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Research paper

1,3,5-triazaspiro[5.5]undeca-2,4-dienes as selective *Mycobacterium tuberculosis* dihydrofolate reductase inhibitors with potent whole cell activity

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ABSTRACT

The emergence of multi- and extensively-drug resistant tubercular (MDR- and XDR-TB) strains of mycobacteria has limited the use of existing therapies, therefore new drugs are needed. Dihydrofolate reductase (DHFR) has recently attracted much attention as a target for the development of anti-TB agents. This study aimed to develop selective *M. tuberculosis* DHFR inhibitors using rationale scaffolding design and synthesis, phenotype-oriented screening, enzymatic inhibitory study, whole cell on-target validation, molecular modeling, and *in vitro* DMPK determination to derive new anti-TB agents. 2,4-diamino-1-phenyl-1,3,5-triazaspiro[5.5]undeca-2,4-dienes **20b** and **20c** were identified as selective *M. tuberculosis* DHFR inhibitors, showing promising antimycobacterial activities (MIC_{50}: 0.01 μM and MIC_{90}: 0.025 μM on *M. tuberculosis* H37Rv). This study provided compelling evidence that compound **20b** and **20c** exerted whole cell antimycobacterial activity through DHFR inhibition. In addition, these two compounds exhibited low cytotoxicity and low hemolytic activity. The *in vitro* DMPK and physiochemical properties suggested their potential *in vivo* efficacy.

1. Introduction

Tuberculosis (TB) is one of the leading causes of death worldwide and this has become a global public health issue. In 2015, WHO estimated 10.4 million cases of new TB infections and reported that 1.8 million deaths were TB related [1]. The statistics from recent reports have shown that the prevalence of TB has escalated to an epidemic proportion. The emergence of multi and extensively drug-resistant TB (MDR-TB and XDR-TB) strains of *Mycobacterium tuberculosis* has limited the use of existing therapies, thus exacerbating the epidemic further. In 2015, the mortality cases due to MDR-TB were estimated at 0.25 million while among the people who were diagnosed with MDR-TB, an estimated 9.5% of them were suffering from XDR-TB [2]. In addition, within the HIV positive population of patients, opportunistic infection due to TB is contributing to increasing morbidity and mortality [3]. The epidemiology of the disease has raised deep concerns among clinicians and scientists therefore active efforts are underway to address this public health related issue.

The current treatment regimen for drug-sensitive TB consists of a combination of four first-line drugs namely, isoniazid, pyrazinamide, rifampicin and ethambutol [4]. The patients are required to adhere to the combination therapy for at least 6 months. In case of drug-resistant strains, the treatment period can

Abbreviations: DHFR, dihydrofolate reductase; DMPK, drug metabolism and pharmacokinetics; FRLM, female rat liver microsomes; HBA, hydrogen bond acceptors; HBD, hydrogen bond donors; MDR, multi-drug resistant; MRLM, male rat liver microsomes; *Mtb*, *M. tuberculosis*; MTX, methotrexate; NMR, nuclear magnetic resonance; SI, selectivity index; TB, tuberculosis; TPSA, topological polar surface area; XDR, extensively-drug resistant.

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be extended to 18–24 months with the inclusion of some second-line drugs such as fluoroquinolones, streptomycin, linezolid, *para*-aminosalicylic acid and others [5]. Apart from the high pill burden and the lengthy treatment, the therapies for TB can also be costly. Many second-line drugs are known to be toxic and less efficient, posing a significant barrier to patient compliance [6]. Hence, more efforts are needed to broaden the availability of treatment options by designing and synthesizing new anti-TB agents that exert different mechanism of action to circumvent the current drug resistance issue.

Dihydrofolate reductase (DHFR), a key enzyme in the folate pathway, is responsible for reducing dihydrofolic acid to tetrahydrofolic acid. Tetrahydrofolate is involved in one carbon transfer reactions that lead to the biosynthesis of nucleic acids and amino acids. Thus, the inhibition of DHFR is able to arrest cell growth by depleting the cellular pool of DNA, RNA and protein synthesis precursors [7,8]. DHFR has been recognized as a validated drug target for antibacterial agents [9]. However, few potent and selective DHFR inhibitors have so far been reported that exhibits anti-mycobacterial activity despite on-going research work in the scientific community [10]. For example, by using a well-known DHFR inhibitor, methotrexate (**MTX**) as lead compound, two esters of methotrexate (**JSF-1187** and **JSF-1183**) have been reported to demonstrate potent pathogenic DHFR inhibition activity (Fig. 1) by Nixon et al. [11]. Nevertheless, these two compounds have not shown significant selectivity for *M. tuberculosis* DHFR (*Mtb* DHFR) due to their close similarity with **MTX**. Besides the pteridine scaffold, three hits (**PDP-1**, **PQD-1** and **Dpt-1**, Fig. 1) representing other chemical scaffolds were identified by Kumar et al. through a phenotypic screening against live H37Rv strain of *Mtb*, and DHFR inhibitory activity evaluation [12]. However, only **Dpt-1** exhibited selectivity against pathogenic DHFR. Recently, Nilesh and colleagues have developed two 2,4-diamino-1,3,5-triazine derivatives (Fig. 1) that showed selective *Mtb* DHFR inhibition, and these two compounds were found to possess promising antitubercular activity and lower cytotoxicity in human [13]. Collectively, these reports suggested that there is potential pharmacotherapeutic significance in using DHFR inhibitors for the treatment of tuberculosis. The core structural feature for the inhibition of DHFR is known to be a diamino-substituted N-containing single or fused polycyclic heterocycle such as pyrimidine, quinazoline or pteridine. Many of these scaffolds have been extensively studied and demonstrated to possess antibacterial activity against various microorganisms [7,14,15]. Nonetheless, few studies were carried out on diamino-substituted N-containing spiro scaffold [16].

