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Synthesis and docking studies of pyrazine-thiazolidinone hybrid scaffold targeting dormant tuberculosis.

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Abstract: The persistence of *Mycobacterium tuberculosis* (MTB) in dormant stage assists the pathogen to develop resistance against current antimycobactrial drugs. To address this issue, we report herein the synthesis of N-(4-oxo-2 substituted thiazolidin-3yl) pyrazine-2-carbohydrazide derivatives designed by following the molecular hybridization approach using pyrazine and thiazolidenone scaffolds. The compounds were evaluated against MTB H37Ra and *Mycobacterium bovis* BCG in dormancy model. Most of the compounds had IC_{50} values in 0.3-1 µg/ml range. The active compounds were further tested for anti-proliferative activity against THP-1, Panc –1, A549, and MCF – 7 cell lines using MTT assay and exhibited no significant cytotoxicity. We also report molecular docking studies using active analogs and MTB - Decaprenylphosphoryl-β-D-ribose-2'-epimerase (DprE1) to rationalize the biological activity and to provide an insight into the probable mechanism of action and binding mode of hybridized structures. The results obtained validate the use of molecular hybridization approach and also suggest that reported compounds can provide a novel pharmacophore to synthesize lead compounds against dormat MTB.

Key words: Tuberculosis, dormant, pyrazine, thiazolidenone, hybrid design, docking

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (MTB) is prevalent in all parts of the world. It is one of the world's deadliest communicable diseases due to high virulence and ability of MTB to enter into a dormant state which can subsequently undergo reactivation. The long-term persistence of MTB in dormant stage assists the pathogen to develop resistance against current antimycobactrial drugs. Thus, there is need for development of drugs which can target the dormant MTB and help in eradication of the disease. [1-3] *Mycobacterium bovis* (*M.bovis*) is also considered to be a disease causing strain in humans exposed to infected material or immunodeficiency. Hence identification of Bacillus Calmette-Guerin (BCG), an attenuated derivative of virulent strain of *M.bovis* is also considered clinically significant. [4]

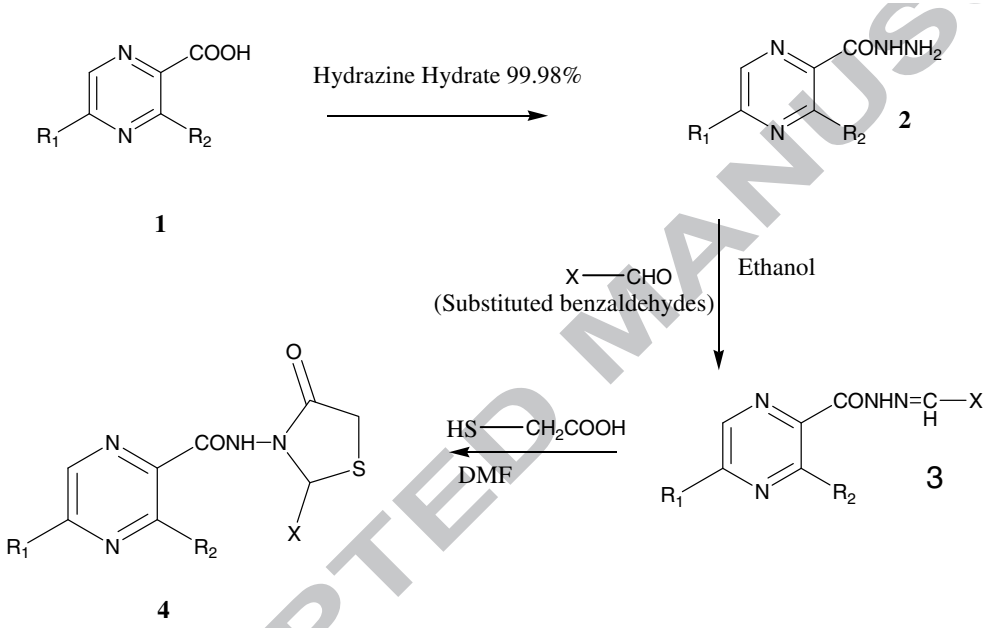
Molecular hybridization approach has been an area of interest towards development of agents against MTB. Most important hybridized structures include clinically used drugs such as rifamycin, ethambutol and isoniazid clubbed with other hydrophobic structure such as cinnamyl acid derivatives.[5-11] In this letter, we report novel chemical structures as antitubercular agents based on the hybridization of pyrazine and thiazolidine derivatives.

