8-Mercaptoguanine-based inhibitors of Mycobacterium tuberculosis dihydroneopterin aldolase: synthesis, in vitro inhibition and docking studies

Alexia de Matos Czeczota,b, Candida Deves Rotha, Rodrigo Gay Ducati,a,d, Kenia Pissinatea, Raoni Scheibler Ramboa, Luís Fernando Saraiva Macedo Timmersd, Bruno Lopes Abbadi,a, Fernanda Souza Macchia,b, Victor Zajaczkowski Pestana, Luiz Augusto Bassoa,b,c, Pablo Machadoa,b, and Cristiano Valim Bizarroa,b,c

a, Instituto Nacional de Ciência e Tecnologia em Tuberculose, Centro de Pesquisas em Biologia Molecular e Funcional, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Brazil; b, Programa de Pós-Graduação em Biologia Celular e Molecular, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Brazil; c, Programa de Pós-Graduação em Medicina e Ciências da Saúde, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Brazil; d, Programa de Pós-Graduação em Biotecnologia, Universidade do Vale do Taquari, Lajeado, Brazil

ABSTRACT

The dihydroneopterin aldolase (DHNAA, EC 4.1.2.25) activity of FolB protein is required for the conversion of 7,8-dihydropterin (DHNP) to 6-hydroxymethyl-7,8-dihydropterin (HP) and glycolaldehyde (GA) in the folate pathway. FolB protein from Mycobacterium tuberculosis (MtFolB) is essential for bacilli survival and represents an important molecular target for drug development. S8-functionalized 8-mercaptoguanine derivatives were synthesised and evaluated for inhibitory activity against MtFolB. The compounds showed IC50 values in the submicromolar range. The inhibition mode and inhibition constants were determined for compounds that exhibited the strongest inhibition. Additionally, molecular docking analyses were performed to suggest enzyme-inhibitor interactions and ligand conformations. To the best of our knowledge, this study describes the first class of MtFolB inhibitors.

Introduction

Tuberculosis (TB) is one of the oldest diseases that remain a health concern worldwide due to high incidence and mortality rates. According to the World Health Organisation (WHO), an estimated 10 million people fell ill in 2019, while 1.4 million people died with TB in the same period. Duration, complexity of treatment, and drug side effects result in poor adherence, suboptimal response, treatment failure, emergence of drug resistance, and continuous disease spread. Therefore, new and more effective treatments are urgently needed.

Folate and its derivatives act as cofactors in the biosynthesis of purines, pyrimidines, and amino acids. Antifolates interrupt the production of folate and its derivatives by inhibiting key enzymes in the folate metabolic pathway. Among the enzymes of this pathway, only dihydropterate synthase (DPHS) and dihydrofolate reductase (DHFR) are currently used as targets for antimicrobial agents. Despite the antimycobacterial activity of antifolates in culture and the use of para-aminosalicylic acid (PAS) as a second-line drug, these molecules are not used in the first-line treatment of TB. The FolB protein, encoded by the folB gene, is a dihydroneopterin aldolase enzyme (DHNA, EC 4.1.2.25), as it converts 7,8-dihydropterin (DHNP) to 6-hydroxymethyl-7,8-dihydropterin (HP) and glycolaldehyde (GA) in the third step of the folate pathway. FolB from Mycobacterium tuberculosis (MtFolB) is also a dihydroneopterin aldolase (DHMP) aldolase, converting DHMP and HP to GA, an epimerase, interconverting DHNP and DHMP, and an oxygenase, producing 7,8-dihydroxopterin (DHXP) from either DHNP or DHMP. This protein is the first of the three enzymes from the folate pathway that are absent in mammals and represents an attractive target for the development of antimicrobial agents.

We have shown previously that the folB gene from M. tuberculosis is essential for bacilli survival under defined conditions and that its essentiality depends on the aldolase and/or epimerase activities of MtFolB protein. This paved the way for the development of MtFolB aldolase/epimerase inhibitors as potential anti-TB agents. Compounds with inhibitory activity against the orthologous enzyme from Staphylococcus aureus (SoFolB) were previously identified in a high-throughput X-ray crystallographic screening using an initial library with 10 000 compounds. In this same study, a new sublibary of approximately 1 000 compounds was constructed, all containing the H2N-C-NH-C=O substructure in common (highlighted in blue in Scheme 1). Several hit compounds with low IC50 values against SoFolB were identified, including 8-mercaptopguanine (8MG), with an IC50 value of 1 μM. X-ray crystallography revealed that the H2N-C-NH-C=O substructure shared by 8-MG, the substrate analogue neopterin and also the product HP presented hydrogen bonds with the same SoFolB residues in these three ligand-protein complexes.

Furthermore, 8-MG inhibits 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase from S. aureus (SoHPPK), another enzyme of the folate pathway, with an IC50 value of 41 μM. Structure-activity relationship (SAR) studies have been performed to identify structural analogues of 8-MG with greater potential to inhibit enzymes from the folate biosynthesis pathway. S8-functionalized derivatives of 8-MG with improved affinity for both SoHPPK and Escherichia coli HPPK (EcHPPK)
Melting points were determined on an Microquímica MQAPF-302 apparatus. IR spectra were recorded on Perkin-Elmer Spectrum 100 FT-IR spectrometer with a Universal ATR sampling accessory. NMR spectra were recorded on an Avance III HD Bruker spectrometer with chemical shifts values (δ) in ppm relative to TMS using the residual DMSO-d6 signal as an internal standard. High-resolution mass spectra (HRMS) were recorded on an LTQ Orbitrap Discovery mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). This system combines an LTQ XL linear ion-trap mass spectrometer and an Orbitrap mass analyser. The analyses were performed by direct infusion of the sample in MeOH/CH₃CN (1:1) with 0.1% formic acid (flow rate of 10 µL/min) in positive-ion mode using electrospray ionisation (ESI). For the elemental composition, the calculations used the specific tool included in the Qual Browser module of Xcalibur (Thermo Fisher Scientific, release 2.0.7) software.

