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Original article

Piperazine derivatives: Synthesis, inhibition of the *Mycobacterium tuberculosis* enoyl-acyl carrier protein reductase and SAR studies

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ABSTRACT

The *Mycobacterium tuberculosis* NADH-dependent enoyl-acyl carrier protein reductase (*Mt*InhA) catalyzes hydride transfer to long-chain enoyl thioester substrates. *Mt*InhA is a member of the mycobacterial type II dissociated fatty acid biosynthesis system, and is the bona fide target for isoniazid, the most prescribed drug for tuberculosis treatment. Here, a series of piperazine derivatives was synthesized and screened as *Mt*InhA inhibitors, which resulted in the identification of compounds with IC_{50} values in the submicromolar range. A structure–activity relationship (SAR) evaluation indicated the importance of the chemical environment surrounding the carbonyl group for inhibition. In addition, the structure of one selected compound was supported by crystallographic studies, and experimental geometrical values were compared with semi-empirical quantum chemical calculations. Furthermore, the mode of inhibition and inhibitory dissociation constants were determined for the nine most active compounds. These findings suggest that these 9*H*-fluoren-9-yl-piperazine-containing compounds interact with *Mt*InhA at the enoyl thioester (2-*trans*-dodecenoyl-CoA) substrate binding site.

1. Introduction

Tuberculosis (TB) is an infectious disease primarily caused by *Mycobacterium tuberculosis* (*M. tuberculosis*), and it remains a major global health concern. According to the World Health Organization, an estimated 8.6 million new TB cases with approximately 1.3 million deaths were reported in 2012 [1]. The emergence of *M. tuberculosis* strains resistant to first- and second-line TB drugs has highlighted the need for novel and effective treatments [2]. Additional major concerns include TB-HIV coinfection and latent TB. In 2012, approximately 1.1 million of the estimated TB cases and 0.3 million deaths were described to occur among people who were HIV-positive [1]. In addition, one-third of the worldwide population has been reported to be at risk for reactivation from latent TB,

Abbreviations: TB, tuberculosis; *M. tuberculosis*, *Mycobacterium tuberculosis*; MDR-TB, multidrug-resistant tuberculosis; HIV, human immunodeficiency virus; FAS, fatty acid synthase; ACP, acyl carrier protein; *Mt*InhA, enoyl-ACP reductase from *M. tuberculosis*; NADH, nicotinamide adenine dinucleotide; INH, isonicotinic acid hydrazide; KatG, catalase-peroxidase; SAR, structure activity relationship; HTS, high-throughput screening; K_{ii}, inhibitory dissociation constant for the ESI complex; K_{is}, inhibitory dissociation constant for the EI complex; MIC, minimal inhibitory concentration; TEA, triethylamine; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; DMF, *N*,*N*-dimethylformamide; DD-CoA, 2-*trans*-dodecenoyl-CoA; DMSO, dimethyl sulfoxide; K_m, Michaelis constant; DMAP, 4-(*N*,*N*-dimethylamino)pyridine; PIPES, 1,4-piperazinediethanesulfonic acid.

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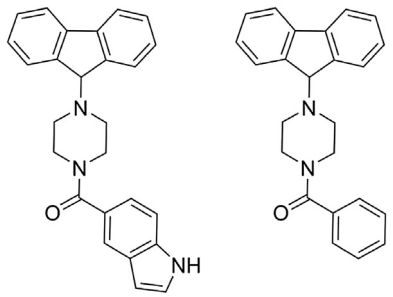
which presents challenges in the eradication of this disease [3]. Within this context, worldwide efforts have been directed at the development of new anti-TB drugs [4]. Following more than 40 years, a new TB drug named bedaquiline was approved for clinical use [5]. However, the restrict indications of this drug, possible side effects [6], and the rapid appearance of new drug-resistant TB strains strengthen the need to continuing efforts towards the development of novel antimycobacterial compounds.

The mycobacterial type II dissociated fatty acid biosynthesis system (FAS-II) has emerged as an attractive, validated target for the development of novel anti-TB molecules [7]. The FAS-II system elongates acyl fatty acid precursors yielding the long carbon chain of the meromycolate branch of mycolic acids, the hallmark of mycobacteria [8,9]. Mycolic acids are high-molecular-weight α-alkyl, β-hydroxy fatty acids, which appear mostly as bound esters in tetramycolylpentaarabinosyl cluster in the mycobacterial cell wall [10,11]. These mycolic acids have been associated with *M. tuberculosis* virulence [9], the ability of *M. tuberculosis* to survive and replicate inside macrophages and with the inability of many antimycobacterial compounds to penetrate into the *M. tuberculosis* cytosol [8,10]. The fatty acid precursors required for *M. tuberculosis* mycolic acid biosynthesis are synthesized by successive rounds of elongation and reduction reactions via the type I and type II fatty acid synthase (FAS-I and FAS-II) systems to yield, respectively, the α-branch and the meromycolate chain [10,11]. Encoded by the *inhA* gene, *M. tuberculosis* enoyl-ACP reductase (*Mt*InhA) catalyzes the final essential enzymatic step in fatty acid elongation in the FAS-II pathway, converting 2-*trans*-enoyl-ACP to acyl-ACP via a hydride transfer from the 4*S* hydrogen of NADH to the C3 position of the 2-*trans*-enoyl-CoA(ACP) substrate. *Mt*InhA has been identified as the primary target of isoniazid (INH), which is the frontline drug for TB chemotherapy, thereby validating this target for anti-TB drug discovery [12–14]. As a prodrug, INH requires oxidation by mycobacterial *katG*-encoded catalase-peroxidase (KatG) [15,16], which leads to the formation of the final covalent INH-NAD adduct that has been shown to be a slow, high-affinity competitive inhibitor of *Mt*InhA [13,17]. Notably, mutations in the *katG* gene have been linked to clinical resistance in 25–50% of newly diagnosed cases of INH-resistant TB [18,19]. Therefore, compounds able to directly inhibit *Mt*InhA, without KatG-assisted activation, could in theory target INH-resistant *M. tuberculosis* strains harboring *katG* gene mutations. We have thus been investigating compounds bound to transition metal complexes as self-activating species in the whole mycobacterial cell context with some encouraging results [20,21]. Moreover, virtual screening and pharmacophore-based approaches have resulted in the discovery of new *Mt*InhA inhibitors in the micromolar range [22]. In contrast to the recently reported methyl thiazoles that interact with *Mt*InhA in a "Tyr158-out" binding mode