Previous studies have indicated promising antitubercular activity for pyrazine and thiazolidine derivatives against active strain. [12-19] Pyrazinamide mannich bases, pyrazine 2-carbohydrazides and pyrazine-2-carboxamide derivatives have been reported to have MIC values in lower micromolar range. Similarly thiazolidinone derivative have been reported to have MIC values in the micromolar range with some compounds having activity in submicromolar range.

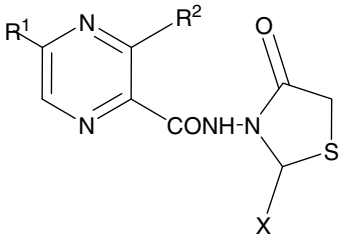
Thus, it was of interest to evaluate the activity of hybrids of pyrazine and thiazolidine derivatives against dormant mycobacterium. Since some of our compounds demonstrated promising activity we also investigated their probable mechanism by performing molecular docking study using Decaprenylphosphoryl-β-D-ribose-2'-epimerase (DprE1) as the target receptor.[20]

The starting materials, pyrazine-2-carboxylic acid/ 3-aminopyrazine – 2-carboxylic acid/ 5-methylpyrazine-2-carboxylic acid were purchased from Spectrochem (Mumbai, India) and their identity was confirmed by IR, ^{1}H NMR and Mass spectra. N-(4-oxo-2 substituted thiazolidin-3yl) pyrazine-2-carbohydrazide derivatives (4a-4w) were synthesized in three steps as shown in

scheme 1. Briefly, substituted pyrazine-2-carboxylic acid derivative were converted to carbohydrazides using hydrazine hydrate. Substituted pyrazine-2-carbohydrazides were then converted to their respective hydrazine using substituted benzaldehydes. Lastly, carbohydrazones were reacted with with thioglycolic acid in the presence of catalytic amount of $ZnCl_2$ to obtain final hybrid compounds.[14,19,21] In total 23 compounds were synthesized (4a- 4w; Fig. 1). Final compounds were purified using column chromatography and structures of all the compounds were confirmed by IR, ^{1}H NMR and mass spectroscopy (supplementary data). Purity of the compounds was determined by TLC/HPLC. The melting points were found in the range of 145-167 ^{0}C.



Scheme 1. Synthetic route for N-(4-oxo-2 substituted thiazolidin-3yl) pyrazine-2-carbohydrazide derivatives (4a-4w)



Sr. No	Comp code	X	R^1	R^2	Sr. No	Comp code	X	R^1	R^2
1	4a	$-C_6H_5$	-H	$-NH_2$	13	4m	3- $NO_2C_6H_5$	$-CH_3$	-H
2	4b	4- ClC_6H_5	-H	$-NH_2$	14	4n	4- $NO_2C_6H_5$	$-CH_3$	-H
3	4c	2,4- ClC_6H_5	-H	$-NH_2$	15	4o	4- OHC_6H_5	$-CH_3$	-H
4	4d	3- $NO_2C_6H_5$	-H	$-NH_2$	16	4p	3-OC_2H_5,4 -OHC_6H_5	$-CH_3$	-H
5	4e	4- $NO_2C_6H_5$	-H	$-NH_2$	17	4q	4-OCH_3 C_6H_5	$-CH_3$	-H
6	4f	4- OHC_6H_5	-H	$-NH_2$	18	4r	3,4-OCH_3 C_6H_5	$-CH_3$	-H
7	4g	3- OC_2H_5,4 -OHC_6H_5	-H	$-NH_2$	19	4s	3- OCH_3,4 -OHC_6H_5	$-CH_3$	-H
8	4h	3,4-OCH_3 C_6H_5	-H	$-NH_2$	20	4t	4- $N(CH_3)_2$ C_6H_5	$-CH_3$	-H
9	4i	4- FC_6H_5	-H	$-NH_2$	21	4u	4- OHC_6H_5	-H	-H
10	4j	4- $N(CH_3)_2$ C_6H_5	-H	$-NH_2$	22	4v	4- ClC_6H_5	-H	-H
11	4k	$-C_6H_5$	$-CH_3$	-H	23	4w	4- $NO_2C_6H_5$	-H	-H
12	4l	4- ClC_6H_5	$-CH_3$	-H					

