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Synthesis and biological evaluation of substituted *N*-alkylphenyl-3,5-dinitrobenzamide analogs as anti-TB agents†

Gurunadham Munagala,[ab] Kushalava Reddy Yempalla,[ab] Sravan Kumar Aithagani,[ab] Nitin Pal Kalia,[c] Furqan Ali,[c] Intzar Ali,[c] Vikrant Singh Rajput,[ac] Chitra Rani,[ac] Reena Chib,[c] Rukmankesh Mehra,[d] Amit Nargotra,[d] Inshad Ali Khan,*[ac] Ram A. Vishwakarma*[ab] and Parvinder Pal Singh*[ab]

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Here, a medicinal chemistry study of an *N*-alkylphenyl-3,5-dinitrobenzamide (DNB) scaffold as a potent anti-TB agent is presented. A series of chemical modifications were performed and forty-three new molecules were synthesized to study the structure–activity relationship (SAR) by evaluating against a sensitive strain ($H_{37}Rv$) of *Mycobacterium tuberculosis* (MTB). Potent DNB analogs **4b**, **7a**, **7c**, **7d**, **7j**, **7r** and **9a** were further tested against resistant strains of MTB. Their intracellular as well as bactericidal potential was also evaluated. Cytotoxicity and *in vivo* pharmacokinetic studies suggested that DNB analogs have an acceptable safety index, *in vivo* stability and bio-availability. From the present work, two compounds **7a** and **7d** have shown nanomolar to sub micro-molar MIC in extracellular and intracellular assays.

Introduction

Tuberculosis (TB) remains a leading infectious disease worldwide and infects about one-third of the world's population. The World Health Organization (WHO) reported that TB caused more than 8 million cases of illness and 1.4 million deaths globally in 2011.[1] Emergence of multidrug resistant TB (MDR-TB) and extensively drug resistant TB (XDR-TB) has further complicated the world situation.[2–4] The 2012 WHO Global tuberculosis report suggested that the situation is even worse in India where the largest incidence of drug-resistant MTB has appeared in the last two to three years. The current situation necessitates the discovery and development of new anti-tuberculosis agents with low toxicity profiles and having potency against both drug-susceptible and drug-resistant MTB. In addition to this, new chemical entities should be capable of shortening the current duration of therapy and could be used in conjunction with first/second line anti-TB drugs and other drugs used to treat secondary infections such as cancer and HIV.[1]

Recently, the USFDA approved bedaquiline (also known as TMC-207, developed by Johnson & Johnson), the first drug against MDR-TB which works by inhibiting ATP-synthase. The approval of TMC-207 is being seen as a starting point in a new era of TB treatment.[5] Even though several other candidates such as OPC-67683,[6–8] PA-824,[9,10] Moxifloxacin,[11] SQ109,[12] Sutezolid,[13] AZD5847 and Linezolid[14] are presently being actively pursued in Phase II/III clinical trials still there is a need for novel scaffolds which should be effective against sensitive and resistant MTB.[15] In search of novel anti-TB scaffolds, we initiated a discovery

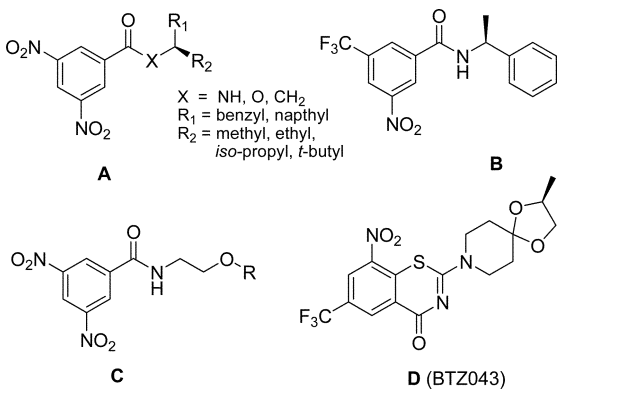

Fig. 1 Structure of known dinitrobenzamide (DNB) based anti-tubercular agents.

