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Design and synthesis of a focused library of diamino triazines as potential *Mycobacterium tuberculosis* DHFR inhibitors

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Keywords: Diamino triazine, Dihydrofolate Reductase, *Mycobacterium tuberculosis*, Molecular modeling, Enzyme assay, Synergy, Selectivity.

Abstract

We report design of a series of 2,4-diamino triazines as *Mycobacterium tuberculosis* (*Mtb*) dihydrofolate reductase inhibitors. The synthesized compounds were evaluated against *Mtb* ($H_{37}Rv$ and Dormant stage $H_{37}Ra$), their cytotoxicity was assessed (HepG2 and A549 cell lines) and selectivity towards *Mtb* was evaluated by testing against other bacterial strains. Some derivatives showed promising activity along with low cytotoxicity. The most potent compound in the whole cell assay (MIC 0.325μM against $H_{37}Rv$) showed selectivity in the enzyme assay and exhibited synergy with second line anti-TB agent *p*-amino salicylic acid.

This study therefore provides promising molecules for further development as anti-tuberculosis DHFR inhibitors.

Folate metabolism represents an important and attractive target for chemotherapy. The importance of this pathway in chemotherapy of infectious diseases arises from its function in DNA biosynthesis and cell replication. Tetrahydrofolate, the central component of folate metabolism, is a critical one-carbon unit donor. Owing to this, it plays an essential role in the biosynthesis of purines and pyrimidines and therefore in the nucleic acid biosynthesis for all the living organisms and is thus directly or indirectly involved in the processes of cell reproduction.[1] Numerous enzymes in the cell reproduction cycle use folate either as a cofactor or as a substrate. Differences in the enzymatic constitution of the microorganisms and mammals, either with respect to the enzyme types or with respect to architecture of common enzymes, have enabled devising an attack strategy towards pathogens.[2] These differences provide the basis for design of selective inhibitors devoid of toxicity to human cells. Dihydrofolate reductase (DHFR), a key enzyme of this pathway plays an important role in the cell growth and proliferation, as it is the sole source of tetrahydrofolate, and thus acts as an Achilles' heel for rapidly proliferating cells.[3] DHFR is found in many pathogenic microorganisms including *Mycobacterium tuberculosis (Mtb)*. However, it remains relatively unexplored in *Mtb*, an obligate pathogen. In the year 2014, World Health Organization has estimated 6.1 million cases of tuberculosis (TB).[4] A rigid cell wall barrier along with the ability to remain dormant has made TB treatment difficult. The issue of subclinical persistence and resistance development highlights the need for new molecules, particularly in immune-compromised individuals.[5] Thus, new anti-tuberculosis agents are required to which could act *via* unique mechanisms and therefore show minimum cross resistance with the existing drugs.

Researchers have identified nitrogen heterocycles[6-8], boron containing carboranes[9] and a tripeptide as *Mtb* DHFR inhibitors.[10] Our research group has been actively involved in the search of DHFR inhibitors and has synthesized 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 *Saccharomyces cerevisiae* strains and purified it using affinity chromatography with a novel epoxy bound resin linked to the inhibitor, methotrexate.[18,19]

In our earlier efforts to identify *Mtb* DHFR inhibitors, various diamino triazines had been synthesized. These studies resulted in hits with *Mtb* inhibition in the micromolar range.[12] This encouraged us to focus our efforts towards strategically modifying the diamino triazine scaffold, using various molecular modeling techniques, with the goal to achieve sub-micromolar activity. The current work therefore deals with identification of pharmacophoric features using the interactions of known DHFR inhibitors and alignment of our earlier developed analogues. Further virtual screening was carried out based on the obtained pharmacophoric features to generate hits. On the basis of these molecular modeling studies, we report a new series of diamino triazines as *Mtb* DHFR inhibitors. These derivatives were evaluated for whole cell *Mtb* inhibition (active and dormant) and cytotoxicity on liver and lungs cell lines to evaluate their toxicity. These were also tested against other bacterial strains to assess selectivity towards *Mtb*. The most active derivative was further evaluated for inhibition of DHFR in an enzyme assay and synergy with the second line agent, *para* amino salicylic acid (PAS), which acts upstream to DHFR in the folate pathway.