General procedure for synthesis of compounds 2a–h

Compounds 2a–h were synthesised as previously described. In brief, 8-mercaptopurine (scaffold molecule 1–8-MG) (0.2 g, 1.09 mmol) was dissolved in 0.5 M NaOH (5.5 ml) resulting in a real solution. To this solution was added the respective benzyl bromide (1.2 mmol) in ethanol (0.9 ml). The reaction was stirred for 4h at 25 °C and the precipitated formed was collected by vacuum filtration affording the title compound as a white-yellowish amorphous solid.

Materials and methods

Chemical synthesis

Reagents, chemicals, starting materials and solvents were obtained from commercial sources and used without further purification.

![Scheme 1](image-url)
2-(2-Amino-8-oxo-6,9-dihydro-1H-purin-8-yl)thio)-N-phenylacetamide (4a)

10% yield. MP = 291–293°C. 1H NMR (400 MHz, DMSO-d6): δ: 10.53 (bs, 2H), 7.57 (d, J = 8.0 Hz, 2H), 7.30 (t, J = 7.9 Hz, 2H), 7.05 (t, J = 7.4 Hz, 1H), 6.32 (bs, 2H), 4.07 (s, 2H). IR-ATR (cm⁻¹): 3321, 3145, 3070, 1665. HRMS (ESI): calc. for [C₁₅H₁₂N₂O₅S + H]+: 317.0815; obt.: 317.0817.

2-(2-Amino-6-oxo,6,9-dihydro-1H-purin-8-yl)thio)-2-Amino-8-((2,4-dichlorobenzyl)thio)-1,9-dihydro-6H-purin-6-one (2g)

76% yield. MP = 290–291°C. 1H NMR (400 MHz, DMSO-d6): δ: 10.64 (bs, 1H), 7.63 (s, 1H), 7.54 (d, J = 8.2 Hz, 1H), 7.33 (d, J = 8.2 Hz, 1H), 6.33 (bs, 2H), 4.37 (s, 2H). IR-ATR (cm⁻¹): 3413, 3276, 3146, 1677. HRMS (ESI): calc. for [C₁₂H₁₂ClN₅OS + H]+: 341.9978; obt.: 341.9971.

General procedure for synthesis of compounds 3a–c and 4a–h

Compounds 3a–c and 4a–h were synthesised according to an already reported protocol with minor modifications. In brief, 8-MG (1) (0.2 g, 1.09 mmol) was dissolved in 0.5 M NaOH (5.5 ml), and to the resulting solution was added the respective bromoacetamide or 2-bromoacetophenone (1.2 mmol) in ethanol (0.9 ml). The reaction was stirred for 24 h at 25°C, then 1% acetic acid was added until pH = 5. The mixture was extracted with ethyl acetate, the organic layers were combined, dried with MgSO₄ and evaporated under reduced pressure. The residue was purified by flash column chromatography eluting a mixture of chloroform and methanol (9:1 to 1:1) or recrystallized in methanol to give the title compound as a white-yellowish amorphous solid.

2-Amino-8-((2-oxo-2-phenylethyl)thio)-1,9-dihydro-6H-purin-6-one (3a) 31% yield. MP > 310°C. 1H NMR (400 MHz, DMSO-d6): δ: 12.51 (bs, 1H), 10.55 (bs 1H), 8.20–7.26 (m, 2H), 7.78–7.47 (m, 3H), 6.29 (bs, 2H), 4.85 (s, 2H). IR-ATR (cm⁻¹): 3322, 3098, 2919, 1651. HRMS (ESI): calc. for [C₁₃H₁₁N₅O₂S + H]+: 320.0706; obt.: 320.0706.¹³

2-Amino-8-((2-oxo-2-phenylethyl)thio)-1,9-dihydro-6H-purin-6-one (3b) 53% yield. MP = 300–316°C. 1H NMR (400 MHz, DMSO-d6): δ: 12.53 (bs, 1H), 10.82 (bs, 1H), 8.04 (d, J = 8.3 Hz, 2H), 7.63 (d, J = 8.2 Hz, 2H), 6.52 (bs, 2H), 4.88 (d, J = 37.0 Hz, 2H). IR-ATR (cm⁻¹): 3094, 1673, 1586. HRMS (ESI): calc. for [C₁₃H₁₁N₅O₂S + H]+: 336.0316; obt.: 336.0325.¹⁴

