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DESIGN AND SYNTHESIS OF NOVEL *MYCOBACTERIUM TUBERCULOSIS* DHFR INHIBITORS

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Dihydrofolate Reductase, *Mycobacterium tuberculosis*, Diaminotriazine, Enzyme assay, Selectivity.

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ABSTRACT: A series of 2,4-diaminotriazines were synthesized as *Mycobacterium tuberculosis*(*Mtb*) dihydrofolatereductase (DHFR) inhibitors. These derivatives were evaluated in whole cells by employing resazurinmicrotitre plate assay (REMA) against *Mtb*H_{37}Rv. Further, these were tested against other gram positive and gram negative bacterial strains to check specificity for *Mtb*. Cytotoxicity assessment was performed using HepG2 cell line and the compounds were found to be non-cytotoxic. The results indicated that some of the derivatives exhibited promising activity. The most active compound in the REMA assay was selected for DHFR enzyme assay against both the *Mtb* and human enzymes. The enzyme assay results indicated that this derivative exhibited selectivity towards the pathogenic enzyme. The most active compound in the whole cell assay against *Mtb*H_{37}Rv showed low cytotoxicity, was specific towards *Mtb* and displayed selectivity in the DHFR enzyme assay. Thus this study provides promising insight for design of potent and selective *Mtb* DHFR inhibitors.

INTRODUCTION: Folate pathway is an attractive and widely explored target for chemotherapy of various bacterial and protozoal infections, along with cancer. It is vital for nucleic acid biosynthesis and therefore DNA formation, and cell replication. [1] The differences in the enzymatic constitution of the microorganisms and mammals provide the basis for design of selective inhibitors with low toxicity to human cells. [2]

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dihydrofolatereductase (DHFR) is a crucial enzyme in the folate pathway that links folate synthesis to the production of Tetrahydrofolate (THF). DHFR inhibition directly halts cellular replication by starving the cell of crucial cellular precursors. [3, 4] DHFR is found in many pathogenic microorganisms including *Mycobacterium tuberculosis (Mtb)*. However, it remains relatively unexplored in *Mtb*, an obligate pathogen. World Health Organization has estimated 6.1 million cases of tuberculosis in the year 2014.[5] A rigid cell wall barrier and the ability to remain dormant are major hurdles in the treatment of TB. Issues such as subclinical persistence and development of resistance emphasize the need for new molecules which could act via unique mechanisms and

therefore show minimum cross resistance with existing drugs.

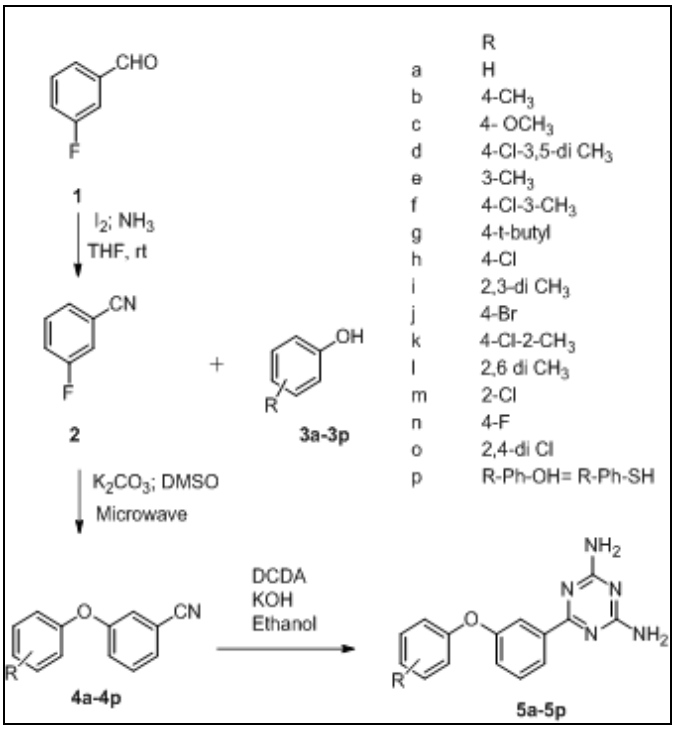
Various nitrogen heterocycles, [6-8] boron containing carboranes [9] and a tripeptide compound have been reported as *Mtb* DHFR inhibitors.[10] Our research group has been actively involved in the synthesis of several novel, diverse inhibitors of DHFR for *Mtb* [11, 12] and opportunistic pathogens, [13-15] including *Mycobacterium avium*. [16, 17]Additionally, we have isolated *Mtb* DHFR enzyme from recombinant *Saccharomycescerevisiae* strains and purified it using a novel affinity column.[18, 19]

