)ethylidene]acetohydrazide (7t)

Yield: 68%; m.p.: 211 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 2.14 (s, 3H), 3.96 (s, 2H), 5.33 (s, 2H), 7.22-8.18 (m, 12H), 12.0 (s, 1H); ^{13}C NMR (DMSO-d_6) δ (ppm): 186.8, 170.6, 165.6, 161.7, 158.7, 141.3, 132.3, 131.2, 130.7, 129.8, 129.2, 127.6, 127.1, 122.7, 120.5, 115.5, 38.9, 15.2; Calculated for $C_{25}H_{19}BrF_2N_4O_2$: C, 57.16; H, 3.65; N, 10.66; found: C, 57.13; H, 3.66; N, 10.70.

2-[3-(4-Bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phtha-lazinyl]-*N*'-[1-(4-bromophenyl)ethylidene]acetohydrazide (7u)

Yield: 67%; m.p.: 228 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 2.14 (s, 3H), 3.96 (s, 2H), 5.33 (s, 2H), 7.27-8.18 (m, 12H), 12.0 (s, 1H); ^{13}C NMR (DMSO-d_6) δ (ppm): 186.8, 170.6, 161.7, 158.7, 141.3, 133.6, 132.3, 131.8, 131.2, 130.7, 129.8, 129.2, 127.6, 127.1, 125.6, 122.7, 120.5, 38.9, 15.2; Calculated for $C_{25}H_{19}Br_2FN_4O_2$: C, 51.22; H, 3.27; N, 9.56; found: C, 51.20; H, 3.31; N, 9.59.

2-[3-(4-Bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phtha-lazinyl]-*N'*-[1-(2-hydroxyphenyl)ethylidene]acetohydra-zide (7v)

Yield: 52%; m.p.: >250 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 2.14 (s, 3H), 3.96 (s, 2H), 5.33 (s, 2H), 7.17-8.19 (m, 12H), 11.65 (s, 1H), 12.0 (s, 1H); ^{13}C NMR (DMSO-d_6) δ (ppm): 186.8, 170.6, 161.7, 160.9, 158.7, 141.3, 132.3, 131.2, 130.7, 129.8, 129.2, 127.6, 127.1, 122.7, 121.9, 120.5, 118.9, 116.9, 38.9, 15.2; Calculated for $C_{25}H_{20}BrFN_4O_3$: C, 57.37; H, 3.85; N, 10.71; found: C, 57.39; H, 3.80; N, 10.67.

2-[3-(4-Bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phtha-lazinyl]-*N'*-[1-(4-hydroxyphenyl)ethylidene]acetohydra-zide (7w)

Yield: 45%; m.p.: >250 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 2.15 (s, 3H), 3.96 (s, 2H), 5.33 (s, 2H), 7.16-8.18 (m, 12H), 11.62 (s, 1H), 12.0 (s, 1H); ^{13}C NMR (DMSO-d_6) δ (ppm): 186.8, 170.6, 161.7, 160.9, 158.7, 141.3, 132.3, 131.2, 130.7, 129.8, 129.2, 127.6, 127.1, 126.6, 122.7, 120.5, 116.8, 38.9, 15.2; Calculated for $C_{25}H_{20}BrFN_4O_3$: C, 57.37; H, 3.85; N, 10.71; found: C, 57.32; H, 3.84; N, 10.70.

2-[3-(4-Bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phtha-lazinyl]-*N*'-[1-(4-methyl phenyl)ethylidene]acetohydra-zide (7x)

Yield: 70%; m.p.: 186 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 2.14 (s, 3H), 2.34 (s, 3H), 3.96 (s, 2H), 5.33 (s, 2H), 7.12-8.18 (m, 12H), 12.0 (s, 1H); ^{13}C NMR (DMSO-d_6) δ (ppm): 186.8, 170.6, 161.7, 158.7, 141.3, 140.5, 132.3, 131.2, 130.7, 129.8, 129.2, 127.6, 127.1, 122.7, 120.5, 38.9, 24.5, 15.2; Calculated for $C_{26}H_{22}BrFN_4O_2$: C, 59.89; H, 4.25; N, 10.75; found: C, 59.92; H, 4.27; N, 10.78.