2-Amino-8-((2-oxo-2-phenylethyl)thio)-1,9-dihydro-6H-purin-6-one (3c) 72% yield. MP = 309–311°C. 1H NMR (400 MHz, DMSO-d6): δ: 12.51 (bs, 1H), 10.54 (bs, 1H), 7.76–7.50 (m, 2H), 7.21 (d, J = 8.5 Hz, 1H), 7.05 (td, J = 7.5, 1.0 Hz, 1H), 6.35 (bs, 2H), 4.71 (s, 2H), 3.93 (s, 3H). IR-ATR (cm⁻¹): 3322, 3097, 1673, 1652. HRMS (ESI): calc. for [C₁₄H₁₃N₃O₂S + H]+: 332.0812; obt.: 332.0820.¹³

2-(2-Amino-6-oxo,6,9-dihydro-1H-purin-8-yl)thio)-N-phenylacetamide (4a)

50% yield. MP = 270–271°C. 1H NMR (400 MHz, DMSO-d6): δ: 12.54 (s, 1H), 10.55 (bs, 1H), 8.71 (s, 1H), 7.33–7.17 (m, 5H), 6.39–6.32 (m, 2H), 4.30 (d, J = 6.0 Hz, 3H), 3.92 (s, 2H). IR-ATR (cm⁻¹): 3303, 3164, 2987, 2879, 1663. HRMS (ESI): calc. for [C₁₄H₁₄N₄O₂S + H]+: 331.0972; obt.: 331.0966.

2-(2-Amino-6-oxo,6,9-dihydro-1H-purin-8-yl)thio)-N-benzylacetamide (4c)

59% yield. MP = 243–245°C. 1H NMR (400 MHz, DMSO-d6): δ: 11.08 (bs, 1H), 7.76–7.54 (m, 2H), 7.27–7.08 (m, 2H), 6.39 (bs, 1H), 4.01 (d, J = 2.9 Hz, 2H). IR-ATR (cm⁻¹): 3073, 1615, 1507. HRMS (ESI): calc. for [C₁₃H₁₁N₅O₂S + H]+: 335.0721; obt.: 335.0726.

2-(2-Amino-6-oxo,6,9-dihydro-1H-purin-8-yl)thio)-N-(4-fluorophenyl)acetamide (4d)

38% yield. MP = 306–308°C. 1H NMR (400 MHz, DMSO-d6): δ: 12.49 (bs, 1H), 10.69 (s, 1H), 7.72–7.53 (m, 2H), 7.46–7.30 (m, 2H), 6.71–6.55 (m, 2H), 4.10 (s, 2H). IR-ATR (cm⁻¹): 3073, 1615, 1507. HRMS (ESI): calc. for [C₁₃H₁₁C₆N₄O₂S + H]+: 351.0425; obt.: 351.0428.

2-(2-Amino-6-oxo,6,9-dihydro-1H-purin-8-yl)thio)-N-(4-chlorophenyl)acetamide (4e)

84% yield. MP > 310°C. 1H NMR (400 MHz, DMSO-d6): δ: 10.75 (bs, 1H), 7.62–7.54 (m, 2H), 7.53–7.46 (m, 2H), 6.37 (bs, 2H), 4.09 (s, 2H). IR-ATR (cm⁻¹): 3310, 3124, 1668. HRMS (ESI): calc. for [C₁₃H₁₁BrN₄O₂S + H]+: 394.9912; obt.: 394.9920.

2-(2-Amino-6-oxo,6,9-dihydro-1H-purin-8-yl)thio)-N-(4-propylphenyl)acetamide (4g)

80% yield. MP = 299–301°C. 1H NMR (400 MHz, DMSO-d6): δ: 10.41 (bs, 1H), 7.48 (d, J = 8.1 Hz, 2H), 7.11 (d, J = 8.4 Hz, 2H), 6.48 (bs, 3H), 4.09 (s, 2H), 2.55–2.41 (m, 2H), 1.53 (p, J = 7.3 Hz, 2H), 0.86 (t, J = 7.3 Hz, 3H). IR-ATR (cm⁻¹): 2927, 1669, 1600, 1513. HRMS (ESI): Calc. for [C₁₆H₁₆N₄O₂S + H]+: 359.1285; obt.: 359.1287.
Expression, purification, and continuous fluorescence-based enzyme activity assay

The expression and purification of recombinant MfOIB were performed as previously described\(^\text{10}\). A continuous fluorescence-based enzyme activity assay was optimized\(^\text{24}\) for monitoring the aldolase reaction of MfOIB (conversion of DHNP to HP and GA) by an increase in fluorescence due to HP formation on an RF-5301 spectrofluorophotometer (Shimadzu) with an excitation wavelength of 365 nm and fluorescence emission at 525 nm. The slits were 10 and 15 nm for excitation and emission, respectively. To determine the apparent steady-state kinetic constants, MfOIB activity was monitored at varying concentrations of DHNP (0.10–10 \(\mu\)M) using 300 nM MfOIB in 25 mM Tris, 50 mM NaCl, 5% glycerol pH 8.0 at 25°C in 6 min for a final volume of 1.0 ml. Control reactions (buffer only, buffer + substrate, buffer + enzyme) were performed under the same conditions to subtract fluorescence intensities not coming from the reaction product. The data were fitted to Equation (1) for a saturation curve, in which \(V\) is the initial velocity, \(V_{\text{max}}\) is the maximum velocity, \(E_0\) is the initial total enzyme concentration, \(S_t\) is the initial total substrate concentration, and \(K_M\) is the Michaelis–Menten constant for the substrate used\(^\text{2}\). A calibration curve ranging from 0.020 to 15 \(\mu\)M of HP was performed. The slope of the fluorescence emission at 525 nm as a function of HP concentration was applied to obtain the catalytic constant (\(k_{\text{cat}}\)) values for the aldolase reactions:

\[
V = \frac{V_{\text{max}} (E_0 + S_t + K_M) - \sqrt{(E_0 + S_t + K_M)^2 - 4 \times E_0 \times S_t}}{2 \times E_0}
\]  

