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New 1-hydroxy-2-thiopyridine derivatives active against both replicating and dormant *Mycobacterium tuberculosis*

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ABSTRACT

Tuberculosis (TB) treatment is confounded by the range of metabolic states displayed by *Mycobacterium tuberculosis*, by the long duration required and by the increasing prevalence of drug-resistant strains. Latent TB infection is especially difficult to treat due to the phenotypic antibiotic resistance of non-replicating *M. tuberculosis*. Therefore, the development of new drugs effective against both active and latent TB infection is needed. New 1-hydroxy-2-thiopyridine derivatives were synthesized and found to be highly effective *in vitro* against both actively growing and dormant non-culturable *M. tuberculosis*. Such compounds are leads for the development of new drugs for all forms of TB including latent infection.

Mycobacteria have plagued humanity for several millennia by causing major diseases like tuberculosis (TB), leprosy, and Buruli ulcer. In terms of disease burden and mortality, TB is incontestably the most important and challenging threat to human health, as evidenced by the fact that one third of the world's population is infected with *Mycobacterium tuberculosis* without overt disease symptoms. Because of the increasing prevalence of primary resistance to the current drugs and the wide distribution of latent TB infection, there is a growing need for new drugs with a novel mode of action [1], and these may also find application in treating other mycobacterial diseases.

In the past 20 years, drug-resistant TB has reached an alarming level. From the 1990s, there was increasing concern about multidrug-resistant (MDR) *M. tuberculosis*, which acquired resistance to the main front-line drugs, isoniazid and rifampicin. Currently, there are an estimated 500,000 cases of MDR-TB worldwide, of which ~70,000 occur in Europe [2,3]. Latently infected individuals have a 5% lifetime risk of disease reactivation, which increases to around 10% per year in immuno-compromised patients [4]. *M. tuberculosis* associated with latent TB in humans is considered to be in a non-replicating (dormant) state characterized by greatly reduced metabolism and the development of antibiotic tolerance [5]. Therefore, there is an urgent need for new efficient drugs to treat both active and latent TB.

Several types of cyclic oxide nitrogen compounds such as an aspergillic acid or pyritions are known to possess antimycobacterial properties [6,7]. We studied how transformation of oxide nitrogen moiety to corresponding hydroxy derivatives and introduction of substituted isothioureas to the second position of the pyridine ring may affect antimycobacterial activity. Here, we report a new series of 1-hydroxy-2-thiopyridinederivatives with extremely high antitubercular activity against both actively growing and dormant *M. tuberculosis* cells.

Minimal inhibitory concentrations (MIC) of compounds against *M. tuberculosis* H37Rv were determined in 96-well plates by the resazurin reduction assay as described previously [8,9]. DMSO (1%) and rifampicin (0.5 μg/ml) were used as negative and positive controls, respectively. In addition, MIC values were measured

Abbreviations: MDR, multidrug-resistant; MIC, minimal inhibitory concentration; NC, non-culturable; CFU, colony forming units; DMSO, dimethylsulfoxide; DMEM, Dulbecco's Modified Eagle's medium; FCS, fetal calf serum; MPN, most probable numbers.

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against the isoniazid-resistant clinical isolate CN-40 [10], bearing selective resistance to isoniazid, but susceptible to rifampicin, ethambutol and streptomycin, which was isolated from a TB patient at Central Institute for Tuberculosis (Moscow, Russia). 1-Hydroxy-2-thiopyridine derivatives were also tested for activity against the streptomycin-dependent *M. tuberculosis* strain 18b after streptomycin removal as ss18b (streptomycin starved 18b) represents a well-established model for the non-replicating state [9] and has been used extensively for drug testing [11,12].

Bactericidal activity of 1-hydroxy-2-thiopyridine derivatives against both replicating and dormant non-culturable (NC) *M. tuberculosis* obtained under potassium deficiency [7], as well as dormant *M. tuberculosis* cells in the Wayne hypoxia model [13] and Betts starvation model [14] was examined using the most probable number (MPN) assay [15]. Briefly, 2×10^7 *M. tuberculosis* bacilli were treated with 25 μM of each of the most active compounds for 7 days then 10-fold serial dilutions were prepared and employed in triplicate for the MPN assay in 48-well plates to obtain an estimate of the proportion of potentially resuscitable cells. Plates were incubated at 37 °C for 30 days then wells with visible bacterial growth were counted as positive. The number of bacteria (MPN values) was calculated using standard statistical methods [15] and compared with that of untreated cells in order to measure the bactericidal effect of each compound against replicating and dormant bacilli.

To assess their stability *in vivo* compounds were injected into the tail vein of mice at a dose of 25 mg/kg, then blood was collected into sterile tubes 5 or 30 min after injection and allowed to clot at room temperature for 20 min. The clot was removed by centrifuging at 1000–2000× *g* for 10 min in a refrigerated centrifuge and the serum transferred into polypropylene tubes and stored at −35 °C. Different volumes of serum (10, 20 or 40 μl) were added to 1 ml of *M. tuberculosis* H37Rv culture (1×10^4 cells/ml) and incubated for 7 days when 10-fold serial dilutions were prepared and employed in triplicate for CFU counting.

To determine possible cytotoxicity of the synthesized compounds, HepG2 human hepatocellular carcinoma cells and A549 lung epithelial cells were seeded in 96 well plates at a concentration of 4×10^4 cells/ml in DMEM (without phenol red) supplemented with 10% fetal calf serum (FCS). Cells were incubated in the presence of different concentrations of compounds in a humidified incubator with 5% CO_2 at 37 °C for 3 days, then cell viability was measured by fluorescence spectroscopy 6 h after adding resazurin.