[a]*Academy of Scientific and Innovative Research, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu 180 001, India. E-mail: ppsingh@iiim.res.in; ram@iiim.res.in; iakhan@iiim.res.in; Fax: +91-191-2569333; Tel: +91-191-2569111*

[b]*Medicinal Chemistry Division, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu 180 001, India*

[c]*Clinical Microbiology, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu 180 001, India*

[d]*Discovery Informatics, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu 180 001, India*

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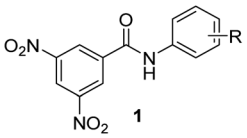
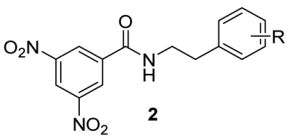
programme and a diverse library of 20 000 drug-like molecules procured from the Chembridge were screened against the $H_{37}Rv$ strain of *Mycobacterium tuberculosis* (MTB), wherein one of the hit series contains an *N*-(alkyl/alkylphenyl)-3,5-dinitrobenzamide scaffold. A literature survey revealed that *N*-(substituted)-3,5-dinitrobenzamide derivatives (**A**, Fig. 1) were known to have anti-TB activity[16] and recently it was reported that the nitrobenzamide derivatives **A**, **B** and **C** resemble a very potent anti-TB candidate BTZ043 **D** in their mode of action and inhibit the same target, heterodimeric decaprenyl-phosphoribose-2′-epimerase (DprE1).[17–20] From our screening of the 20 000 Chembridge library, eleven compounds containing the *N*-(alkyl/alkylphenyl)-3,5-dinitrobenzamide skeleton showed potent MIC against both sensitive ($H_{37}Rv$) as well as rifampicin-resistant (Rif^R) strain of MTB (data given in Fig. 2). This scaffold inhibits novel anti-Tb targets and is effective against both sensitive and resistant strains of MTB, but unfortunately not much work has been done on this scaffold except for Christophe *et al.*[21] findings where very closely related *N*-(substituted)-3,5-dinitrobenzamide derivatives of formula **C** were evaluated in detail for anti-Tb activity. In this direction, we have started a medicinal chemistry programme around the *N*-(alkyl/alkylphenyl)-3,5-dinitrobenzamide scaffold and a series of chemical modifications were made to understand the SAR by evaluating the synthesized compounds for anti-TB potential.



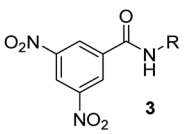
1

Entry	R	$H_{37}Rv$ μM	MIC (Rif^R) μM
a	4-*iso*-propyl	12.14	48.58
b	4-F	1.63	13.04
c	2-F	26.08	13.04
d	3,5-Di-Cl	22.53	45.07
e	2-CN	6.40	3.20

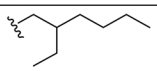


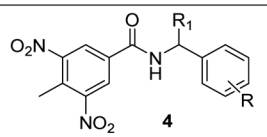
2

a	**H**	**0.79**	**<0.19**
b	4-OMe	1.45	<0.17



3

a		**0.19**	**<0.19**
b		**0.9**	**0.9**



4

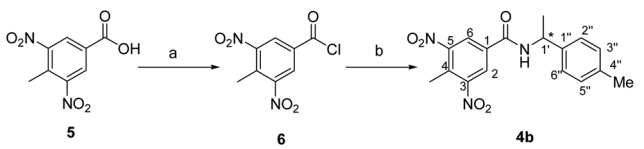
	R	R_1		
a	H	H	**6.35**	**3.17**
b	**4-Me**	**Me**	**<0.17**	**<0.17**

Fig. 2 Hits identified from screening of 20 000 small drug-like molecules.

Results and discussion

Among the 11 identified hits, di-nitrobenzamide (DNB) analogs **3a** and **4b** have shown potent MIC against sensitive $H_{37}Rv$ and Rif^R MTB. In order to re-validate these results, we first synthesized the most active hit **4b** following Scheme 1[22] and confirmed the activity results. We designed a medicinal chemistry strategy for structural modification as presented in Fig. 3. As the most potent hit **4b** contains a chiral centre, we synthesized both optically pure *R*- and *S*-enantiomers by coupling benzoyl chloride **6** with optically pure amines (Fig. 4). Between *R*- and *S*-enantiomers, *R*-enantiomer **7a** has shown more potent MIC (0.09 μM) as compared to *S*-enantiomer **7b** (11.66 μM).[23]

To know the effect of substituents present on the aromatic ring B, a series of optically pure (both *R*- and *S*-) analogs from corresponding un/substituted (F, Cl, OMe) amines **7c–i** were synthesized and tested against the $H_{37}Rv$ strain of MTB. MIC data revealed that *R*-enantiomers (**7e** and **7g**) were more active than *S*-enantiomers (**7f** and **7h**) except for fluoro substituted



Scheme 1 Synthesis of the most active hit **4b**.