N-(4-oxo-2 substituted thiazolidin-3yl) pyrazine-2-carbohydrazide derivatives were screened for *in vitro* antitubercular activity against MTB H37Ra (ATCC 25177) and *M.bovis* BCG (ATCC 35743). Preliminary antitubercular screening was performed at concentrations, 30, 10 and 3µg/mL (Supplementary material). The XTT Reduction Menadione assay (XRMA) which is a well established anti-tubercular screening protocol (for dormancy model) was used for screening the compounds, against MTB H37Ra. [22] *For M.bovis* BCG, Nitrate reductase assay protocol was used .[3] The antitubercular drugs, rifampicin, pyrazinamide and isoniazid were used as reference standards. The MIC and IC_{50} are presented only for compounds which showed more than 90% inhibition at 30µg/ml concentration (Table 1) in dormant assay for MTB H37*Ra* and *M. bovis* BCG.[3] To evaluate the selectivity towards mycobacterium, the derivatives were tested (Supplementary data Table 2) against *Mycobacterium smegmatitis* as *well as* antibacterial screening using two Gram Positive (*S. aureus* and *B.* subtillus) and two Gram Negative bacteria (*P. flurescence* and *E.coli)* was carried out. [23] The compounds, 4a, 4g, 4h, 4m, 4n, 4p,4s, 4t, 4v and 4w showed more than 90 % inhibition of MTB H37Ra and *M.bovis* BCG. Compound 4p, displayed MIC = 26.56 µg/mL and IC50 value of 0.337 µg/mL against MTB H37Ra, whereas 4v

showed, MIC = 28.82 µg/mL and IC50 value of 3.26 µg/mL against *M.bovis* BCG respectively. None of the derivatives showed inhibition of *Mycobacterium smegmatitis.* According to the data, substitution in phenyl ring with hydroxyl and ethoxy groups has favored activity against MTB H37Ra and presence of 4-Chloro has shown more activity against *M.bovis* BCG. In general presence of 4-Chloro, 4- Nitro and substitution with hydroxyl and ethoxy/methoxy groups in ortho positions on phenyl ring have seem to give better activity. Remaining 13 compounds have shown % inhibition in a range of around 50 to 80% and hence were not studied further. The mentioned 10 compounds were then further examined for toxicity in a non adherent, human monocytic (THP-1) cell line, in adherent human pancreas epithelioid *(*Panc –1), adherent human lung epithelial (A549) cell line and in human breast epithelial (MCF – 7) cell line by MTT assay (Table 1). [24]

Table 1

Selected derivatives with their antimycobacterial activity and cytotoxicity.

Comp. code	*M.tuberculosis*		*M.bovis BCG*		THP 1 cell line		Panc 1 cell line		A 549 cell line		MCF 7	
	[a]MIC (µg/ml)	[a]IC_{50} (µg/ml)	[b]MIC (µg/ml)	[b]IC_{50} (µg/ml)	[c]MIC (µg/ml)	[c]IC_{50} (µg/ml)	[d]MIC (µg/ml)	[d]IC_{50} (µg/ml)	[e]MIC (µg/ml	[e]IC_{50} (µg/ml)	[f]MIC (µg/ml)	[f]IC_{50} (µg/ml)
4a	28.03	0.909	24.4	12.9	>100	89.65	>100	>100	>100	77.04	>100	81.33
4g	28.64	3.46	29.42	23.42	>100	68.88	>100	>100	>100	84.17	>100	80.77
4h	25.82	0.718	29.23	23.45	>100	85.41	>100	>100	>100	80.02	>100	86.17
4m	29	4.29	18.52	9.89	>100	>100	>100	>100	>100	>100	>100	>100
4n	27.53	0.528	28.8	23.45	>100	>100	>100	>100	>100	>100	>100	>100
4p	26.56	0.337	15.92	9.46	>100	>100	>100	>100	>100	>100	>100	>100
4s	27.87	0.997	12.25	5.1	>100	86.88	>100	>100	>100	62.26	>100	70.22
4t	28.402	2.31	29.4	21.8	>100	>100	>100	>100	>100	>100	>100	>100
4v	28.82	1.092	8.48	3.26	>100	>100	>100	>100	>100	>100	>100	>100
4w	29.02	1.466	9.38	4.93	>100	97.66	>100	>100	>100	>100	>100	65.6