(1)

Enzyme inhibition studies

Enzyme inhibition studies were performed using an RF-5301 spectrofluorophotometer (Shimadzu), monitoring an increase in fluorescence at 525 nm for HP formation for 6 min. The presence of time-dependent inhibitory activity was evaluated for 8-MG (1) and the compounds synthesised. For this analysis, 300 nM (final concentration) of recombinant MfOIB was preincubated with a fixed inhibitor concentration defined for each compound (final concentrations of 360 nM for 8-MG (1); 500 nM for 3b and 4h; 625 nM for 4g, 4f, and 4e; 830 nM for 4b and 4d; 1.00 \(\mu\)M for 3a; 1.25 \(\mu\)M for 3c, 2g and 2f; 1.65 \(\mu\)M for 2b and 2h; 2.5 \(\mu\)M for 4c; 5 \(\mu\)M for 4a; or 7 \(\mu\)M for 2d, 2c and 2a), which was then added at different times (up to 40 min) to the reaction mixture (DHNP at \(K_M\) value, 25 mM Tris, 50 mM NaCl, 5% glycerol pH 8.0 and final concentration of 2% DMSO). The change in initial velocity as a function of time was monitored and the percentage of inhibition was calculated. As a control, MfOIB was preincubated with DMSO alone at a maximum final concentration of 2% and added to the reaction mixture. All experiments were performed at 25°C.

The IC\(_{50}\) values for the compounds were determined in the reaction conditions aforementioned. We fixed DHNP at a non-saturating concentration (~ \(K_M\) value) and dissolved the compounds in DMSO at varied concentrations (8-MG (1): 0.1–1.0 \(\mu\)M; 3b and 4h: 0.1–1.2 \(\mu\)M; 4b: 0.2–3.0 \(\mu\)M; 4e and 4g: 0.2–2.5 \(\mu\)M; 4f: 0.2–2.0 \(\mu\)M; 3a: 0.35–5 \(\mu\)M; 4d: 0.4–4.0 \(\mu\)M; 4a: 0.5–10.0 \(\mu\)M; 3c: 0.6–3.0 \(\mu\)M; 2f: 1.0–7.0 \(\mu\)M; 2g: 1.0–15 \(\mu\)M; 2b: 0.6–5.0 \(\mu\)M; 2h: 1.5–15 \(\mu\)M; 4c: 3.0–40 \(\mu\)M; 2d, 2c and 2a 2.0–30 \(\mu\)M; 2e 2.0–15 \(\mu\)M). The maximal rate of the enzymatic reaction (100% of MfOIB activity) was determined with 2% DMSO in the absence of inhibitor. IC\(_{50}\) values were estimated using Equation (2), where \(V_I\) and \(V_0\) are, respectively, the reaction velocity in the presence and in the absence of inhibitor (I):

\[
\frac{V_I}{V_0} = \frac{1}{1 + \frac{[I]}{IC_{50}}}
\]  

(2)

The determination of the mode of inhibition (competitive, non-competitive, or uncompetitive) and the inhibition constants \((K_I, and/or \ K_0)\) were performed for each selected inhibitor with an IC\(_{50}\) value < 0.50 \(\mu\)M. We consider as competitive inhibitors compounds that bind only the free enzyme, as non-competitive inhibitors the ones that bind both the enzyme-substrate complex and the free enzyme (but not necessarily with the same binding affinity), and uncompetitive inhibitors the compounds that bind exclusively the enzyme-substrate complex\(^\text{14}\).

The inhibition studies were carried out at varying concentrations of DHNP until saturation, and fixed-varied inhibitor concentrations. For 8-MG (1) the fixed-varied concentrations were 0.15 \(\mu\)M, 0.30 \(\mu\)M (varying DHNP 0.25–10 \(\mu\)M) and 0.45 \(\mu\)M (varying DHNP 0.35–10 \(\mu\)M). For 3b the fixed-varied concentrations were 0.20 \(\mu\)M (varying DHNP 0.35–10 \(\mu\)M), 0.40 \(\mu\)M (varying DHNP 0.35–15 \(\mu\)M), and 0.80 \(\mu\)M (varying DHNP 0.5–20 \(\mu\)M). For 4f the fixed-varied concentrations were 0.30 \(\mu\)M (varying DHNP 0.35–10 \(\mu\)M), 0.50 \(\mu\)M and 0.70 \(\mu\)M (varying DHNP 0.5–15 \(\mu\)M). For 4h the fixed-varied concentrations were 0.30 \(\mu\)M, 0.50 \(\mu\)M (varying DHNP 0.35–10 \(\mu\)M), and 0.70 \(\mu\)M (varying DHNP 0.5–15 \(\mu\)M). The enzyme concentration was constant at 300 nM throughout the assays. The mode of inhibition of compounds was determined from the straight-line patterns, and \(K_I\) and/or \(K_0\) values towards DHNP were estimated using Equation (3) or Equation (4), which describe a non-competitive and competitive inhibition, respectively. Data were fitted to the following equations:

\[
V_0 = \frac{V_{\text{max}} [S]}{[S] + K_M \left(1 + \frac{[I]}{K_I}\right)}
\]  

(3)

\[
V_0 = \frac{V_{\text{max}} [S]}{[S] + K_M \left(1 + \frac{[I]}{K_0}\right)}
\]  

(4)

where [I] is the inhibitor concentration, [S] is the substrate concentration, \(K_M\) and \(V_{\text{max}}\) are the Michaelis–Menten constant and maximum velocity, respectively, \(K_I\) is the overall inhibition constant for the enzyme–substrate–inhibitor complex and \(K_0\) is the overall inhibition constant for the enzyme–inhibitor complex\(^\text{16}\).