We successfully developed a three-step method of chemical synthesis of original 1-hydroxypyridines with an isothiourea moiety in the second position of the pyridine ring (eleven compounds, **4a**–**k**). This method allows the synthesis of different 1-hydroxypyridines and enables substituents to be made in the pyridine ring as well as in the thiourea group (Fig. 1). In addition, we synthesized the corresponding 1-oxido-2-R-thiopyridines (six compounds, **3a**–**f**) without a carbodiimide group. The general procedure for the synthesis and analytical data are presented in the Supplementary File 1. All the synthesized compounds were >98% pure, and stable both in the solid form and in aqueous solution.

The anti-TB activity was evaluated for all the synthesized compounds and minimal inhibitory concentration (MIC) values were determined by the resazurin reduction assay [8]. It was found that 1-oxido-2-R-thiopyridines **3a**–**f** were not active on *M. tuberculosis* H37Rv cells (MIC > 100 μg/ml). However, transformation to 1-hydroxypyridines by inclusion an isothiourea moiety in the second position of the pyridine ring resulted in compounds with significant antitubercular activity (derivatives of group **4**). We also investigated structure-activity relationships such as the effect of different substituents both in the isothiourea moiety and in position 5 of the pyridine ring for compounds of group **4**. No activity (MIC > 100 μg/ml) was observed for the compounds with H, Me, or Cl at position 5 of the pyridine ring (compounds **4a**–**c,** Suppl. File 1). Nonetheless, derivatives bearing electron-withdrawing groups in this position (compounds **4d**–**k**), such as alkoxycarbonyl or trifluoromethyl groups, were found to exhibit high antitubercular activity (Table 1). The most active compounds **4d,e,g,h,i,k** displayed MIC values from 0.031 to 0.063 μg/ml, and moderate cytotoxicity with selectivity indexes (IC_{50}/MIC_{99}) up to 123.87 for HepG2 cells and 265.8 for A549 cells (Table 1). It is interesting that compounds **4f** and **4j,** with tetrahydropyrimidine residues, exhibited less pronounced antitubercular activity in comparison to their close analogues compounds **4e** and **4i** with smaller cycle size (Table 1). We also found that the compounds **4d,e,g,h,i,k** revealed a comparable level of activity against the clinical isolate CN-40 with MIC values of 0.063 μg/ml.

All the 1-hydroxy-2-thiopyridine derivatives **4d**–**k** listed in Table 1 were highly active against the non-replicating streptomycin-starved *M. tuberculosis* 18b strain [9] as indicated by low MIC values in the range of 0.31–0.63 μg/ml. Moreover, compounds **4d**–**k** exhibited strong bactericidal activity against both replicating and dormant non-culturable (NC) *M. tuberculosis* H37Rv bacilli with a 'zero-CFU' phenotype, obtained under potassium deficiency [7]. These dormant bacteria were characterized by a high recovery potential being able to resuscitate into a fully culturable state after reintroducing of K^+ ions [7]. To calculate the viability decline for both replicating and dormant bacilli we compared MPN values after a 7-day exposure to the compounds with MPN values of untreated cells. The most pronounced viability decline was obtained for the compounds **4d,e,g,i,k** (Table 1). For further studying the effect of 1-hydroxy-2-thiopyridines on dormant *M. tuberculosis* H37Rv bacilli we selected one of the most active compound **4k** and performed two other *in vitro* dormancy models: the Wayne hypoxia [13] and the Betts starvation [14] models. Similarly, the MPN assay was applied for estimation of the corresponding cell viability decline. The compound **4k** displayed prominent activity for dormant cells obtained in both Wayne and Betts models: 2.62 ± 0.43 and 2.78 ± 0.59 logs of viability decline, respectively. Thus, 1-hydroxy-2-thiopyridines are strongly active against both replicating and dormant *M. tuberculosis*.

Although recent advancement of new anti-TB drugs [16–18] cannot be overstated, none of them displayed prominent activity against dormant tubercle bacilli, as many of prospective molecular targets are evidently inactive on dormancy. However, recently we have reported thienopyrimidine derivatives [19] which are another example of highly active against dormant *M. tuberculosis* compounds. The mode of action for both 1-hydroxy-2-thiopyridines and thienopyrimidines is now under detailed study.

The activity of the most active compound **4k** was also tested in a serum inhibition titration assay [20] after intravenous (i.v.) injection of the compound at a dose of 25 mg/kg. Mouse serum collected 5 min after injection was active against *M. tuberculosis* while this activity disappeared after 30 min (Table 2). When the compound was administered orally (p.o.) we did not find any significant activity in serum possibly due to rapid metabolism of the compound in the mouse liver, as N-hydroxy moiety is supposed to be quickly reduced by various reductases, that is one of the most probable scenario of metabolic inactivation of the compound in the host [17]. Further SAR studies may shed light on the ways for finding new 1-hydroxy-2-thiopyridine derivatives with pronounced *in vivo* activity.

In conclusion, the present study reports new 1-hydroxy-2-thiopyridine derivatives with strong bactericidal activity against both actively growing and dormant tubercle bacilli thereby indicating that drug-like scaffold has promise for further development of an all purpose anti-TB drug.