Rifam-picin	0.0021	0.0014	0.04	0.004	-	-	-	-	-	-	-	-
Isoni-azid	0.075	0.0025	0.045	0.0023	-	-	-	-	-	-	-	-
Pyrazinamid-e	>10	ND*	>100	ND	-	-	-	-	-	-	-	-

a Compounds were tested against MTB H37Ra strains using XTT-Menadione assay; b. Compounds were tested against *M.bovis* BCG using Nitrate reductase assay; c. Cytotoxicity studies on THP-, Panc 1, A549, and MCF 7 cell line using MTT assay.
ND*- not determined

Further, with the aim of rationalizing the antitubercular activity, in the absence of available resources to carry out the enzyme-based experimental studies, a molecular docking study was carried out for the most active analogues against Decaprenylphosphoryl-β-D-ribose-2'-epimerase (DprE1) as the target receptor. [25,26,27] DprE1 is an essential component for cell growth and survival, making it a potential target. Following the discovery of the benzothiazinones (nitrobenzothiazinone-BTZ043) that binds covalently to DprE1 there has been a growing interest in this target.[28]

Molecular docking study revealed that N-(4-oxo-2 substituted thiazolidin-3yl) pyrazine-2-carbohydrazide derivatives investigated herein could snugly fit into the active site of DprE1 with very similar orientations occupying positions very close to that of the native ligand in the crystal structure and their resulting complexes were stabilized by a network of steric and electrostatic interactions (Fig.2). Their binding energies were also found to be negative ranging from -51.558kcal/mol to -36.395kcal/mol while the docking score ranged from -7.83 to -6.00 (native ligand: -7.953). The docking score for currently known DprE1 inhibitor – nitrobenzothiazinone (BTZ043) was found to be -8.621 with a binding energy of -55.288kcal/mol. A statistically significant correlation was observed as well between the experimental antitubercular activity and the molecular docking scores wherein the active analogues exhibited higher docking scores while those with relatively low inhibition were also predicted to have a lower score. Furthermore a detailed per-residue interaction analysis between the enzyme and the docked pyrazine-2-

carbohydrazides was carried out to identify the most significantly interacting residues through which we can speculate regarding the detailed binding patterns in the cavity. For the sake of brevity we have illustrated these results only for the most active analogue 4p while the results for 4a, 4g, 4h, 4m, 4n, 4s, 4t, 4v and 4w and their binding modes as, Figures 3S-11S respectively are provided in the supplementary material.

