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# Preparation, biological evaluation and molecular docking study of imidazolyl dihydropyrimidines as potential *Mycobacterium tuberculosis* dihydrofolate reductase inhibitors

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ABSTRACT

A series of novel dihydropyrimidine derivatives bearing an imidazole nucleus at C-4 position were synthesized in excellent yields via Biginelli multi-component reaction. The newly synthesized compounds were characterized by IR, $^{1}$H NMR, $^{13}$C NMR and Mass spectroscopy. In vitro antitubercular evaluation of all the newly synthesized compounds **4a**–**p** against *Mycobacterium tuberculosis* (Mtb) $H_{37}Rv$ showed, **4j** (MIC: 0.39 μg/mL; SI: >25.64), **4m** (MIC: 0.78 μg/mL; SI: >12.82) and **4p** (MIC: 0.39 μg/mL; SI: 24.10) as the most promising lead analogues. Compounds **4j**, **4m** and **4p** displayed effective reduction in residual Mtb growth within the tuberculosis-infected macrophage model. Further, molecular docking study of active molecules **4j**, **4m** and **4p** against *Mycobacterium tuberculosis* dihydrofolate reductase (Mtb DHFR) proved their potency as Mtb DHFR inhibitors acting as potential leads for further development. Pharmacokinetic properties leading to drug-likeness were also predicted for most active molecules **4j**, **4m** and **4p**.



Tuberculosis (TB) is a global epidemic caused by pathogenic bacterium *Mycobacterium tuberculosis* (Mtb).[1] As per the latest World Health Organization (WHO) TB report, in the year 2014, there were estimated 9.6 million new TB cases and 1.5 million deaths from it, which included 400,000 deaths among HIV infected patients.[2] Majority of TB cases were reported in the Asian, African and western Pacific regions. Two Asian countries, China and India together accounted for 40% of total TB cases worldwide.[2] In addition, emergence of multi drug resistant (MDR-TB) and extremely drug resistant (XDR-TB), and its synergy with human immunodeficiency virus (HIV) has become a major threat to humankind.[3–6] All the above facts necessitate a crucial need to design and develop new, potent and quick acting antitubercular agents with enhanced biological properties.[7–9] It is believed that molecular hybridization, a new concept in drug design and development based on the combination of pharmacophoric moieties of different bioactive substances to generate new hybrid molecules with improved affinity and efficacy is considered as one of the best tool to develop newer antitubercular agents.[10,11]

The quest for discovering novel antitubercular agents may be categorized into at least eleven categories as per their mechanism of action. These eleven categories are: inhibitors of DNA-based processes, arabinogalactan and peptidoglycan biosynthesis inhibitors, fatty acid biosynthesis inhibitors, dihydrofolate reductase (DHFR) inhibitors or siderophore biosynthesis inhibitors, protein synthesis inhibitors, branched-chain amino acid biosynthesis nucleoside inhibitors, proton pump $F_0F_1$ $H^+$ ATPase inhibitors, Mtb cytochrome P450 mono-oxygenase inhibitors, FtsZ protein targeting compounds, monophosphate kinase inhibitors and signaling kinase inhibitors.[12]

With regards to the above mentioned molecular targets, dihydrofolate reductase (DHFR) is considered as a promising drug target for the treatment of Mtb infections. DHFR is an essential enzyme in the folate cycle[13,14] supplying one-carbon units, derived from the serine hydroxymethyltransferase's action[15,16] on L-serine, for the biosynthesis of deoxythymidine monophosphate (dTMP). The folate cycle inhibition results in the interruption of the supply of thymidine and thus in inhibition of DNA biosynthesis and proliferation of cells. Inhibition of proliferation is an important goal in bacterial and protozoal infections therapy.[17]

One of the most well-known and highly potent DHFR inhibitor is methotrexate, which is an analogue of the substrate

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dihydrofolate. Methotrexate has diaminopteridine as the central core and is referred to as classical inhibitor. However, the full pteridinediamine structure is not required and non-classical inhibitors such as trimethoprim (TMP), pyrimethamine and methylbenzoprim were subsequently developed that contain pyrimidine-2,4-diamines or analogues as the central cores (Fig. 1). In this context, dihydropyrimidines contain a nitrogen-based heterocyclic core which is reminiscent of both classical and non-classical DHFR inhibitors. DHFR may not be a novel target, but it cannot be ignored as there is ample enthusiasm for the development of DHFR inhibitors, particularly with regard to mycobacteria.[18–24] This distinctive feature of DHFR makes it an ideal target for rational and effective drug design for antitubercular agents.

On the other side, imidazole derivatives are known to possess significant antimicrobial properties attributed mainly to presence of aryl–azolyl–ethane moiety present in many azole antifungal drugs serving as pharmacophore in compounds having antimycobacterial properties.[25–27] Apart from antifungal activity, various azole derivatives display substantial antitubercular activity,[28–31] leading to identification of azoles as sterol demethylase inhibitors, a mixed-function oxidase involved in sterol synthesis in eukaryotic organisms.[32] The unraveling of *Mycobacterium* genome sequence has shown that Mtb possesses a protein having homology to one of the above mixed oxidase function.[33] Additionally, PA-824, a nitroimidazopyran displayed potent in vitro activity against Mtb, a narrow spectrum of activity limited primarily to the Mtb complex with no noticeable cross-resistance to a wide range of antituberculosis drugs.[34] Keeping all these facts in mind and in continuation of our previous endeavor,[35–42] it was envisaged to design, synthesize and investigate in vitro efficacy of new prototypes including the advantage of dual pharmacophore of dihydropyrimidine and imidazole in a single molecular platform. Furthermore, molecular docking studies of most active compounds against the active site of the Mtb DHFR enzyme helped in revealing the potential mode of action through their interactions.