Molecular docking protocol

Molecular docking simulations were carried out to evaluate the orientation and binding affinity of 8-MG (1) and its derivatives into the binding pocket of MfOIB. Prior to this, we performed a redocking procedure, using the crystallographic structures of MfOIB as an octamer (PDB ID 1NBU)\(^\text{17}\) aiming to verify whether our protocol could reproduce the ligand location found in the experimental structure. The flexible docking simulations were performed using Pyrx-0.9.4\(^\text{18}\), where the AutoDock 4.2
Mycobacterium tuberculosis inhibition assay

The inhibitory potential of the compounds was evaluated against M. tuberculosis H37Rv reference strain (ATCC 27294) by the resazurin reduction microplate assay (REMA) as previously described. Stock solutions (0.5 mg mL\(^{-1}\)) for 8-MG (1) and 2 mg mL\(^{-1}\) for all other test compounds were made in neat DMSO (Sigma-Aldrich) and aliquots were stored at \(-20^\circ\)C. The assays were performed in Difco\textsuperscript{TM} Middlebrook 7H9 broth (Becton Dickinson – BD) supplemented with 10% (v/v) BBLTM Middlebrook ADC enrichment (albumin, dextrose and catalase – BD) and 2.5% (v/v) DMSO. The maximum concentration tested varied among compounds due to differences in solubility (2.5 – 40 μg mL\(^{-1}\)). The minimal inhibitory concentration (MIC) was determined by performing 10-point 2-fold serial dilutions for each compound. Three independent experiments were performed, and MIC was considered as the lowest compound concentration that prevented the resazurin (Sigma-Aldrich) colour conversion from blue (inhibition) to pink (growth). The MIC values stated for the compounds were the most frequent values among the three experiments, or the highest value observed.

Results and discussion

The synthesis of compounds 2a-h, 3a-c, and 4a-h was accomplished through S-alkylation in a nucleophilic substitution reaction. Our strategy was to attach hydrophobic side chains to 8-mercaptoguanine (1) to obtain enzymatic inhibitors with physicochemical properties that could facilitate the permeability of the molecules and increase the chance of obtaining structures with potent antimycobacterial activity. The alkylating agents were chosen from different aryl(naphthyl) groups containing electron-donating and electron-withdrawing groups as substituents.

The dihydro-purinones 2a-h were obtained from the reaction of 8-mercaptopurinone (1) and benzyl bromides in the presence of sodium hydride (NaOH) as a base and ethanol (EtOH) as the solvent. The reactants were stirred for 4h at 25 °C, leading to products 2a-h with 55–82% yields (Scheme 1). Using the same conditions for 24h, the compounds 3a-c were synthesised by the reaction of 8-mercaptopurinone (1) and 2-bromo-1-arylethanones, with 43 – 72% yields (Scheme 1). Finally, using the same procedure described above, the dihydro-purinones 4a-h were obtained from the reaction of 8-mercaptopurinone (1) and bromoacetamides, with 10 – 84% yields (Scheme 1). In general, the presence of a carbonyl group in the alkylating agent provided products in lower yields when compared to the reactions using benzyl bromides.

The synthesised compounds 2a-h, 3a-c, and 4a-h were evaluated as inhibitors of MfFoIB aldolase activity using a continuous fluorescence assay. The Michaelis-Menten constant (K\(_{\text{m}}\)) was determined at varying concentrations of DHNP until enzyme saturation (Figure S1, Supplementary Material). K\(_{\text{m}}\) and K\(_{\text{cat}}\) values of 1.42 ± 0.13 μM and 0.011 ± 0.0003 s\(^{-1}\) were obtained, respectively. The values determined here differ from the values previously reported for this enzyme (K\(_{\text{m}}\) = 0.165 ± 0.026 μM and K\(_{\text{cat}}\) = 0.0054 ± 0.0002 s\(^{-1}\))\textsuperscript{8}. This should be attributed to differences in the method of enzyme purification and the buffer and pH of the enzyme activity assay; changes in solution conditions can affect the apparent value of K\(_{\text{m}}\) influencing the ability of the enzyme to combine with substrate\textsuperscript{16}.

The inhibitory potential of 8-mercaptopurinone (8-MG (1)) and the synthesised compounds was evaluated against MfFoIB. No time dependence was demonstrated up to 40 min of preincubation with MfFoIB (data not shown). The initial screening of 20 compounds showed inhibition with IC\(_{50}\) values ranging from 0.3 to 12.3 μM (Scheme 1).