In the present work, docking interactions of known DHFR inhibitors methotrexate (MTX) and trimethoprim (TMP) with *Mtb* DHFR (PDB: 1DF7) were studied which revealed necessary interactions such as hydrogen bonding interaction with Ile94, Ile5 and Asp27 along with presence of a tertiary nitrogen containing heterocycle (**Figure 1a**). Therefore diamino

substituted ring features were short-listed for manual pharmacophore generation (**Figure 1c**). Simultaneously, Pharmacophore Alignment and Scoring Engine (PHASE) module of Schrödinger suite was used to align the 26 in-house *Mtb* DHFR inhibitors, having 2,4-diamino triazine scaffold,[12] and identify additional features which could enhance the activity. It was observed that the phenyl ring, which is directly attached to the triazine ring, shows a good overlap in the alignment (**Figure 1b**).

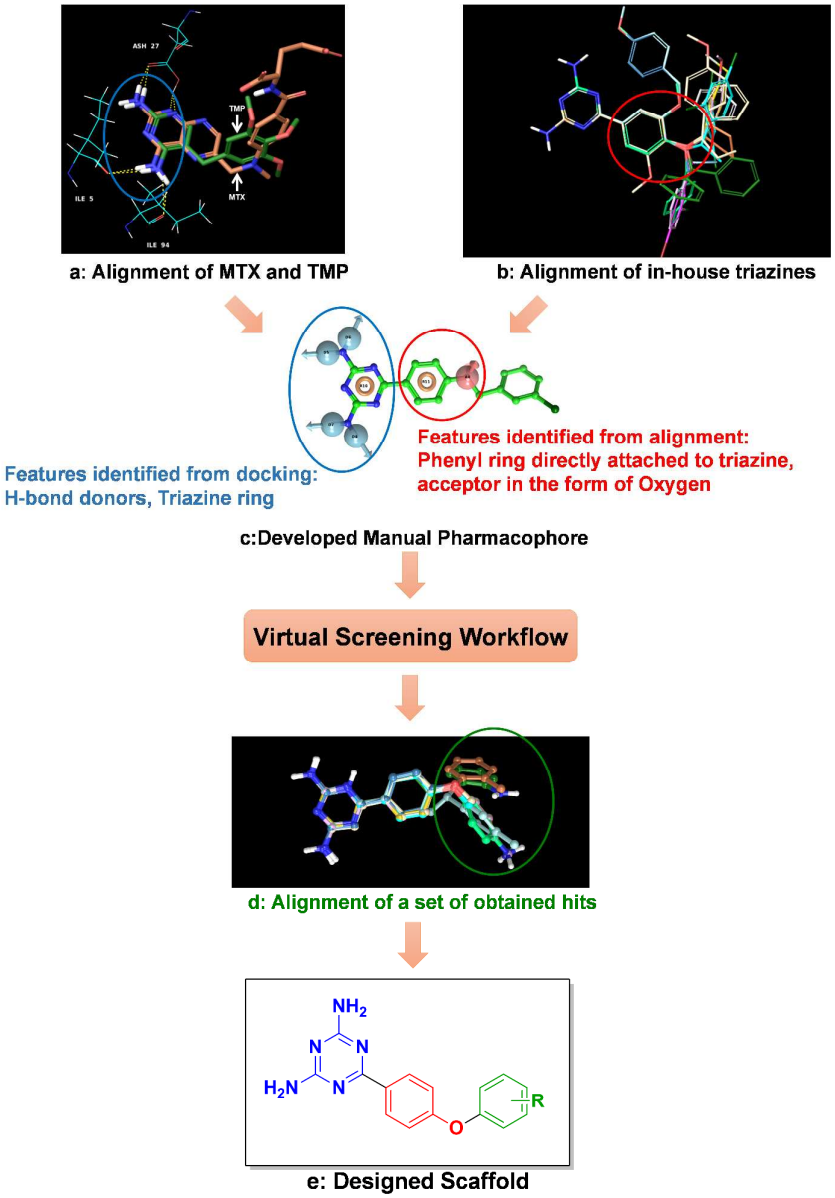


Figure 1. Design of **5a-5p**.