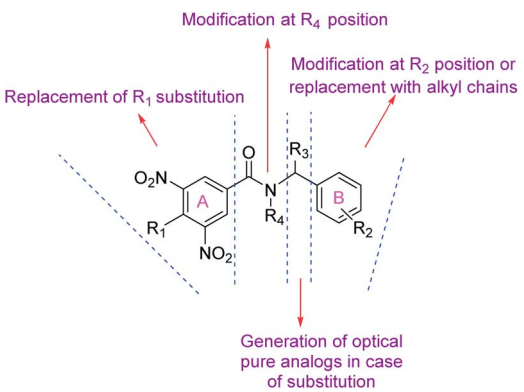
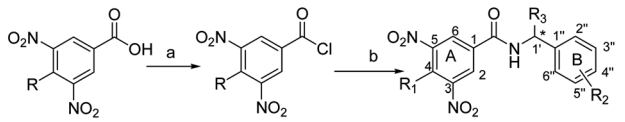


Fig. 3 Strategy for functional SAR.



5 (R=Me or H) 6 7

Scheme 2: Reaction and Conditions: **a**) $SOCl_2$, benzene, reflux, 4 h; **b**) (*R*) or (*S*)-substitedamine triethylamine, DMAP, CH_2Cl_2, rt, 12 h.

Entry	Reactant **5** or **6**	Product **7**				$H_{37}R_v$
	R	R_1	R_2	R_3	*	μM
a	Me	Me	4-Me	Me	R	0.09
b	Me	Me	4-Me	Me	S	11.66
c	Me	Me	4-F	Me	R	0.17
d	Me	Me	4-F	Me	S	0.04
e	Me	Me	4-Cl	Me	R	1.38
f	Me	Me	4-Cl	Me	S	>5.51
g	Me	Me	4-OMe	Me	R	0.7
h	Me	Me	4-OMe	Me	S	1.39
i	Me	Me	H	Me	R	0.36
j	H	H	4-Me	Me	R	0.05
k	H	H	4-Me	Me	S	0.76
l	H	H	4-F	Me	R	>12.01
m	H	H	4-F	Me	S	3
n	H	H	4-Cl	Me	R	0.17
o	H	H	4-Cl	Me	S	1.43
p	H	H	4-OMe	Me	R	2.9
q	H	H	4-OMe	Me	S	0.35
r	H	H	H	Me	R	0.19
s	H	H	H	Me	S	>3.17
t	H	H	H	Et	R	0.76
u	H	H	H	Et	S	3.04
v	H	H	H	Pr	R	2.91
Rifampicin						0.07

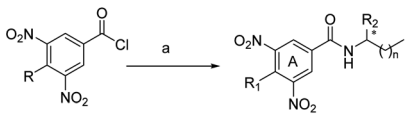
Fig. 4 Synthesis and screening result of *N*-benzyl DNB analogs (**a–v**).

analogs where *S*-enantiomer **7c** was more active than *R*-enantiomer **7d**. Among all these six analogs, both enantiomers of fluoro-substituted DNB analogs **7c** and **7d** had shown an MIC of 0.17 and 0.04 μM, respectively. The activity result suggested that the presence of substituents on ring B plays a critical role in modulating the anti-TB activity.

To know the role of the methyl group at ring A, we synthesized a series of DNB analogs **7j–s** without the methyl substituent but with varying substituents at ring B and tested (the result is given in Fig. 4). Again, *R*-enantiomers were found to be more active than *S*-enantiomers except for fluoro and methoxy substituted analogs. Among all these synthesized analogs **7j–s**, three compounds **7j**, **7n** and **7r** had shown a potent MIC of 0.05 μM, 0.17 μM and 0.19 μM, respectively. The activity results suggested that the presence of the methyl group at aromatic ring A is not very critical. On the other hand, replacement of the methyl group at the chiral centre with other groups such as ethyl **7t–u** and *n*-propyl **7v** diminished the activity (Fig. 4). This activity pattern suggested that methyl is more preferable over other alkyl groups.