Based on IC\(_{50}\) values, benzyl-containing compounds 2a-h showed lower activity than 8-MG (1) (Scheme 1). The unsubstituted compound 2a exhibited an IC\(_{50}\) of 7.1 μM whereas 8-MG (1) has an IC\(_{50}\) of 0.3 μM for MfFoIB. Compared to the benzyl derivative 2a, the molecular volume increase with the use of the naphthyl group in the dihydro-purinone 2b improved the activity more than 5-fold, leading to an IC\(_{50}\) of 1.3 μM. By contrast, the presence of a fluorine atom at the 2- (2c) or 4- (2d) position of the benzene ring led to molecules with reduced activities (IC\(_{50}\) = 8.0 μM). Once more, increasing molecular volume with change of fluorine by chlorine atom improved the inhibitory activity towards MfFoIB, resulting in an IC\(_{50}\) of 4.2 and 2.2 μM for compounds 2e and 2f, respectively. Substitution with chlorine atoms at position 3 and 4 of the benzyl ring reduced the inhibitory activity of MfFoIB. 3,4-Dichlorophenyl-substituted 2g showed an IC\(_{50}\) of 5.5 μM, which was 2.5-fold higher than its monosubstituted analog, 2f. Additionally, the 4-bromophenyl-substituted 2h exhibited an IC\(_{50}\) of 3.0 μM, denoting that the classic bioisosteric replacement between the chlorine and bromine was able to maintain similar and reduced potencies.

In the second round, carbonyl-containing compounds 3a-c were evaluated as inhibitors of MfFoIB activity. The presence of this hydrogen bond acceptor group could lead to more potent structures when compared to benzyl derivatives 2a-h. Indeed, dihydro-purinones 3a-c were more potent than their counterparts 2a-h. The phenyl derivative 3a exhibited an IC\(_{50}\) of 0.9 μM. When chlorine atom was positioned at 4-position of the benzene ring in the compound 3b, the capacity to inhibit the MfFoIB was increased. The IC\(_{50}\) presented by structure 3b was 0.3 μM. This IC\(_{50}\) value indicated an equipotent activity compared to that presented by 8-MG (1). The presence of the methoxy group attached at the 2-position of the benzene ring yielded compound 3c, which exhibited an IC\(_{50}\) of 1.3 μM. This result demonstrates that this electron-donating group reduced in more than 4-fold the inhibitory capacity of this molecule when compared to the activity presented by structure 3b.

In view of these results, our research focus was directed to the insertion of an amide group to the molecules. If ketone carbonyl groups were responsible for the increase in activity, the presence of a more potent hydrogen bond acceptor (amide) could lead to more potent inhibitors. Such hypothesis started to be evaluated by the unsubstituted derivative 4a which showed an IC\(_{50}\) of 2.2 μM. Similar to that observed with dihydro-purinones 2a-h, the presence of the naphthyl group significantly increased the
inhibitory activity. The compound 4b exhibited an IC<sub>50</sub> of 0.7 μM. Interestingly, the use of methylene as a spacer in the 4c reduced the activity to a great extent. Structure 4c presented IC<sub>50</sub> of 12.3 μM which was near 5.6-fold less active than phenyl derivative 4a. This result denotes that the amide planarity can be crucial for the activity shown by the synthesised compounds. Dihydro-purinone 4d, containing a fluorine atom at the 4-position of benzene ring, showed an IC<sub>50</sub> of 2.0 μM. When fluorine atom was changed by 4-chloro, the capacity to inhibit MtFolB increased. The IC<sub>50</sub> value of the compound 4e was 0.8 μM which indicated a 2.5-fold increase in the inhibitory activity compared to that exhibited by 4-fluor-substituted structure 4d. The bromine atom attached at the 4-position of 4f yielded a molecule with IC<sub>50</sub> of 0.5 μM. Once more, increasing the volume of the substituent in this portion of the molecule seems to favour its inhibitory activity towards MtFolB. Finally, positioning propyl (4g) and heptyl (4h) groups at position of the benzene ring led to structures with IC<sub>50</sub> of 0.9 and 0.4 μM, respectively. Interestingly, bulky heptyl group provided similar activity to that presented by bromo-substituted 4f. This finding indicates that there may be an important hydrophobic pocket surrounding this portion of the molecule after binding.

Using an IC<sub>50</sub> value < 0.50 μM as threshold, the mode of inhibition of four compounds (8-MG (1), 3b, 4f and 4h) was determined from Lineweaver – Burk plots. The data was fitted to the appropriate equations to give values for the inhibition constants (K<sub>i</sub> and/or K<sub>i</sub>) (Table 1). For 8-MG (1), 3b and 4h, the double-reciprocal plots resulted in a set of lines that intercept on the left of the y-axis (Figure 1(A–C)), indicating a non-competitive inhibition mode. The in vitro inhibition constant values K<sub>i</sub> and K<sub>i</sub> for these compounds were determined fitting to Equation (3), where K<sub>i</sub> ranged from 0.3 – 0.5 μM and K<sub>i</sub> ranged from 0.6 – 1.2 μM. This analysis was consistent with a typical effect of a non-competitive inhibitor with K<sub>i</sub> < K<sub>i</sub>. Therefore, the inhibitory profile suggests that these three compounds inhibit both the free enzyme and the enzyme – DHNP binary complex, being more effective inhibitors towards the free enzyme<sup>16</sup>. For 4f, the double-reciprocal plots resulted in a set of lines that intercept at the y-axis (Figure 1(D)), indicating a competitive inhibition mode. This inhibitor binds to the free enzyme, disrupting substrate binding<sup>16</sup>. Importantly, compound 4f was found to have a lower Ki value (0.1 ± 0.03 μM) than 8-MG (1) (0.3 ± 0.1 μM) (see Table 1), indicating that this derivative is a more potent inhibitor than the scaffold molecule 1 (8-MG).