2-[3-(4-Bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phtha-lazinyl]-*N*'-(diphenylmethylene)acetohydrazide (7y)

Yield: 49%; m.p.: >250 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 2.14 (s, 3H), 3.96 (s, 2H), 5.33 (s, 2H), 7.24-8.18 (m, 17H), 12.0 (s, 1H); ^{13}C NMR (DMSO-d_6) δ (ppm): 186.8, 161.7, 158.7, 156.6, 141.3, 140.1, 133.2, 132.3, 131.2, 130.7, 129.8, 129.2, 128.8, 127.6, 127.1, 122.7, 120.5, 38.9; Calculated for $C_{30}H_{22}BrFN_4O_2$: C, 63.28; H, 3.89; N, 9.84; found: C, 63.31; H, 3.87; N, 9.83.

2-[3-(4-Bromo-2-fluorobenzyl)-4-oxo-3,4-dihydro-1-phtha-lazinyl]-*N*'-[(4-bromophenyl)(phenyl)methylidene]aceto-hydrazide (7z)

Yield: 55%; m.p.: >250 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 2.14 (s, 3H), 3.96 (s, 2H), 5.33 (s, 2H), 7.24-8.19 (m, 16H), 12.0 (s, 1H); ^{13}C NMR (DMSO-d_6) δ (ppm): 186.8, 161.7, 158.7, 156.6, 141.3, 140.1, 132.3, 131.9, 131.4, 131.2, 130.7, 129.8, 129.2, 128.8, 127.6, 127.1, 125.5, 122.7, 120.5, 38.9; Calculated for $C_{30}H_{21}Br_2FN_4O_2$: C, 55.58; H, 3.26; N, 8.64; found: C, 55.62; H, 3.25; N, 8.66.

ANTIMYCOBACTERIAL ACTIVITY IN LOG-PHASE CULTURES

Compounds were screened for their *in vitro* antimycobacterial activity against log-phase cultures of MTB, MDR-TB and NTM species like *M. smegmatis* ATCC 14468, *M. microti* MTCC 1727, *M. vaccae* MTCC 997, *M. phlei* MTCC 1724, *M. fortuitum* MTCC 951, and *M. kansasii* MTCC 3058 in Middlebrook 7H11agar medium supplemented with OADC by agar dilution method similar to that recommended by the National Committee for Clinical Laboratory Standards for the determination of MIC in triplicate. The MDR-TB clinical isolate was obtained from Tuberculosis Research Center, Chennai, India, and was resistant to isoniazid, rifampicin, and ciprofloxacin. The minimum inhibitory concentration (MIC) is defined as the minimum concentration of compound required to give complete inhibition of bacterial growth.

ANTIMYCOBACTERIAL ACTIVITY IN 6-WEEK STARVED CULTURES

For starvation protocol, MTB cells were grown in Middlebrook 7H9 medium supplemented with 0.2% (vol/vol) glycerol, 10% (vol/vol) Middlebrook oleic acid-albumin-dextrose-catalase (OADC) enrichment, and 0.025% (vol/vol) Tween 80 at 37°C with constant rolling at 2 rpm until they reached an optical density at 600 nm of ~0.6. The culture were then washed twice and re-suspended in phosphate-buffered saline (PBS) at the same cell density. 50 ml of culture were then incubated at 37°C for an additional 6 weeks in 1-liter roller bottles. Compounds, dissolved in DMSO in different concentrations, were added to 1 ml PBS containing ~1x10^7 starved MTB cells at various concentrations. Cultures were incubated in 15-ml conical tubes at 37°C with constant shaking for 7 days and then washed twice in PBS before dilutions were inoculated on Middlebrook 7H11 plates supplemented with 0.2% (vol/vol) glyc-

erol, 10% (vol/vol) Middlebrook OADC enrichment, and 0.025% (vol/vol) Tween 80, containing no antibiotics. Bacterial growth was determined after incubation for 4 weeks at 37°C. The minimum inhibitory concentration (MIC) is defined as the minimum concentration of compound required to give complete inhibition of bacterial growth. All values were determined in triplicate.

PROTEIN INDUCTION OF ISOCITRATE LYASE

The recombinant MTb ICL was expressed in expression host, *Escherichia coli* by incubating them together in Luria Bertani(LB) Broth with the antibiotic, ampicillin (0.02% w/v) at 37°C for overnight. The expression host with the ICL construct was then further incubated in LB broth at 37°C till an Optical Density (OD) of 0.5-0.6 was attained at 324nm. This was then followed by the addition of 1mM Isopropyl β-D-1- thiogalactopyranoside (IPTG) and further incubated for another 4 h at 37°C. The culture was pelletized by centrifuging at 10000 × g for 20 min at 4°C; the supernatant discarded and the pellet washed with 200µl of 100mM Tris (pH 8.0) and then centrifuged again at 10000 × g for 20 min at 4°C. The pellet is further resuspended in 200 µl Tris (pH 8.0) and 1mM PMSF and is sonicated in ice for 30 s with pulses of 10 s each at 100W with cooling period of 10s in between. It is further centrifuged at 10000× g for 20 min at 4°C and the supernatant is collected as soluble fraction to be loaded on the Ni^{+2} column.