In our earlier efforts to identify *Mtb* DHFR inhibitors, various diaminotriazines had been synthesized providing valuable insights for design of new derivatives. The current work deals with identification of key features necessary for binding to the active site of DHFR, using the interactions of known inhibitors and alignment of our earlier developed analogues. On the basis of these molecular modeling studies, we report a series of diaminotriazines as *Mtb* DHFR inhibitors. These derivatives were evaluated for whole cell *Mtb* inhibition. Cytotoxicity assessment was carried out to evaluate their toxicity using HepG2 cell lines. The molecules were also tested against other bacterial strains to assess selectivity towards *Mtb*. The most active derivative was further evaluated for inhibition of *Mtb* and human DHFR in an enzyme assay.

MATERIALS AND METHODS:

In the present work, docking interactions of known DHFR inhibitors methotrexate (MTX) and trimethoprim (TMP) with *Mtb* DHFR (PDB: 1DF7) were studied using Glide molecular docking protocol of Schrödinger LLC, NY.[20] Simultaneously, the Pharmacophore Alignment and Scoring Engine (PHASE) module of Schrödinger suite was used to align the 26 in-house *Mtb* DHFR inhibitors, having 2,4-diaminotriazine scaffold, [11] and identify additional features. The designed derivatives were docked into *Mtb* DHFR active site to investigate the *in-silico* binding interactions.

The synthesis of these designed compounds was carried out according to the reactions depicted in **Scheme1**.



SCHEME 1: SYNTHESIS OF DESIGNED DIAMINOTRIAZINE DERIVATIVES 5a-5p

Conversion of 3-fluorobenzaldehyde to its corresponding nitrile was carried out using iodine in ammonia water. Various phenols were condensed with this 3-fluorobenzonitrile in DMSO with K_2CO_3 as base using microwave irradiation giving intermediates **4a-4p**. These intermediates were reacted with dicyandiamide in ethanol using KOH as a base to give corresponding triazine derivatives. [21]

The synthesized derivatives were tested for their anti-tuberculosis activity against *Mycobacterium tuberculosis* $H_{37}Rv$ using two fold dilution technique of the resazurinmicrotitre assay (REMA). [22, 23] DHFR enzyme is present in both pathogens as well as humans. Hence to assess the cytotoxicity of these derivatives in the host, the compounds were tested using HepG2 cell lines (**Table 1**). [24] Additionally whole cell assay was carried out against *S. aureus* and *E. coli* as representatives of gram positive and gram negative organisms to determine selectivity towards the *Mtb*.

DHFR enzyme assay was carried out for the most active derivative in the whole cell assay. [25, 26] Enzyme activity was determined spectrophotometrically by monitoring the

decreasing absorbance for two min at 340 nm and 37°C. Standard assay mixture (1.0 mL) contained 50 mM potassium phosphate (pH 7.4), 5 mM DTT, and 60 µM NADPH and the reaction was started after 3 min of incubation by the addition of 45 µM DHF. A unit of the enzyme activity is defined as the amount of the enzyme converting 1.0 µmol of DHF and NADPH to THF and $NADP^+$ per min. This is calculated from the change in absorbance at 340 nm under specified assay condition.

RESULTS AND DISCUSSION: Docking study of MTX and TMP revealed necessary interactions such as hydrogen bonding interaction with Ile94, Ile5 and Asp27 along with presence of a tertiary nitrogen containing heterocycle (**Fig. 1a**). Simultaneously, in the alignment studies of the earlier reported derivatives it was observed that the phenyl ring directly attached to the triazine ring shows a good overlap as seen in **Fig. 1b.**

Further, the alignment revealed scope for metaphenoxy substitution taking into account the relative spatial orientation of the terminal phenyl rings near meta oxygen atom of the phenyl ring directly attached to triazine ring. (**Fig.1b**).