The interaction modes of compound 8-MG (1) and its derivatives at the active site of MtFolB were evaluated using molecular docking studies. The predicted stabilities of the octameric form of MtFolB bound to inhibitors were determined for 8-MG (1) and all derivatizations by docking simulations (Table S1). These data are presented together with IC<sub>50</sub> values determined in this study. From the four compounds with IC<sub>50</sub> values equal or lower than 0.5 μM, compound 4f is the most potent inhibitor, with a K<sub>i</sub> of 0.1 ± 0.03 μM (Table 1). It is indeed the only derivatized compound found to be a more potent inhibitor than compound 8-MG (1) (K<sub>i</sub>: 0.3 ± 0.1 and K<sub>i</sub>: 0.6 ± 0.1 – Table 1) and the only one to display a competitive inhibition mode (Figure 1). The predicted interactions of both 8-MG (1) and compound 4f with MtFolB active site were compared (Figure 2). According to our results from the docking simulations, the inhibitors are associated to the binding pocket mainly by hydrogen bonds, π-π stacking, and hydrophobic interactions. In the compound 8-MG (1), the amino group attached at 2-position of dihydro-purinone ring established hydrogen bonds with Tyr52D and Glu74A at distances of 2.9 and 2.7 Å, respectively.

![Figure 1](image-url)
Similar distances were observed in another two hydrogen bonds involving the 3-N and 9-NH with Tyr54D and Asp53D residues. While the NH group was positioned at a distance of 2.7 Å from Asp53D, the 3-N formed a hydrogen bonding donor-acceptor pair with a distance of 2.9 Å from Tyr54D. The complex formed between compound 8-MG (1) and MtFolB was also stabilised by \( p \)–\( p \) stacking interactions between phenyl group of the Tyr54D and the dihydro-purinone ring.

Following the same pattern presented by compound 8-MG (1), the main contacts between dihydro-purinone 4f and MtFolB were performed by the heterocyclic ring. The amino group (2-NH\(_2\)) acted as a hydrogen bonding donor with distances of 2.7 and 2.8 Å from Glu74H and Tyr52F, respectively. The residue Ile73A also establishes a hydrogen bond with the carbonyl group of the dihydro-purinone ring, at the same distance of 3.0 Å found in both compounds 4f and 8-MG (1). Moreover, the Tyr54 residue establishes both \( \pi \)–\( \pi \) stacking interactions and hydrogen bonds with the dihydro-purinone ring of the three compounds. A hydrogen bond of Glu74A with 1-NH of the dihydro-purinone ring (2.8 Å) also present for compound 4f (but not 8-MG (1)) and another hydrogen bond of Asp53D with 9-NH of the same ring (2.8 Å) shared with compound 8-MG (1) (but not 4f) completes the set of interactions shared among these compounds. Finally, the 4-chlorophenyl portion of compound 3b establishes hydrophobic interactions with Val18A residue.

Figure 2. Predicted binding mode of compounds 4f (A,B) and 8-MG (1) (C,D) into the binding pocket of MtFolB. (A,C) 2D-interaction diagrams of the binding models of 4f (A) and 8-MG (1) (C) with MtFolB residues, with hydrogen bonds and \( \pi \)–\( \pi \) stacking interactions shown in dashed lines. (B and D) Predicted docking orientations of 4f (B) and 8-MG (1) (D) into the binding pocket of MtFolB (PDB ID: 1NBU).
The structural pose of compound 4h bound to the octameric form of MtFolB reveals a completely different binding mechanism. None of the interactions described above shared by compounds 8-MG (1), 4f and 3b within the binding pocket of MtFolB are found in the case of compound 4h. In fact, this compound is bound in an inverted orientation relative to the others (Figure 3(C,D)). The bulky heptyl group attached at 4-position of the benzene ring in the derivatized portion of this molecule undergo extensive hydrophobic interactions with six different amino acids (Val55D, Leu72A, Val18A, Leu48D, Ile73A and Tyr54D). The Tyr54D is also establishing π–π stacking interactions with the ligand, but in this case with the benzene ring, and not with the dihydro-purinone group, as with compounds 8-MG (1), 4f and 3b. Due to its change in orientation, the dihydro-purinone group of 4h is not found with the same interactions shared by the other three compounds. It is hydrogen bonded with only Asp53D and Tyr19A.

Interestingly, MtFolB octamer bound to compound 4h has the worst predicted free energy of binding for the derivatizations (−7.14 kcal/mol – Table S1) and experimentally was found to be the least potent of the four compounds whose mode of inhibition was investigated ($k_i$: 0.5 ± 0.1 μM; $k_{ic}$: 1.2 ± 0.2 μM – Table 1). The different modes of binding obtained from docking simulations described above could be a structural explanation for the predicted reduced stability of the inhibitor-protein complex which in turn could result in a weaker inhibition, as observed experimentally.