PROTEIN PURIFICATION

The Ni-NTA superflow cartridge prefilled with 5 ml of Ni-NTA Superflow resin (Qiagen) was fixed onto a stand and 10ml MilliQ water was passed through it followed by 10ml of Strip Buffer (Composition: 20mM Tris Buffer, 100mM EDTA, 500mM NaCl) and 20 ml of Charge Buffer (50mM $NiSO_4$). Further 20 ml of Binding Buffer (Composition: Tris pH 7.5-20mM, NaCl-200mM, MgCl2- 5mM, β-Mercaptoethanol-2mM, Imidazole- 10mM) was passed through it at a flow rate of 5ml/min and then the soluble fraction was loaded after filtering it thrice through 0.45 µm membrane filter. Further 20 ml Binding Buffer was passed through it followed by 30 ml of Wash buffer(Composition-Same as that of Binding buffer except for Imidazole which is present in the concentration of 50mM) and 5ml of Elution Buffer, which consists of 200mM Imidazole and all the other ingredients similar to that of Binding Buffer. After a standing period of 15 min the His-tagged proteins were eluted out and collected and loaded on for SDS-PAGE for analysis.

SDS-PAGE

For the SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) tank buffer consisting of Tris-HCl, SDS, Glycine, and Distilled water was used. The stacking gel consisted of Distilled water, 1.5M Tris (pH 6.8), Acrylamide (30%), SDS (20%), Ammonium per sulfate (10%), and TEMED. The resolving gel had similar composition as that of the Stacking gel except for Tris which had a pH of 8.8. The gel was stained with Comassie brilliant blue R-250 and then destained with a solution of acetic acid and methanol in water.

DIALYSIS

The elutant fraction was dialyzed against Dialysis buffer comprising of Tris-Cl(pH 7.5) 100mm and EDTA 2mM overnight at 4°C.This enzyme fraction was then stored at -70^{o}C nitrogen.

ICL ENZYME ASSAY

Isocitrate lyase activity was determined at 37 ^{0}C by measuring the formation of glyoxylate-phenylhydrazone at 324 nm. The reaction mixture contains 100 µL of 0.5mM potassiumphosphate buffer, 1.2µL of 1mM magnesium chloride, 24 µL of 100 mM 2-merceptoethanol, 7µL of 4mM phenylhydrazine hydrochloride, 6µL of 50 mM trisodiumisocitricacid and ICL enzyme (usually 3 to 6µL). This mixture is made up to 200µL with MilliQ water. At the end of the 10th minute this reaction mixture is made up to 1mL and UV absorbance is measured at 324nM which serves as a control. For the test compounds 3µL of 100mM 3-NPA used and in case of the candidate molecules 10µL of 10mM concentration added with the above mentioned reaction mixture. At the end of the 10th minute this reaction mixture is made up to 1mL and UV absorbance is measured at 324nM which serves as a test. The % inhibition is calculated by the formulae control absorbance minus test absorbance divided by control absorbance multiply by 100.

CYTOTOXICITY

Some compounds were further examined for toxicity (IC_{50}) in a mammalian Vero cell line at single concentration of 62.5 µg/mL by serial dilution method. 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.

IN VIVO STUDIES

O ne compound was tested for efficacy against MTB at a dose of 25 mg/kg in six-week-old female CD-1 mice six per group. In this model, the mice were infected intravenously through caudal vein approximately 10^7 viable *M. tuberculosis* ATCC 35801. Drug treatment by intra peritoneal route began after 10 days of inoculation of the animal with microorganism and continued for 10 days. After 35 days post infection the spleens and right lungs were aseptically removed and ground in a tissue homogenizer, the number of viable organisms was determined by serial 10-fold dilutions and subsequent inoculation onto 7H10 agar plates. Cultures were incubated at 37°C in ambient air for 4 weeks prior to counting. Bacterial counts were measured, and compared with the counts from negative controls (vehicle treated) in lung and in spleen.