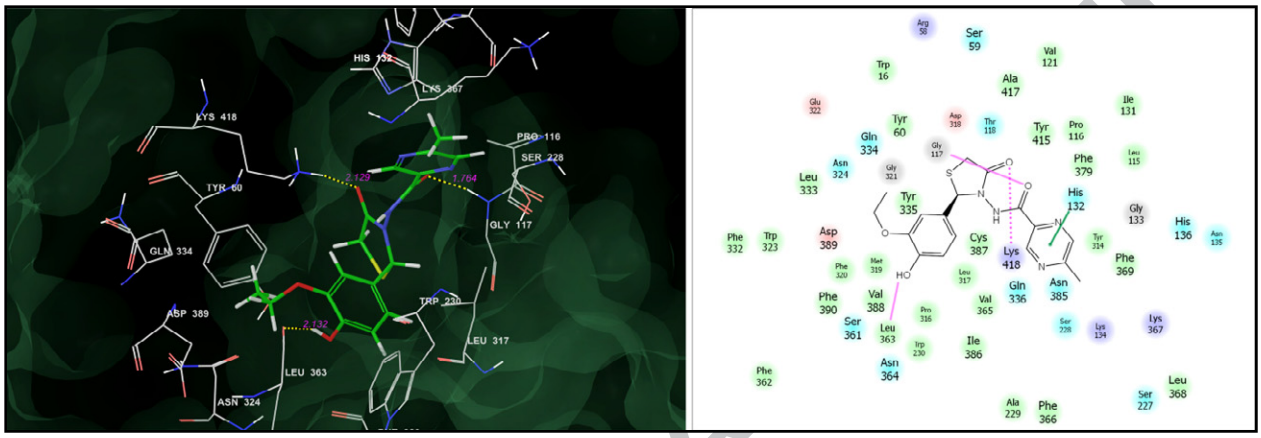


Figure 2. Binding mode of 4p into the active site of DprE1 enzyme (the dotted lines signify the hydrogen bonding interactions and the pi-pi stacking interactions are represented by green lines while).

The lowest energy docked conformation of 4p (Fig. 2) revealed that the compound binds with an overall binding energy of -51.558 kcal/mol making intimate contacts with the residues lining the active site of the target enzyme DprE1 through significant bonded and non-bonded interactions. The per residue-ligand interaction energy distribution showed an extensive network of favorable van der Waals interactions with Lys418(-2.65 kcal/mol), Thr118(-1. 85 kcal/mol), Gly117(-2.936 kcal/mol), Pro116(-2.164 kcal/mol) and Tyr60(-2.12 kcal/mol) residues through the central thiazolidinone ring; with Lys367(-1.82 kcal/mol), Val365(-4.01 kcal/mol), Gln336(-2.27 kcal/mol), Leu317(-3.92 kcal/mol), His132(-3.33 kcal/mol) through the pyrazine heterocycle while 3-ethoxy-4-hydroxyphenyl component of 4p was engaged in significant van der Waals interactions with Asp389(-1.93 kcal/mol), Cys387(-3.04 kcal/mol), Leu363(-1.95 kcal/mol), Asn324(-1.90 kcal/mol), Gly321(-1.82 kcal/mol), Phe320(-2.79 kcal/mol), Trp230(-1.96 kcal/mol) residues in the active site. Furthermore significant electrostatic interactions were observed with Lys418 (-2.30 kcal/mol), Gly117 (-2.10 kcal/mol) and Pro116 (-1.73 kcal/mol) via

thiazolidinone ring; with Asp389 (-2.08 kcal/mol) and Leu363 (-2.18 kcal/mol), via 3-ethoxy-4-hydroxyphenyl ring contributed to the enhanced binding affinity of 4p. This enhanced binding affinity can also be attributed to the three prominent hydrogen bonding interactions: first between oxygen (O) of amide linker and Gly117 residue at a distance of 1.76Å; the second interaction was between oxygen (O) of thiazolidinone scaffold and Lys418 residue with a bonding distance of 2.12Å while the third hydrogen bond was formed between hydroxy (OH) group of the 3-ethoxy-4-hydroxyphenyl ring and Leu363 with a bonding distance of 2.13Å. The complex of 4p with DprE1 was additionally stabilized by a crucial pi-pi stacking interactions observed between the pyrazine heterocycle of 4p and His132 residue in the active site. These type of hydrogen-bonding and the pi-pi (π- π) stacking interactions function as an "anchor", guiding the 3D orientation of the ligand in its active site and aid the steric and electrostatic interactions within. Thus a strong network of thermodynamic interactions observed between 4p and DprE1 account for its good *insilico* binding and provides a clue for its significant *invitro* antitubercular activity. These type of interactions were consistently observed for 4a, 4g, 4h, 4m, 4n, 4s, 4t, 4v and 4w as well but decreasing gradually with their observed anti-tubercular activity. Further, Figure 12S presents an overlay of the binding mode of 4p over BTZ043 to provide a comparison of their binding mode into the active site of DprE1 (please refer supplementary material).