The alignment revealed the scope for substituting the oxygen atom attached to the phenyl ring with various hydrophobic groups. Therefore the phenyl ring and the oxygen atom were selected as ring and acceptor feature respectively and combined with the earlier identified features to generate the final manual pharmacophore hypothesis (**Figure 1c**). The manual pharmacophore generated was used for virtual screening, on *Mtb* DHFR (PDB: 1DF7), using the Phase Database to obtain virtual hits (**Figure 1d).** On the basis of the obtained hits, 2,4-diamino triazines were designed as potential *Mtb* DHFR inhibitors (**Figure 1e**). The designed derivatives were docked into *Mtb* DHFR active site to investigate the *in-silico* binding interactions. All the designed diamino triazines showed favourable binding interactions with crucial active site residues of *Mtb* DHFR. 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. Additional π-π stacking interactions with amino acid residue Phe31 were observed in many derivatives. Docking interaction of a representative molecule is depicted in **Figure 2**.

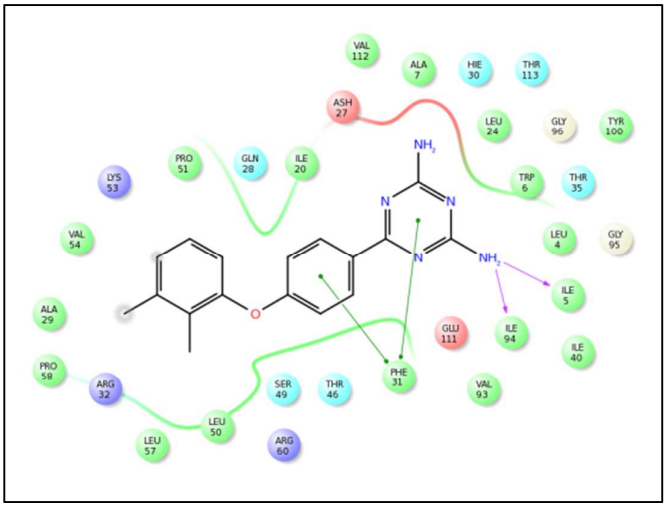
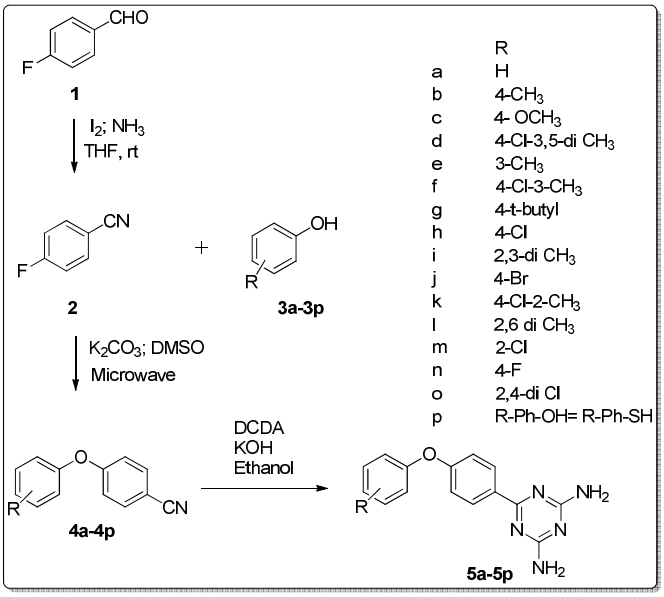


Figure 2. Ligand interaction diagram of a representative derivative **5i**.

The designed molecules were subjected to *in silico* ADME prediction and solubility estimation to assess drug likeness and were found to be drug like.