ACKNOWLEDGEMENT

The authors are thankful to Department of Biotechnology (BT/01/COE/05/06/01), Government of India for their financial assistances.

REFERENCES

[1] World Health Organization. Global TB Control Report, **2008**.
[2] Coninx, R. Tuberculosis in complex emergencies. *Bull. World Health Organ*., **2007**, *85*, 637.

[3] Del Olmo, E.; Barboza, B.; Ybarra, M.I.; Lopez-Perez, J.L.; Carron, R.; Sevilla, M.A.; Boselli, C.; San Feliciano, A. Vasorelaxant activity of phthalazinones and related compounds. *Bioorg. Med. Chem. Lett.,* **2006**, *16*, 2786.

[4] Madhavan, G.R.; Chakrabarti, R.; Kumar, S.K.; Misra, P.; Mamidi, R.N.; Balraju, V.; Kasiram, K.; Babu, R.K.; Suresh, J.; Lohray, B.B.; Lohrayb, V.B.; Iqbal, J.; Rajagopalan, R. Novel phthalazinone and benzoxazinone containing thiazolidinediones as antidiabetic and hypolipidemic agents. *Eur. J. Med. Chem.,* **2001**, *36*, 627.

[5] Bedoya, L.M.; del Olmo, E.; Sancho, R.; Barboza, B.; Beltran, M.; Garcıa-Cadenas, A.E.; Sanchez-Palomino, S.; Lopez-Perez, J.L.; Munoz, E.; Feliciano, A.S.; Alcamıa, J. Anti-HIV activity of stilbene-related heterocyclic compounds. *Bioorg. Med. Chem. Lett.,* **2006**, *16*, 4075.

[6] Cockcroft, X.L.; Dillon, K.J.; Dixon, L.; Drzewiecki, J.; Kerrigan, F.; Loh, V.M.; Martin, N.M. Phthalazinones 2: Optimisation and synthesis of novel potent inhibitors of poly(ADP-ribose)polymerase. *Bioorg. Med. Chem. Lett.,* **2006**, *16*, 1040.

[7] Mey, V.M.; Bommele, K.M.; Boss, H.; Hatzelmann, A.; Slingerland, V.M.; Sterk, G.J.; Timmerman, H. Synthesis and structure-activity relationships of cis-tetrahydrophthalazinone/pyridazinone hybrids: a novel series of potent dual PDE3/PDE4 inhibitory agents. *J. Med. Chem.,* **2003**, *46*, 2008.

[8] Yamaguchi, M.; Koga, T.; Kamei, K.; Akima, M.; Maruyama, N.; Kuroki, T.; Hamana, M.; Ohi, N. Novel antiasthmatic agents with dual activities of thromboxane A2 synthetase inhibition and bronchodilation. IV. 2-[2-(1-Imidazolyl)ethyl]-4-(3-pyridyl)-1(2H)-phthalazinones. *Chem. Pharm. Bull.,* **1994**, *42*, 1850.

[9] Kumar, R.R.; Perumal, S.; Senthilkumar, P.; Yogeeswari, P.; Sriram, D. Discovery of antimycobacterial spiro-piperidin-4-ones: an atom economic, stereoselective synthesis, and biological intervention. *J. Med. Chem.,* **2008**, *51*, 5731.

[10] Dinakaran, M.; Senthilkumar, P.; Yogeeswari, P.; China, A.; Nagaraja, V.; Sriram, D. Synthesis, antimycobacterial activities and phototoxic evaluation of 5H-thiazolo[3,2-a]quinoline-4-carboxylic acid derivatives. *Med. Chem.,* **2008**, *4*, 482.

[11] Dinakaran, M.; Senthilkumar, P.; Yogeeswari, P.; China, A.; Nagaraja, V.; Sriram, D. Novel ofloxacin derivatives: synthesis, antimycobacterial and toxicological evaluation. *Bioorg. Med. Chem. Lett.,* **2008**, *18*, 1229.

[12] Senthilkumar, P.; Dinakaran, M.; Banerjee, D.; Devakaram, R.V.; Yogeeswari, P.; China, A.; Nagaraja, V.; Sriram, D. Synthesis and antimycobacterial evaluation of newer 1-cyclopropyl-1,4-dihydro-6-fluoro-7-(substituted secondary amino)-8-methoxy-5-(sub)-4-oxoquinoline-3-carboxylic acids. *Bioorg. Med. Chem.,* **2008**, *16*, 2558.