Over the years, in the main author's laboratory, the spiro heterocycle 1,3,5-triazaspiro[5.5]undeca-2,4-diene has demonstrated behaviours that can be exploited for selective DHFR inhibitory activity against pathogenic bacteria. For instance, **7s** (Fig. 2) was demonstrated to inhibit the growth of *Mycobacterium smegmatis* [16], while **P-I-23** (Fig. 2) was found to have no inhibitory action on recombinant human DHFR, and was also found to be non-toxic to A549 cell line human lung carcinoma [17]. Taken together, it was hypothesized that with appropriate substitution on the saturated nitrogen of this spiro heterocycle, selective mycobacterial DHFR inhibitor with low human cytotoxicity could be developed. The saturated nitrogen was selected as a point for side chain extension because it was believed that the side chain would be accommodated in a channel at the active site [13,18,19]. This paper reports a study that entailed the synthesis of new compounds having extension made to the saturated nitrogen (N1) of the 1,3,5-triazaspiro[5.5]undeca-2,4-diene by inserting either a methylene or a phenyl attachment. The side chain was elongated using click chemistry. This conjunctive approach was applied to search for new compounds with DHFR inhibitory activity. It is also reported that in the distal end of the side chain, there are amino acid residues that may offer hydrophobic and hydrogen-bonding interactions [18]. It was therefore proposed to have phenyl alkyl group, benzoyl methyl group, and coumarin alkyl group inserted into the distal end in an attempt to interact with this part of the enzyme (Fig. 2).

2. Results and discussion

2.1. Chemistry

All the compounds were designed and synthesized using strategies that extended the side chain at position N1 of the spiro heterocycle (Fig. 2). Based on compound **7s**, one strategy was to link N1 of 1,3,5-triazaspiro[5.5]undeca-2,4-diene and the 1,2,3-triazole with a methylene group to evaluate if a rotatable alkyl linker would be favour DHFR inhibition that might lead to anti-mycobacterial activity. This strategy resulted in the design of compounds **7a-7c**, **11a-11c** and **14** (Scheme 1). The second strategy was derived from compound **P-I-23** by attaching 4-methyleneoxy phenyl ring at N1 of 1,3,5-triazaspiro[5.5]undeca-2,4-diene and linking it to the triazole ring. This strategy afforded compounds **19a-19c**, **20a-20c** and **21** (Scheme 2A, 2B and 2C). In these two strategies, the three-component one-pot synthesis described by Modest's was used to generate the alkyne intermediates **4** and **18** as hydrochloric salt [20]. The intermediates were reacted with selected azido derivatives to give the target compounds.

2.1.1. Synthesis of target compounds *7a-7c, 11a-11c and 14*

As shown in Scheme 1, the modified three-component one pot synthetic strategy was adopted to generate intermediate **4** by refluxing propargylamine hydrochloride **1**, cyanoguanidine **2**, and cyclohexanone **3** in the presence of catalytic amount (0.2 equivalent) of concentrated hydrochloric acid in ethanol [20]. In the following step, the transformation of benzylbromides **5a-5c** into the corresponding azides **6a-6c** was achieved via a nucleophilic substitution of the bromide with three equivalents of sodium azide under reflux condition. The azide intermediate was used in the next step without further purification. The freshly prepared azides **6a-6c** were coupled to intermediate **4** via a 1,3-dipolar addition reaction in the presence of catalytic amount (0.1 equivalent) of copper sulfate. The reaction of intermediate **4** and azide **6a-6c** was monitored by TLC (MeOH: DCM = 1: 9) using UV as detector. The target products **7a-7c** were formed in reasonably good yield (61–68%) when the reflux was conducted in a solvent system of THF and water at volume ratio of 1:1. For the synthesis of compounds **9a-9c**, the ethereal spacer was furnished via Williamson synthesis by refluxing the corresponding dibromoalkanes and compound **8** in acetonitrile under basic condition. Potassium carbonate was used to deprotonate the phenolic group on umbelliferon **8**. Azide derivatives **10a-10c** were formed when compounds **9a-9c** were treated with sodium azide under reflux. The coupling reaction of the azide derivatives **10a-10c** and intermediate **4** produced the desired target products **11a-11c**. The synthesis of product **14** was similar to the preparation of products **7** and **11**. The acetophenone **12** was treated with sodium azide to afford the corresponding azide **13**, which was followed by coupling with intermediate **4** to furnish the desired target product **14**. The yield for the final-step reaction was further improved by dissolving the azide derivative in tetrahydrofuran and followed by dropwise addition of this azide solution into the reaction mixture with constant stirring.

2.1.2. Synthesis of target compounds *19a-19c, 20a-20c, and 21*

The synthetic schemes for compounds **19a-19c**, **20a-20c** and **21** are illustrated in Scheme 2A, 2B and 2C respectively. Prop-2-ynyloxy aniline **17** was prepared by a two-step reaction. Firstly, 4-