One of the identified potent hit **3a** contains an alkyl chain instead of ring B and in order to know the role of aromatic ring B, a series of six compounds **8a–f** were synthesized wherein un/substituted alkyl amines were placed instead of substituted benzylamine (Fig. 5). None of the *N*-alkyl containing compounds was found to be better than the *N*-benzyl containing compounds.

Furthermore, the effects of chiral centre and length of the alkyl chain between N-atom and aromatic ring B were also studied and a series of compounds **9a–h** and **10a–e** were synthesized (Fig. 6 and 7). Activity data revealed that the

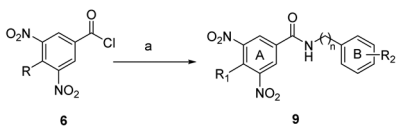


6 8

Scheme 3: Reaction and Conditions: **a**) alkylamenes, triethylamine, DMAP, CH_2Cl_2, rt, overnight.

Entry	Reactant **5** or **6**	Product **8**				$H_{37}R_v$
	R	R_1	R_2	*	n	μM
a	H	H	Me	R	1	1.87
b	H	H	Me	S	1	>3.74
c	H	H	Me	R	4	1.62
d	H	H	Me	S	4	1.62
e	H	H	H	-	4	1.69
f	H	H	H	-	14	0.57
Rifampicin						0.07

Fig. 5 Synthesis and screening result of *N*-alkyl DNB analogs **8(a–f)**.

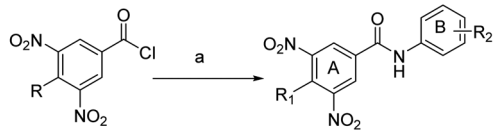


6 9

Scheme 4: Reaction and Conditions: **a**) substituted arylalkylamenes, triethylamine, DMAP, CH_2Cl_2, rt, overnight.

Entry	Reactant **5** or **6**	Product **9**			$H_{37}R_v$
	R	R_1	R_2	n	μM
a	H	H	H	2	0.79
b	Me	Me	H	1	0.38
c	Me	Me	4-Me	1	0.09
d	H	H	4-Me	1	3.17
e	H	H	H	1	0.8
f	H	H	4-Me	2	1.52
g	H	H	H	3	1.52
h	H	H	H	4	0.35
Rifampicin					0.07

Fig. 6 Synthesis and screening result of *N*-alkylphenyl DNB analogs **9(a–h)**.



6 10

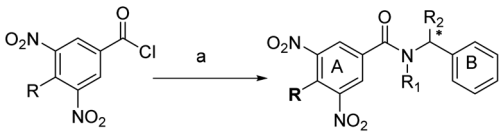
Scheme 5: Reaction and Conditions: **a**) substited anilines, triethylamine, DMAP, CH_2Cl_2, rt, overnight.

Entry	Reactant **5** or **6**	Product **10**		$H_{37}R_v$
	R	R_1	R_2	μM
a	H	H	4-F	>3.28
b	H	H	4-OCF_3	2.7
c	H	H	4-CF_3	2.82
d	H	H	2-CF_3	1.41
e	H	H	3-F	3.28
Rifampicin				0.07

Fig. 7 Synthesis and screening result of *N*-phenyl DNB analogs **10**(a–e).

presence of alkyl is also critical and the absence or increase in the chain length lowers the activity. We also synthesized *N*,*N*-di-substituted compound **11a** which showed an MIC value of 1.52 μM (Fig. 8). The presence of *N*,*N*-di-substitution also lowers the activity.

Among all the tested compounds, seven potent DNB analogs **4b**, **7a**, **7c**, **7d**, **7j**, **7r** and **9a** were further screened against rifampicin-resistant (Rif^R), isoniazid-resistant (INH^R) and multi-drug resistant (MDR) strains of MTB[23] and results are shown in Fig. 9. Four DNB analogs *viz.*, **7a**, **7d**, **7j** and **7r** had shown an MIC of <0.05 μM against Rif^R MTB and one derivative **7j** had shown an MIC of <0.05 μM against INH^R MTB. Two DNB derivatives **7a** and **7d** had also shown a potent MIC against the MDR strain of MTB.