The synthesis of these designed compounds was carried out according to the reactions depicted in **Scheme 1**.



Scheme 1: Synthesis of designed diamino triazine derivatives 5a-5p

4-fluoro benzaldehyde **1** (4g, 0.0032mol) was dissolved in THF; ammonia (30%) (20 mL) was added and reaction mixture was stirred at room temperature for 25-30mins. To the white suspension formed, iodine (4.92g, 0.00384mol) was added in small portions which resulted in dark colouration of the reaction mass. The reaction mixture decolourized in 30-60 mins hr and it was further stirred at room temperature for 2-3 hours. After completion of the reaction, as indicated by TLC, saturated solution of sodium thiosulphate followed by 15 mL EtOAc was added. The organic layer was separated and the aqueous layer was extracted further with 2 x 10 mL of EtOAc. The combined organic layers were washed with brine (15 mL), dried over sodium sulphate and concentrated *in-vacuo* to obtain 4-fluoro benzonitrile **2.2** (2g, 0.00165 mol) and various phenols (**3a-3p**) (0.0198 mol) were dissolved in 15 mL DMSO.

K_2CO_3 (0.34g, 0.0025 mol) was added and the reaction mixture was subjected to three cycles of microwave irradiation, power 120 Watts, temperature 100°C for 15 mins with an intermittent cooling cycle. After completion, the reaction mass was poured into 20 mL of ice water and extracted thrice with 10 mL of EtOAc. The combined organic layer was treated with brine (15 mL), dried over sodium sulphate and concentrated *in-vacuo* to obtain derivatives **4a-4p**. Dicyandiamide (DCDA, 0.15g, 0.0018 mol), KOH (0.13 g, 0.00225 mol) were added to solution of **4a-4p**, (0.0015mol) in EtOH (10 mL). The resultant reaction mixture was refluxed in an oil bath for 10-18 hours. After completion of reaction, precipitated solid was filtered, washed with EtOH (5 mL) followed by hot water (25 mL) and dried to afford the corresponding diamino triazine derivatives **5a-5p**. The purity of the synthesized derivatives was determined by HPLC, melting point and appropriate spectral characterization using IR, NMR and Mass spectrometry.

The synthesized derivatives were tested for their anti-TB activity against both active[20,21] and latent forms.[22] The objective was to assess the selectivity towards mycobacteria as use of broad spectrum anti-infective agents may result in development of resistance and cross resistance. Therefore, the synthesized derivatives were tested against *S. aureus* and *E. coli* as representative gram positive and gram negative bacteria respectively. (**Table 1**)

The derivative **5i** showed better activity than both the known DHFR inhibitors MTX and TMP. Furthermore, it showed superior activity compared to the first line anti-TB drugs isoniazid (INH 0.73μM), ethambutol (MIC 19.58μM)[23] and streptomycin (MIC 3.44μM)[23]and near equivalent activity with rifampicin (MIC 0.24μM).[23] Derivative **5k** showed activity comparable to MTX. Additionally, derivatives **5j, 5l, 5m** and **5o** exhibited activity between the range of MTX and TMP (**Table 1**).

Table 1: Biological activity studies of the synthesized derivatives

Comp. ID	R	MIC against *Mtb* $H_{37}Rv$ (µM)	% inhibition of *Mtb* $H_{37}Ra$ (Dormant Stage) at 10 µg/ml	MIC against *S. aureus* (µM)	MIC against *E. coli* (µM)
5a	-H	109.56	19.36	447.55	447.55
5b	4-CH_3	>417.63	-	426.16	426.16
5c	4-OCH_3	>396.03	37.54	404.11	404.11
5d	4-Cl-3,5-diCH_3	>369.38	14.50	365.72	365.72
5e	3-CH_3	>447.46	15.04	426.16	426.16
5f	4-Cl-3-CH_3	>381.37	7.27	381.37	381.37
5g	4-t butyl	>380.14	42.19	372.69	372.69
5h	4-Cl	>398.42	81.06	398.42	398.42
5i	2,3-di CH_3	0.325	16.10	406.70	406.70
5j	4-Br	3.56	83.04	348.98	348.98
5k	4-Cl-2-CH_3	1.96	87.19	381.37	381.37
5l	2,6-di CH_3	20.34	96.86	406.70	406.70
5m	2-Cl	20.72	27.62	398.42	398.42