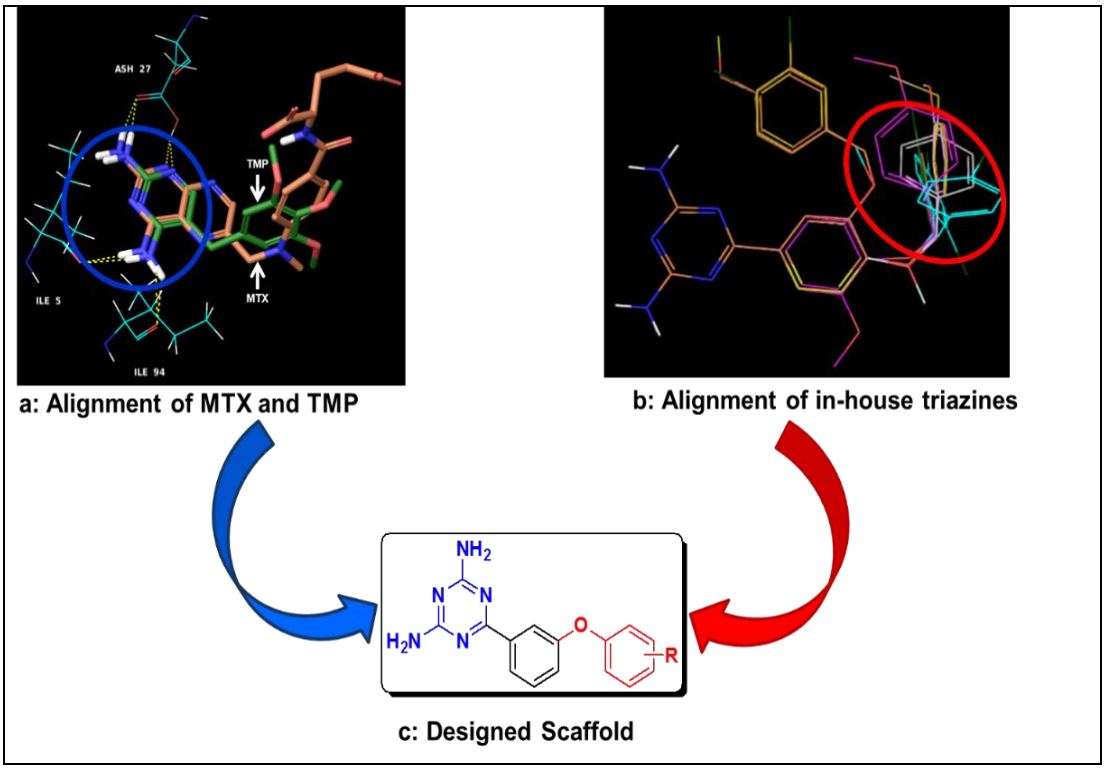


FIG. 1: DESIGN OF 5a-5p.

In the docking study of the designed diaminotriazine derivatives, favourable binding interactions with crucial active site residues of *Mtb* DHFR were observed. The amino group of the triazine ring showed H-bond interaction with the hydrophilic residues Asp27, Ile5 and Ile94 at the bottom of the active site cavity while the distal aromatic ring exhibited hydrophobic interactions with residues at the mouth of the active site tunnel. Docking interaction of a representative molecule is depicted in **Fig. 2**.

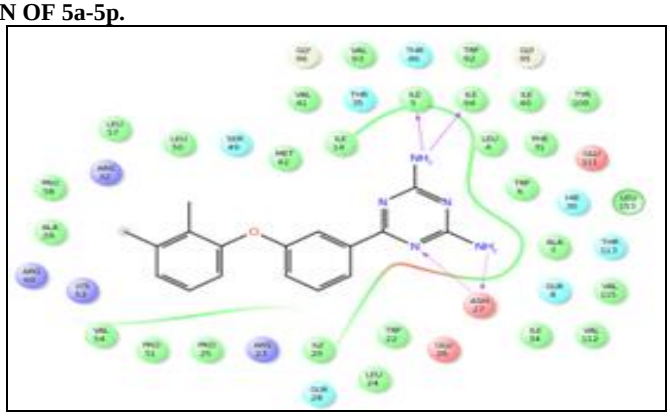


FIG. 2: LIGAND INTERACTION DIAGRAM OF A REPRESENTATIVE DERIVATIVE 5i.

Promising *in-vitro* biological testing results were obtained as indicated by the activity of derivatives

5h, **5k** and **5n**. Derivative **5k** showed moderate activity while derivatives **5b** and **5d** were inactive against *Mtb* H_{37}Rv. All the derivatives were found to be inactive against *S. aureus* and *E. coli* at concentration of 125 µg/ml indicating selectivity towards mycobacteria. *In-vitro* cytotoxicity testing indicated non-toxic nature of these derivatives. (**Table 1**).