6 11

Scheme 6: Reaction and Conditions: **a**) (*R*)- *N*-methyl-1-phenylethan-1-amine triethylamine, DMAP, CH_2Cl_2, rt, overnight.

Entry	Reactant **5** or **6**	Product **11**				$H_{37}R_v$
	R	R	R_1	R_2	*	μM
a	H	H	Me	Me	R	1.52
Rifampicin						0.07

Fig. 8 Synthesis and screening result of *N*,*N*-disubstituted DNB analog **11a**.

Compounds	MIC (Rif^R) μM	MIC (INH^R) μM	MIC (MDR) μM
4b	<0.17	#	#
7a	<0.04	0.35	0.35)
7c	11.53	11.53	5.76
7d	<0.04	0.72	0.72
7j	<0.05	<0.05	12.15
7r	<0.047	0.79	12.69
9a	1.59	0.795	>12.69
Rifampicin	256	#	64
Isoniazid	#	64	16
Ethambutol	#	#	16

not determined

Fig. 9 Screening of active hits against resistant strains.

Compounds	MBC (μM)	MIC (μM) (Intracellular)	IC_{50} (μM) in J-774	SI= IC_{50}/MIC	clogp	PSA
4b	<0.04	0.72	>58.25	>1333	2.978	121.443
7a	0.72	1.44	>58.25	>666	3.021	121.45
7c	0.17	0.71	>57.62	>333	2.899	121.443
7d	**<0.04**	**<0.64**	**>57.62**	**>1333**	**2.941**	**121.239**
7j	0.20	0.80	>60.77	>1333	2.592	124.219
7r	0.19	0.76	>63.47	>333	2.273	124.166
9a	0.79	3.16	>63.47	>80	2.273	126.702

Fig. 10 Bactericidal, intracellular and safety potential of active hits.

DNB analogs have shown bactericidal potential at their MIC value (Fig. 10). As we know, tubercle bacilli survive and multiply within macrophages and killing the intracellular tubercle bacilli is the key requirement for efficient tuberculosis treatment, the seven DNB analogs were screened for intracellular killing of MTB inside the macrophages.[24] Five DNB derivatives **4b**, **7c**, **7d**, **7j** and **7r** have shown a sub-micromolar MIC of 0.72, 0.71, <0.64, 0.80 and 0.76 respectively in intracellular assay (Fig. 10).

The cytotoxic effects of seven potent DNB analogs have been determined on the macrophage J-774 cell line[25] (Fig. 10), wherein none of the compounds had shown any cytotoxicity at 20 μg ml^{-1} which suggested their high safety index.[29]

Compound	7a		7d	
Dose (mg/Kg)	2.5	1	2.5	1
Route	IP	IV	IP	IV
C_{max} (nM)	177.08	672.8	216.5	711.1
t_{max} (h)	0.50		0.25	
$t_{1/2,B}$ (h)	1.63	0.93	1.08	0.90
$AUC_{0\text{-}t}$ (nM*h)	431.06	352.4	351.27	374.31
CL (ml/min/Kg		134		111
%F (IP)	49		35	

[a]values reported are the average of three individual measurements

Fig. 11 *In vivo* PK studies of DNB derivatives **7a** and **7d**.

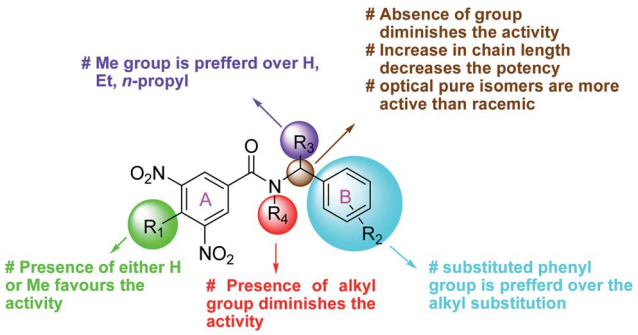


Fig. 12 Structure–activity relationship of DNB analogs wrt the $H_{37}Rv$ strain of MTB.