[13] Dinakaran, M.; Senthilkumar, P.; Yogeeswari, P.; China, A.; Nagaraja, V.; Sriram, D. Antimycobacterial activities of novel 2-(sub)-3-fluoro/nitro-5,12-dihydro-5-oxobenzothiazolo[3,2-a]quinoline-6-carboxylic acid. *Bioorg Med Chem*., **2008**, *16*, 3408.

[14] Dinakaran, M.; Senthilkumar, P.; Yogeeswari, P.; China, A.; Nagaraja, V.; Sriram, D. *Int.* Antimycobacterial and phototoxic evaluation of novel 6-fluoro/nitro-4-oxo-7-(sub)-4H-[1,3]thiazeto [3,2-a]quinoline-3-carboxylic acid. *J. Antimicrob. Agents*, **2008**, *31*, 337.

[15] Sriram, D.; Senthilkumar, P.; Dinakaran, M.; Yogeeswari, P.; China, A.; Nagaraja, V. Antimycobacterial activities of novel 1-(cyclopropyl/tert-butyl/4-fluorophenyl)-1,4-dihydro- 6-nitro-4-oxo-7-(substituted secondary amino)-1,8-naphthyridine-3-carboxylic acid. *J. Med. Chem*., **2007**, *50*, 6232.

[16] Sriram, D.; Yogeeswari, P.; Dinakaran, M.; Thirumurugan, R. Antimycobacterial activity of novel 1-(5-cyclobutyl-1,3-oxazol-2-yl)-3-(sub)phenyl/pyridylthiourea endowed with high activity toward multi-drug resistant tuberculosis. *J. Antimicrob. Chemother*., **2007**, *59*, 1194.

[17] Kumar, R.R.; Perumal, S.; Senthilkumar, P.; Yogeeswari, P.; Sriram, D. An atom efficient, solvent-free, green synthesis and antimycobacterial evaluation of 2-amino-6-methyl-4-aryl-8-[(E)-arylmethylidene]-5,6,7,8-tetrahydro-4H-pyrano[3,2-c]pyridine-3-carbonitriles. *Bioorg. Med. Chem. Lett.,* **2007**, *17*, 6459.

[18] Sriram, D.; Yogeeswari, P.; Thirumurugan, R.; Kumar, P.R. Discovery of newer antitubercular oxazolyl thiosemicarbazones. *J. Med. Chem*., **2006**, *49*, 3448.

[19] Vedejs, E.; Marth, C.F. Synthetic enantiopure aziridinomitosenes: preparation, reactivity, and DNA alkylation studies. *J. Am. Chem. Soc.,* **1988**, *110*, 3948.

[20] Kangani, C.O.; Master, H.E. Preparation of 1-aminoanthraquinone-2-carbohydrazide, 1.3.4-oxadiazoles, pyrazoles, pyrimidines and phthalazines. *Ind. J. Chem.,* **1998**, *37*, 778.

[21] National Committee for Clinical Laboratory Standards. Antimycobacterial susceptibility testing for *Mycobacterium tuberculosis*. Proposed standard M24-T. National Committee for Clinical Laboratory Standards: Villanova, Pa., **1995**.

[22] Katoch, V.M. Infections due to non-tuberculous mycobacteria (NTM). *Ind. J. Med. Res.,* **2004**, *120*, 290.

[23] Schreiber, J.; Burkhardt, U.; Rusch-Gerdes, S.; Amthor, M.; Richter, E.; Zugehor, M.; Rosahl, W.; Ernst, M. Non-tubercular mycobacterial infection of the lungs due to *Mycobacterium smegmatis*. *Pneumologie*, **2001**, *55*, 238.

[24] Geiss, H.K.; Feldhues, R.; Niemann, S.; Nolte, O.; Rieker, R. Landouzy septicemia (sepsis tuberculosa acutissima) due to *Mycobacterium microti* in an immunocompetent man. *Infection*, **2005**, *33*, 393.

[25] Hachem, R.; Raad, I.; Rolston, K.V.; Whimbey, E.; Katz, R.; Tarrand, J.; Libshitz, H. Cutaneous and pulmonary infections caused by *Mycobacterium vaccae*. *Clin. Infect. Dis.,* **1996**, *23*, 173.

[26] Spiegl, P.V.; Feiner, C.M. *Mycobacterium phlei* infection of the foot: a case report. *Foot Ankle Int.,* **1994**, *15*, 680.