5n	4-F	>420.47	4.98	420.47	420.47
5o	2,4-di Cl	3.59	93.62	358.99	358.99
5p	R-Ph-OH= R-Ph-SH	211.61	11.65	423.21	423.21
MTX	-	**0.968**	-	-	-
TMP	-	**28.93**	-	-	-
INH	-	**0.73**	-	-	-

Promising *in-vitro* biological testing results were obtained as indicated by the activity of derivatives **5i**, **5j**, **5k**, **5l**, **5m** and **5o**. It was observed that substitution at 2 position of the terminal phenyl ring with both electron withdrawing or donating group enhances activity against *Mtb* H_{37}Rv. Methyl substitution at position 2 was preferred as observed with the activities of derivatives **5k** and **5o**. Addition of another methyl group at 3 position led to the most active compound of this series, **5i**. Most of the derivatives exhibited moderate to good inhibition of the latent tubercle bacilli at the concentration of 10 μg/ml. All the derivatives were found to be inactive against *S. aureus* and *E. coli* indicating selectivity towards mycobacteria.

In-vitro cytotoxicity testing was carried out using HepG2 cell line (liver)[24] and A549 cell line (lungs).[25] The results indicated that these derivatives were relatively non-toxic.

MTX is very potent albeit a non-selective DHFR inhibitor, toxic to human cells and therefore is not used in anti-infective therapy. Hence, one of the objectives of the current work was to assess the potency and selectivity of the most active derivative **5i** by testing against both *Mtb* and human DHFR. The enzyme assay[26,27] revealed that **5i** showed IC_{50}

values of 25.875 ± 0.006μM and 42 ± 0.063μM against *Mtb* and human DHFR respectively, indicating around two fold selectivity towards the pathogenic enzyme. The IC_{50} values for MTX 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 **5i** is less active than MTX but is 8 times more selective, thus providing promising insight for design of selective, potent *Mtb* DHFR inhibitors.

Synergy studies were carried out for **5i** with second line drug *p*-amino salicylic acid (PAS)[28], acting upstream of DHFR. Synergy of a molecule can be measured from its Fractional Inhibitory Concentration Index i.e. FICI which is determined by using the following formula.

$$\text{FICI} = \frac{\text{MICa combination}}{\text{MICa alone}} + \frac{\text{MICb combination}}{\text{MICb alone}}$$

The FICI value for **5i** was found to be 0.75 indicating synergistic association with PAS. (**Table 2**)

Table 2: Synergy studies of5iwith PAS

Compound	Alone (μg/ml)	In Combination (μg/ml)
5i	0.1	0.025
PAS	1	0.5

To summarize, molecular modelling was used to develop a manual pharmacophore which was further used for virtual screening and design of diamino triazines. Some compounds showed better or comparable activity to the first line anti-TB drugs and known DHFR inhibitors (MTX and TMP). Particularly, **5i** was found to have potent whole cell activity against *Mtb* $H_{37}Rv$ (MIC: 0.325μM) along with low cytotoxicity against both liver and lungs cell lines. Testing against gram positive and gram negative strains indicated

selectivity towards *Mtb*. The enzyme assay results indicated that this derivative showed promising DHFR inhibition and was 8 times more selective than MTX. Additionally, it exhibited synergistic association with PAS. Thus, this study represents a successful attempt to achieve sub-micromolar *Mtb* inhibition using a combined structure and ligand based approach and can provide impetus for further lead development of promising pre-clinical candidates.

ASSOCIATED CONTENT

Supporting Information: Molecular modelling protocols, along with spectral characterization and HPLC purity data for compounds **5a-5p** and details of biological testing.

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