To evaluate whether the compounds inhibit mycobacterial growth in vitro, we performed a REMA experiment against the virulent Mtb H37Rv strain. The compounds presented no activity against Mtb cells (MIC values above the maximum concentration tested for each compound). Further studies will be required to evaluate the reasons for this lack of antimycobacterial activity in REMA experiments. Nevertheless, this study represents the first step towards the development of new drugs targeting FolB enzyme from M. tuberculosis.

Disclosure statement
No potential conflict of interest was reported by the author(s).

Funding
This work was supported by Banco Nacional de Desenvolvimento Econômico e Social (BNDES) [grant number 14.2.0914.1] and the National Institute of Science and Technology on Tuberculosis (CNPq-FAPERGS-CAPES) [grant number 421703-2017-2] and [grant numbers 17–1265-8]. C. V. Bizarro, L. A. Basso, and P. Machado are Research Career Awardees of the National Research Council of Brazil (CNPq). This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil (CAPES), Finance Code 001.

ORCID
Alexia de Matos Czeczot http://orcid.org/0000-0001-9678-7597
Candida Deves Roth http://orcid.org/0000-0002-3560-263X
References

1. World Health Organization. Global tuberculosis report. 2020.
2. Koul A, Arnoult E, Lounis N, et al. The challenge of new drug discovery for tuberculosis. Nature 2011;469:483–90.
3. Pai M, Behr MA, Dowdy D, et al. Tuberculosis. Nat Rev Dis Primers 2016;2:16076.
4. Bertacine Dias MV, Santos JC, Libreros-Zúñiga GA, et al. Folate biosynthesis pathway: mechanisms and insights into drug design for infectious diseases. Future Med Chem 2018;10:935–59.
5. Visentin M, Zhao R, Goldman ID. The antifolates. Hematol Oncol Clin North Am 2012;26:629–48.
6. Minato Y, Thiede JM, Kordus SL, et al. Mycobacterium tuberculosis folate metabolism and the mechanistic basis for para-aminosalicylic acid susceptibility and resistance. Antimicrob Agents Chemother 2015;59:5097–106.
7. Kumar A, Guardia A, Colmenarejo G, et al. A focused screen identifies antifolates with activity on mycobacterium tuberculosis. ACS Infect Dis 2015;1:604–14.
8. Hajian B, Scocchera E, Shoen C, et al. Drugging the folate pathway in mycobacterium tuberculosis: the role of multi-targeting agents. Cell Chem Biol 2019;26:781–91.
9. Czekster CM, Blanchard JS. One substrate, five products: reactions catalyzed by the dihydroleptopterin aldolase from Mycobacterium tuberculosis. J Am Chem Soc 2012;134:19758–71.
10. Falcão VC, Villela AD, Rodrigues-Junior VS, Pissinate K, et al. Validation of Mycobacterium tuberculosis dihydroleptopterin aldolase as a molecular target for anti-tuberculosis drug development. Biochem Biophys Res Commun 2017;485:814–9.
11. Sanders WJ, Nienaber VL, Lerner CG, et al. Discovery of potent inhibitors of dihydroleptopterin aldolase using CrystaLEAD high-throughput X-ray crystallographic screening and structure-directed lead optimization. J Med Chem 2004;47:1709–18.
12. Chhabra S, Dolezal O, Collins BM, et al. Structure of S. aureus HPPK and the discovery of a new substrate site inhibitor. PLoS One 2012;7:e29444.
13. Dennis ML, Chhabra S, Wang ZC, et al. Structure-based design and development of functionalized Mercaptoguanine derivatives as inhibitors of the folate biosynthesis pathway enzyme 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase from Staphylococcus aureus. J. Med. Chem 2014;57:9612–26.
14. Dennis ML, Pitcher NP, Lee MD, et al. Structural Basis for the Selective Binding of Inhibitors to 6-Hydroxymethyl-7,8-dihydropterin Pyrophosphokinase from Staphylococcus aureus and Escherichia coli. J Med Chem 2016;59:5248–63.
15. Dennis ML, Lee MD, Harjani JR, et al. 8-Mercaptoguanine derivatives as inhibitors of dihydropteroate synthase. Chemistry 2018;24:1922–30.
16. Copeland RA, Reversible modes of inhibitor interactions with enzymes. Evaluation of Enzyme Inhibitors in Drug Discovery: a Guide for Medicinal Chemists and Pharmacologists, 2nd ed.; Hoboken, New Jersey: John Wiley&Sons, Inc. 2013.
17. Goulding CW, Apostol MI, Sawaya MR, et al. Regulation by oligomerization in a mycobacterial folate biosynthetic enzyme. J Mol Biol 2005; 349:61–72.
18. Dallakyan S, Olson AJ. Small-molecule library screening by docking with PyRx. Methods Mol Biol 2015;1263:243–50.
19. Morris GM, Huey R, Lindstrom W, et al. AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. J Comput Chem 2009;30:2785–91.
20. Goldberg DE, Genetic algorithms in search, optimization, and machine learning. Arlington Street, MA: Addison-Wesley; Addison-Wesley Longman Publishing Co., Inc.; 1989.
21. Solis FJ, Wets RB. Minimization by random search techniques. Math Oper Res 1981;6:19–30.
22. Giacobbo BC, Pissinate K, Rodrigues-Junior V, et al. New insights into the SAR and drug combination synergy of 2-(quinolin-4-yloxy)acetamides against Mycobacterium tuberculosis. Eur J Med Chem 2017;126:491–501.