We have thus synthesized N-(4-oxo-2 substituted thiazolidin-3yl) pyrazine-2-carbohydrazide derivatives using hybrid approach and evaluated them for antitubercular activity. The synthesized compounds displayed promising antitubercular activity in dormancy model, wherein all the compounds have shown more than 70% inhibition of MTB H37Ra and *M.bovis* BCG at 30µg/ml concentration. Few derivatives have even shown around 70 % inhibition of MTB H37Ra and *M.bovis* BCG at 3µg/ml concentration. The derivative 4p, displayed MIC = 26.56 µg/mL and IC50 value of 0.337 µg/mL against MTB H37Ra, whereas 4v showed MIC = 28.82 µg/mL and IC50 value of 3.26 µg/mL against *M.bovis* BCG. A result of screening against *Mycobacterium smegmatitis* and Gram positive as well as Gram negative bacterial strains, indicated the selectivity of these derivatives towards MTB. Further the most active compounds were evaluated for their cytotoxicity effect against, four human cell lines like, THP-1, Panc –1,

A549 and MCF –7 using MTT assay. The theoretical predictions from molecular docking studies were found to be in harmony with the *in vitro* antitubercular data, suggesting that DprE1 could be a potential target for these pyrazine-thiazolidinone derivatives. It could provide an insights into specific bonded and non-bonded interactions guiding the activity which could be fruitfully utilized for structure based optimization of this scaffold for the discovery of potent DprE1 inhibitors. The compounds could thus be further optimized for lead development.

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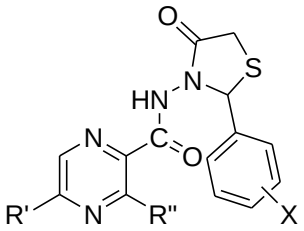

Compound code 4a-4j: R'= -H, R"= -NH_2

Compoundcode 4k-4t: R'= -CH_3, R"= -H

Compound code 4u-4w: R'= H, R"= -H

X = -Cl, -NO_2, -OH, -OCH_3, -NH_2

Figure 1. Structures of synthesized derivatives.

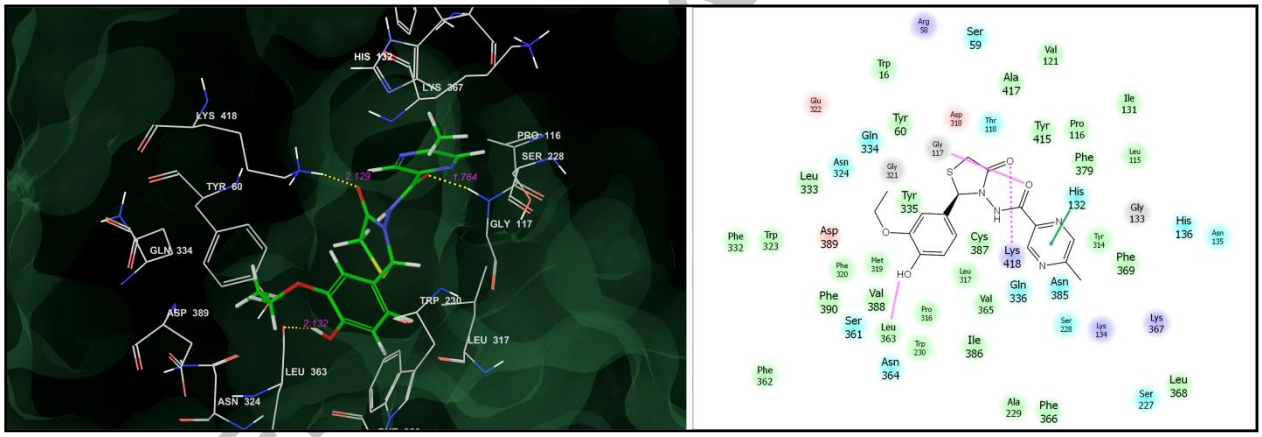

Figure 2. Binding mode of 4p into the active site of DprE1 enzyme (the dotted lines signify the hydrogen bonding interactions and the pi-pi stacking interactions are represented by green lines while).

*Graphical Abstract (for review)