In order to know the *in vivo* stability, *in vivo* pharmacokinetics (PK) of two potent hits **7a** and **7d** were evaluated in male Balb mice *via* intraperitoneal (IP) and intravenous (IV) routes of administration at a dose of 2.5 and 1 mg kg^{-1}, respectively (Fig. 11). PK study revealed that both the compounds achieved maximum plasma concentration C_{max} within half-an-hour and is comparatively higher than their *in vitro* MIC value but their half-lives were found to be short. Both the DNB derivatives had shown good absolute intraperitoneal bioavailability (**7a**, 49%; **7d** 35%, detailed discussion and PK parameters are provided in part B of the ESI†). This is the first report regarding the pharmacokinetic (PK) studies of this class of compounds which suggests that the DNB scaffold is quite stable under *in vivo* conditions. We are presently pursuing the studies towards the oral PK-properties and other pre-clinical parameters that will be reported in due course.

Based on the screening results of DNB analogs against the $H_{37}Rv$ strain of MTB, key structural features essential for anti-TB activity have been identified as depicted in Fig. 12; (i) the *N*-substituted benzyl group is preferred over the *N*-alkyl group, (ii) optically pure isomers are more active than racemic, (iii) an increase in the alkyl chain length or the absence of alkyl chain between N-atom and ring B decreased the activity, (iv) at the chiral centre, the methyl group is preferred over H, Et- and *n*-propyl groups and (v) the presence of *N*,*N*-disubstitution is not preferred. In the case of resistant strains, DNB analogs having the Me-group at the benzylic position are preferred. Moreover, the SAR studies presented here also pave a way towards the designing of more robust and potent analogs with a better PK profile.

Conclusion

In summary, we have presented a first medicinal chemistry attempt towards the exploration of *N*-alkylphenyl-3,5-dinitrobenzamide analogs as potent anti-TB agents. The DNB derivatives possessed sub-micromolar to nanomolar potency not only against extracellular (both sensitive and resistant MTB) but also against intracellular assay (within macrophage). DNB analogs also have an acceptable safety index. *In vivo* pharmacokinetic studies also suggested an interesting PK profile and absolute intraperitoneal bioavailability. Presently, the efforts towards the replacement of the nitro-group with other isosteres as well as detailed investigation of this class of compounds is undergoing in our institute and will be published in due course.

Authors' contribution

PPS, IAK and RAV participated in the design and execution of this study and moreover drafting of the manuscript was done by PPS. GM, KR and SKA performed the chemical syntheses. NPK, FA, IA, VR, CR and RC performed and interpreted bio-activity data. RM and AN helped in the selection of the 20 000 drug like commercial library by selecting various filters of drug-likeness with the help of bio-informatics tools.

Acknowledgements

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22 *Synthesis of N-alkylphenyl-3,5-dinitrobenzamide (DNB) analogs – general procedure*: to a solution of acid **5** (2.6 mmol) in benzene (10 ml) thionyl chloride (2 ml of a 2.0 M CH_2Cl_2 solution; 4.0 mmol) was added. The reaction was stirred under reflux for 5 h and concentrated to give crude acid chloride as a yellow solid. This yellow solid was dissolved in dry CH_2Cl_2 (10 ml) and then added dropwise to the corresponding anilines (2.6 mmol) in pyridine at 0 °C. After being stirred at room temperature for 4 h, the reaction mixture was concentrated. The resulting mixture was partitioned between ethyl acetate and water, the organic layer was separated and washed with brine, dried over sodium sulphate and filtered. The filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography using EtOAc–hexane as eluent and characterized by NMR and mass spectroscopy (details along with spectra are given in the ESI).†

23 *Biological Evaluation – in vitro* activity of compounds against *M. tuberculosis* $H_{37}Rv$ and three clinical isolates (*M. tuberculosis* Rif^R, *M. tuberculosis* INH^R & *M. tuberculosis* MDR): MIC determination: MIC was determined by the broth dilution method against *M. tuberculosis* $H_{37}Rv$ (ATCC 27294; American Type Culture Collection, Manassas, VA,