TABLE 1: BIOLOGICAL ACTIVITY STUDIES OF THE SYNTHESIZED DERIVATIVES

Compound	MIC against *Mtb*H_{37}Rv(µg/ml)*	Inhibition of HepG2 (µg/ml)	MIC against *E.coli*(µg/ml)	MIC against *S.aureus* (µg/ml)
5a	>130	>80	>125	>125
5b	>125	>82	>125	>125
5c	>130	>81	>125	>125
5d	125	>83	>125	>125
5e	>125	>86	>125	>125
5f	25	>89	>125	>125
5g	>125	>84	>125	>125
5h	6.25	>95	>125	>125
5i	30	>92	>125	>125
5j	>125	>85	>125	>125
5k	13.75	>94	>125	>125
5l	25	>93	>125	>125
5m	>125	>87	>125	>125
5n	12.5	>88	>125	>125
5o	27.5	>90	>125	>125
5p	25	>91	>125	>125

* TMP and MTX showed MIC value of 8.4µg/ml and 0.44µg/ml respectively

MTX is a very potent DHFR inhibitor but is non-selective towards the pathogenic enzyme and therefore is not used in anti-infective therapy. Hence, the potency and selectivity of the most active derivative **5h** by was assessed testing against both *Mtb* and human DHFR. The enzyme assay [23, 24] revealed that **5h** showed IC_{50} values of 70 ± 0.011µM and 153 ± 0.042µM against *Mtb* and human DHFR respectively, indicating around two fold selectivity towards the pathogenic enzyme, in contrast to the IC_{50}values for MTX which were 0.00825 ±0.00025µM and 0.0016 ± 0.0003µM against the pathogen and host enzymes respectively with a selectivity of 0.194. The derivative 5h is less active than MTX but is 10 times more selective thus providing promising insight for design of selective, potent *Mtb* DHFR inhibitors.

CONCLUSION: To summarize, molecular modelling was used to design diaminotriazines as *Mtb* DHFR inhibitors. Some compounds showed promising whole cell activity against *Mtb*H_{37}Rv amongst which derivative **5h** was of particular note. It was also found to be non-toxic and specific towards *Mtb*. The enzyme assay results of **5h** indicated that it was 10 times more selective than MTX. Thus, taking in to account our preliminary biological results, our efforts are now focused on developing promising *Mtb* DHFR inhibitors along with series expansion.

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REFERENCES:

1. Gangjee A and Jain H: Antifolates-Past, present and future.Current Medicinal Chemistry. Anti-cancer Agents2004; 4:405-410.
2. Costi M and Ferrari S: Update on antifolate drugs targets.Current Drug Targets2001; 2:135-166.
3. Schweitzer BI, Dicker AP andBertino JR:Dihydrofolate reductase as a therapeutic target.FASEB Journal1990; 4:2441-2452.
4. Schnell JR, Dyson HJ and Wright PE: Structure, dynamics, and catalytic function of dihydrofolate reductase. Annual Reviews of Biophysics and Biomolecular Structure2004; 34:119-140.
5. WHO Global tuberculosis report 2014 (WHO/HTM/TB/2014.08)2014.
6. Kumar A, Zhang M, Zhu L, Liao RP, Mutai C, Hafsat S, Sherman DR and Wang MW: High-throughput screening and sensitized bacteria identify an *M. tuberculosis*