[27] O' Brien, R.J.; Geiter, L.J.; Snider, D.E. The epidemiology of nontuberculous mycobacteria disease in the United States; results from a national survey. *Am. Rev. Respir. Dis.,* **1987**, *135*, 1007.

[28] Tabatabaei, N.; Stout, J.; Goldschmidt-Clermont, P.; Murdoch, D. Central nervous system infection and cutaneous lymphadenitis due to *Mycobacterium kansasii* in an immunocompetent patient. *Infection,* **2007**, *35*, 291.

[29] Xie, Z.; Siddiqi, N.; Rubin, E.J. Differential antibiotic susceptibilities of starved *Mycobacterium tuberculosis* isolates. *Antimicrob. Agents Chemother*., **2005**, *49*, 4778.

[30] Wayne, L.G.; Sohaskey, C.D. Non-replicating persistence of *Mycobacterium tuberculosis*. *Annu. Rev. Microbiol*., **2001**, *55*, 139.

[31] Betts, J.C. Evaluation of a nutrient starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling. *Mol. Microbiol*., **2002**, *43*, 717.

[32] Hu, Y.; Coates, A.R.M.; Mitchison, D.A. Sterilizing activities of fluoroquinolones against rifampin-tolerant populations of Mycobacterium tuberculosis. *Antimicrob. Agents Chemother*., **2003**, *47*, 653.

[33] Betts, J.C.; Lukey, P.T.; Robb, L.C.; Mc Adam, R.A.; Duncan, K. Evaluation of a nutrient starvation model of Mycobacterium tuberculosis persistence by gene and protein expression profiling. *Mol. Microbiol*., **2002**, *43*, 717.

[34] Mc Dermott, W. Microbial persistence. *Yale J. Biol. Med.,* **1958**, *30*, 257.

[35] Zhang, Y. Persistent and dormant tubercle bacilli and latent tuberculosis. *Front. Biosci.*, **2004**, *9*, 1136.

[36] O'Brien, R.J.; Nunn, P.P. The need for new drugs against tuberculosis. Obstacles, opportunities, and next steps. *Am. J. Respir. Crit. Care Med.,* **2001**, *163*, 1055.

[37] Zhang, Y. The magic bullets and tuberculosis drug targets. *Annu. Rev.Pharmacol. Toxicol.,* **2005**, *45*, 529.

[38] McKinney, J.D.; Bentrup, K.H.; Muñoz-Elías, E.J.; Miczak, A.; Chen, B.; Chan, W.T.; Swenson, D.; Sacchettini, J.C.; Jacobs, W.R.; Russell, D.G. Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature,* **2000**, *406*, 735.

[39] Glickman, M.S. A novel mycolic acid cyclopropane synthetase is required for cording, persistence, and virulence of *Mycobacterium tuberculosis. Mol. Cell,* **2000**, *5*, 717.

[40] Dahl, J.L. The role of RelMtb-mediated adaptation to stationary phase in long-term persistence of *Mycobacterium tuberculosis* in mice. *Proc. Natl. Acad. Sci. USA,* **2003**, *100*, 10026.

[41] Park, H.D. Rv3133c/dosR is a transcription factor that mediates the hypoxic response of *Mycobacterium tuberculosis. Mol. Microbiol.,* **2003**, *48*, 833.

[42] Bentrup, K.H.Z.; Miczak, A.; Swenson, D.L.; Russell, D.G. Characterization of activity and expression of isocitrate lyase in *Mycobacterium avium* and *Mycobacterium tuberculosis*. *J. Bacteriol.,* **1999**, *181*, 7161.

[43] Bai, B.; Xie, J.P.; Yan, J.F.; Wang, H.; Hu, C. A high throughput screening approach to identify isocitrate lyase inhibitors from traditional chinese medicine sources. *Drug Dev. Res*., **2006**, *67*, 818.

[44] Gundersen, L.L.; Nissen-Meyer, J.; Spilsberg, B. Synthesis and antimycobacterial activity of 6-arylpurines: The requirements for the N-9 substituent in active antimycobacterial purines. *J. Med. Chem.,* **2002**, *45*, 1383.

[45] Sriram, D.; Yogeeswari, P.; Basha, S.J.; Radha, D.R.; Nagaraja, V. Synthesis and antimycobacterial evaluation of various 7- substituted ciprofloxacin derivatives. *Bioorg. Med. Chem.,* **2005**, *13*, 5774.

Received: 05 May, 2009 **Revised: 29 July, 2009** **Accepted: 29 July, 2009**