Synthetic strategy adopted to obtain the target compounds **4a**–**p** is outlined in Scheme 1. Multicomponent Biginelli reaction of an appropriate *N*-(aryl)-3-oxobutanamide[43] **(1a**–**p)** with 2-butyl-4-chloro-1*H*-imidazole-5-carbaldehyde (**2**) and urea (**3**) in methanol afforded final compounds 4-(2-butyl-4-chloro-1*H*-imidazol-5-yl)-*N*-(aryl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxamide (**4a**–**p**) in excellent yields. Spectral data of the synthesized compounds **4a**–**p** were fully in agreement with their proposed structures. The IR spectra of the synthesized compounds **4a**–**p** showed weak bands in the range of 3200–3220 $cm^{-1}$, 2970–3000 $cm^{-1}$, 2940–2960 $cm^{-1}$ and 2780–2810 $cm^{-1}$ corresponding to –NH stretching of amide, $-CH_3$ attached with C-6 position of 1,2,3,4-tetrahydropyrimidine, $-CH_3$ of butyl chain and $-CH_2-$ respectively and strong absorption bands at around 1700–1720 $cm^{-1}$ and 740–750 $cm^{-1}$ due to >C═O and C—Cl stretching respectively. $^1$H NMR spectra of final compounds **4a**–**p** showed singlets in the range of $\delta$ 2.20–2.30, 5.50–5.65, 9.60–9.90, 10.10–10.30 and 12.60–12.90 corresponding to $-CH_3$ of dihydropyrimidines, proton attached with C-4 position of dihydropyrimidines, –NH of dihydropyrimidines, –NH of amide and –NH of imidazole respectively. The aromatic ring protons were observed at $\delta$ 7.00–8.80. Characteristic peaks at around $\delta$ 122.5–123.5 and 162.5–163.5 in $^{13}$C NMR confirmed the presence of C–Cl and >C═O groups respectively. The mass spectra of compounds (**4a**–**p**) were in full agreement with their proposed structures.

In a standard primary screening, all the newly synthesized dihydropyrimidines **4a**–**p** were evaluated for their in vitro antitubercular potency at 6.25 μg/mL against Mtb$H_{37}$Rv (ATCC 27294; American Type Culture Collection) strain in BACTEC 12B medium using a broth microdilution assay, the microplate alamar blue assay (MABA).[44] The results are reported in Table 1. Compounds **4h**, **4j**, **4m**, **4n** and **4p** displayed inhibition in the range of 90–99% against Mtb. Compounds demonstrating less than 90% inhibition in the primary screening were not evaluated further. The active compounds **4h**, **4j**, **4m**, **4n** and **4p** were retested at and below 6.25 μg/mL to determine the actual MIC by using serial dilution. The Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration effecting a reduction in fluorescence of 90% relative to controls. Compounds **4j**, **4m** and **4p** demonstrated significant inhibitory action against Mtb with MIC of 0.39, 0.78 and 0.39 μg/mL respectively.

After identifying a good number of active antitubercular leads, cytotoxicity ($IC_{50}$) of the most active compounds **4h**, **4j**, **4m**, **4n** and **4p** were tested in VERO cells and their selectivity indexes (SI, the ratio of $IC_{50}$ to the MIC against Mtb $H_{37}$Rv) were measured. The compounds **4j, 4m** and **4p** were somewhat less toxic (SI $\geqslant$ 10) than **4h** and **4n** (SI < 10). Compound with an MIC $\leqslant$ 6.25 μg/mL and an SI $\geqslant$ 10 are considered safe for further screening, and an MIC $\leqslant$ 1 μg/mL in a novel compound class is considered an excellent lead,[45] which makes compounds **4j**, **4m** and **4p** very promising antitubercular compounds.

Mtb is an intracellular parasite and therefore it is crucial for any antitubercular lead to penetrate into macrophages and inhibit the growth of the infecting bacteria. In vitro efficacy of most promising compounds **4j**, **4m** and **4p** were further explored in a tuberculosis-infected macrophage model[46] and their results are summarized in

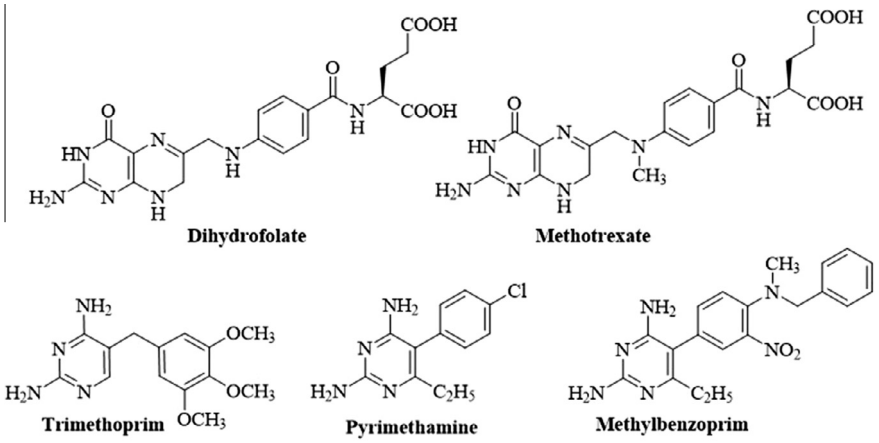


**Figure 1.** Classical and non-classical inhibitors of Mtb DHFR.