dihydrofolate reductase inhibitor with whole cell activity. PLoS One2012; 7:e39961.
7. El-Hamamsy MHRI, Smith AW, Thompson AS and Threadgill MD: Structure-based design, synthesis and preliminary evaluation of selective inhibitors of dihydrofolate reductase from *Mycobacterium tuberculosis*. Bioorganic and Medicinal Chemistry2007; 15:4552-4576.
8. Gerum AB, Ulmer JE, Jacobus DP, Jensen NP, Sherman DR and Sibley CH: Novel *Saccharomycescerevisiae* screen identifies WR99210 analogues that inhibit *Mycobacterium tuberculosis* dihydrofolate reductase. Antimicrobial Agents and Chemotherapy 2002; 46: 3362-3369.
9. Reynolds RC, Campbell SR, Fairchild RG, Kisliuk RL, Micca PL, Queener SF, Riordan JM, Sedwick WD, Waud WR, W KA, Dixon RW, Suling WJ and Borhani DW: Novel boron-containing, nonclassicalantifolates: Synthesis and preliminary biological and structural evaluation. Journal of Medicinal Chemistry 2007; 50:3283-3289.
10. Kumar M, Vijayakrishnan R and Subba Rao G: *In Silico* structure-based design of a novel class of potent and selective small peptide inhibitor of *Mycobacterium tuberculosis* dihydrofolate reductase, a potential target for anti-TB drug discovery. MolecularDiversity2010;14:595-604.
11. Tawari NR, Bag S, Raju A, Lele AC, Bairwa R, Ray MK, Rajan M, Nawale LU, SarkarD and Degani MS: Rational drug design, synthesis and biological evaluation of dihydrofolate reductase inhibitors as antituberculosis agents. Future Medicinal Chemistry2015; 7:979-988.
12. Lele AC, Raju A, Ray MK, Rajan MGR and Degani MS: Design and synthesis of diaminotriazines as anti-tuberculosis DHFR inhibitors. Current Research in Drug Discovery2014; 1:45-50.
13. Bairwa R, Tawari NR, Alate A, Anam S, Degani MS, Ray M and Rajan MGR: Novel 2-hydrazino-pyrimidin-4[3H]-one derivatives with pseudofunctional- similarity to siderophores as potential antimycobacterial agents. Medicinal Chemistry2013; 9:77-83.
14. Bag S, Tawari NR, Degani MS and Queener SF: Design, synthesis, biological evaluation and computational investigation of novel inhibitors of dihydrofolate reductase of opportunistic pathogens. Bioorganic and Medicinal Chemistry2010; 18:3187-3197.
15. Bag S, Tawari NR, Queener SF and Degani MS: Synthesis and biological evaluation of biguanide and dihydrotriazine derivatives as potential inhibitors of dihydrofolate reductase of opportunistic microorganisms. Journal of Enzyme Inhibition and Medicinal Chemistry2010; 25:331-339.
16. Tawari NR, Bag S and Degani MS: a review of molecular modelling studies of dihydrofolate reductase inhibitors against opportunistic microorganisms and comprehensive evaluation of new models. Current Pharmaceutical Design2011; 17:712-751.
17. Bag S, Tawari NR and Degani MS: Insight into inhibitory activity of mycobacterial dihydrofolate reductase inhibitors by *in-silico* molecular modeling approaches. QSAR &Combinatorial Science2009; 28:296-311.
18. Raju A, Degani MS, Khedkar MA and Niphadkar SN: Novel affinity chromatographic technique for purification of dihydrofolate reductase from recombinant yeast. International Journal of Drug Design and Discovery2013; 4:994-997.
19. Raju A, Khedkar MA and Degani, MS: Optimization of *Mycobacterium tuberculosis* DHFR production from recombinant *Saccharomycescerevisiae*. International Journal of Current Microbiology and Applied Sciences2013; 2:70-79.
20. Glide, Version 5.0, Schrödinger, LLC, New York, NY, *User manual*.
21. Bairwa R, Kakwani M, Tawari NR, Lalchandani J, Ray MK, Rajan MGR and Degani MS: Novel molecular hybrids of cinnamic acids and guanylhydrazones as potential antitubercular agents. Bioorganic and Medicinal Chemistry Letters 2010; 20:1623-1625.
22. Bairwa R, Kakwani M, Tawari NR, Lalchandani J, Ray MK, Rajan MGR and Degani MS: Novel molecular hybrids of cinnamic acids and guanylhydrazones as potential antitubercular agents. Bioorganic and Medicinal Chemistry Letters2010; 20:1623-1625.
23. Palomino JC, Martin A, Camacho M, Guerra H, Swings J and Portaels F: Resazurin microtiter assay plate: simple and inexpensive method for detection of drug resistance in mycobacterium tuberculosis. Antimicrobial Agents andChemotherapy2002; 46:2720-2722.
24. Vichai V and Kirtikara K: Sulforhodamine B colorimetric assay for cytotoxicity screening. Nature Protocols2006; 1:1112-1116.
25. White EL, Ross LJ, Cunningham A and Escuyer V: Cloning, expression, and characterization of *Mycobacterium tuberculosis* dihydrofolate reductase. FEMS Microbiology Letters2004; 232:101-105.
26. Hillcoat BL, Nixon PF and Blakley RL: Effect of substrate decomposition on the spectrophotometric assay of dihydrofolate reductase. Analytical Biochemistry 1967;21:178-189

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