1 (Genz-10850) **2**

Fig. 1. Chemical structures of piperazine derivatives identified as potent *Mt*InhA inhibitors through HTS.

[23], direct inhibitors such as pyrrolidine carboxamides [24] and piperazine-indole derivatives [25] have explored polar interactions involving a ribose hydroxyl, the Tyr158 hydroxyl and a hydrogen bond acceptor in the compounds. Consistent with our strategy [22], this substrate–protein–ligand interaction has been observed as a pharmacophoric point in virtual screening campaigns for novel *Mt*InhA inhibitors. However, the structural and electronic requirements for these hydrogen bond donor–acceptor pairs have not been extensively examined.

Therefore, in this study, we evaluated the inhibition of *Mt*InhA by piperazine-based compounds combined with structure activity relationship (SAR) studies. These compounds have been primarily obtained through high-throughput screening (HTS) approaches and exhibit submicromolar inhibition of *Mt*InhA enzyme activity (Fig. 1) [25–27]. Herein, novel piperazine derivatives were synthesized, and the structure of one compound was elucidated using X-ray diffraction. In addition, the mode of inhibition, inhibitory dissociation constants (K_{ii} and/or K_{is}) were determined.

2. Results and discussion

2.1. Chemistry

First, we sought to synthesize 1-(9*H*-fluoren-9-yl)-piperazine derivatives bearing modifications around the carbonyl hydrogen bond acceptor. Using 1-(9*H*-fluoren-9-yl)-piperazine (**3**), compounds **2** and **4**–**8** were synthesized in low to good yields using classical methods (Scheme 1). The amides **2** and **4a**–**j** were obtained through acylation reactions of **3** using synthesized or commercially available benzoyl chlorides in the presence of triethylamine (TEA) as the base and dichloromethane (CH_2Cl_2) as the solvent. The reaction mixture was maintained at 0 °C during the addition of the reactants and then allowed to warm to 25 °C with subsequent stirring for 16 h. The 9*H*-fluoren-9-yl-piperazines **2** and **4a**–**j** were isolated with 7–87% yield. Notably, one-pot synthesis of benzoyl chlorides following acylation reactions resulted in lower product yields in comparison with a direct acylation protocol. In addition, piperazine **4k** was synthesized from an active ester of pyridine-4-carboxylic acid and 1-(9*H*-fluoren-9-yl)-piperazine (**3**). The ester was formed in the presence of 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and TEA using *N*,*N*-dimethylformamide (DMF) as solvent. The reaction mixture was stirred at 45 °C for 4 h to afford **4k** with 83% yield.

Direct sulfonylation was used for the synthesis of benzenesulfonyl derivatives **5a**–**b** to obtain the desired products in 61–82% yield. Additionally, to synthesize piperazine **6**, the cinnamic acid was previously prepared through condensation between benzaldehyde and malonic acid as previously described [28]. Thereafter, the α,β-unsaturated acid was converted to the respective chloride and reacted with 1-(9*H*-fluoren-9-yl)-piperazine (**3**) in a one-pot synthesis to afford **6** with 26% yield. The stereochemistry of the double bond was assigned based on the observed proton coupling constants (*J*) of 15 Hz. Finally, two S_N2 nucleophilic substitution reactions yielded compounds **7** and **8**. The 2-bromo-*N*-phenylacetamide precursor was synthesized as previously described [29] and was reacted with amine **3** in DMF in the presence of potassium carbonate at room temperature for 24 h. Piperazine **8** was synthesized via an identical protocol using (bromomethyl)benzene as the alkylating agent. Products **7** and **8** were obtained with 51% and 55% yield, respectively.

Compounds **10**–**12** were synthesized (Scheme 2) to evaluate the effect of increasing the degrees of freedom of phenyl-like groups inserted into the 1-(9*H*-fluoren-9-yl) ring on *Mt*InhA inhibition. This enhanced conformational flexibility may enable stronger hydrophobic interactions with the enzyme or result in entropy