USA), *M. tuberculosis* MDR (resistant to isoniazid and rifampicin), *M. tuberculosis* INH^R (resistant to isoniazid) and *M. tuberculosis* Rif^R (resistant to rifampicin) using the micro-broth dilution method.[26,27] The bacterial strains were grown for 10 to 15 days in Middlebrook 7H9 broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% (v/v) glycerol, 0.25% (v/v) Tween 80 (Himedia, Mumbai, India), and 10% ADC (albumin dextrose catalase, Becton Dickinson, Sparks, MD) under shaking conditions at 37 °C in 5% CO_2 to facilitate exponential-phase growth of the organism. A bacterial suspension was prepared by suspending *M. tuberculosis* growth in normal saline containing 0.5% Tween 80 and turbidity was adjusted to 1 McFarland standard which is equivalent to 1.0×10^7 CFU ml^{-1}. The 2-fold serial dilutions of compounds were prepared in Middle brook 7H9 (Difco laboratories) for *M. tuberculosis* in100 μl per well in 96-well U bottom microtitre plates (Tarson, Mumbai, India). The above-mentioned bacterial suspension was further diluted 1 : 50 in the growth media and 100 μl volume of this diluted inoculum was added to each well of the plate resulting in the final inoculum of 1.0×10^6 CFU ml^{-1} in the well and the final concentrations of compounds ranged from 0.015 to 32 μg ml^{-1}. The plates were incubated at 37 °C for 3-weeks in 5% CO_2. The plates were read visually and the minimum concentration of the compound showing no turbidity was recorded as MIC. Minimum bactericidal concentration (MBC) was determined by plating 10 μl volume from each well showing no turbidity on Middlebrook 7H10 agar supplemented with 0.5% (v/v) glycerol, 0.25% (v/v) Tween 80, and 10% OADC (oleic acid, albumin dextrose catalase, Becton Dickinson, Sparks, MD, USA).

24 *Intracellular killing activity*: The intracellular killing potential of the *N*-alkylphenyl-3,5-dinitrobenzamide (DNB) analogs was determined on macrophage J774 cell lines. The cells were grown and maintained using RPMI media supplemented with 10% foetal calf serum (FCS). About 10^6 macrophage cells were cultured in each well of a 24 well plate and kept at 37 °C with 5% CO_2 for 24 h. After formation of monolayer cells and before transfection, macrophage cells were washed with growth medium without antibiotics and kept for 1 h at 37 °C. The monolayer was transfected with *M. tuberculosis* $H_{37}Rv$ at a multiplicity of infection (MOI) of 10. Plates were incubated at 37 °C with 5% CO_2 for 2 h. The media was decanted and fresh RPMI media containing amikacin 50 μg ml^{-1} was added and the plate was incubated at 37 °C with 5% CO_2 for 48 h. The antibiotic supplemented media was changed daily. Serial dilutions of the test compounds from MIC to 8XMIC were added in the media and the MTB infected cell lines were incubated at 37 °C with 5% CO_2 for 4 days with a daily change of compounds containing media. Intracellular bacteria, which have been protected from this treatment, were quantified by host-cell lysis in a mild detergent such as 0.25% SDS, and plated on to Middlebrook 7H10 agar supplemented with 0.5% (v/v) glycerol, 0.25% (v/v) Tween 80, and 10% OADC at incubated at 37 °C for 3 weeks for viable colony formation.[28] The activity of the compound was defined as the minimum concentration of the compound exhibiting 2-log killing of intracellular MTB with respect to the untreated control.

25 *Cytotoxicity assay*: the cyto-toxicities of the synthesized compounds were evaluated for mouse macrophage (J-774) cell lines using MTT assay, in a 96 well plate format. Cells were incubated in Roswell Park Memorial Institute (RPMI) containing 10% fetal calf serum (FCS) and supplemented with 75 mg per litre penicillin, 100 mg per litre streptomycin, 110 mg per litre sodium pyruvate, 2.38 g per litre HEPES, 0.05 mM 2 β-mercaptoethanol, and 2 g per litre $NaHCO_3$. Ten 2-fold serial dilutions of *N*-alkylphenyl-3,5-dinitrobenzamide (DNB) analogs starting from 20 μg ml^{-1} were added in the plate, and the plate was incubated for 24 h at 37 °C in a CO_2 incubator. After the completion of incubation 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added and cells were further incubated for 3 h at 37 °C in a CO_2 incubator. Formation of formazan salt by mitochondrial dehydrogenases and was determined by using a Elisa reader at 450 nm (Multiskan Spectrum; Thermo Electron Corporation, USA). The percentage cytotoxicity was calculated with respect to the untreated cells.

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