Design, Synthesis and Antitubercular Activity of 2-(Benzylthio)-1H-benzo[d]imidazoles

Raoni S. Rambo,‡a Etienne C. Waldow,a Bruno L. Abaddi,a Maiele D. Silveira,a Adilio S. Dadda,a Nathalia Sperotto,a Cristiano V. Bizarro,a,b Luiz Augusto Basso,a,b,c and Pablo Machado*,a,b,c

‡Instituto Nacional de Ciência e Tecnologia em Tuberculose, Centro de Pesquisas em Biologia Molecular e Funcional, Pontifícia Universidade Católica do Rio Grande do Sul, 90616-900 Porto Alegre-RS, Brazil
aPrograma de Pós-Graduação em Biologia Celular e Molecular, Pontificia Universidade Católica do Rio Grande do Sul, 90616-900 Porto Alegre-RS, Brazil
bPrograma de Pós-Graduação em Medicina e Ciências da Saúde, Pontificia Universidade Católica do Rio Grande do Sul, 90616-900 Porto Alegre-RS, Brazil

Using molecular simplification and molecular hybridization approaches, a series of 2-(benzylthio)-1H-benzo[d]imidazoles was synthesized and evaluated as in vitro inhibitors of Mycobacterium tuberculosis (M. tuberculosis) growth. Compounds 6p and 6z were considered the lead compounds from this series of molecules, with minimal inhibitory concentration (MIC) values of 6.9 and 3.8 µM against M. tuberculosis H37Rv, respectively. Additionally, the leading compounds were active against multidrug-resistant strains and were devoid of apparent toxicity to Vero and HepG2 cells, from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and neutral red assays. Finally, the compounds presented good aqueous solubility and high plasma stability. These data together indicate that this class of molecules may furnish new anti-tuberculosis drug candidates for future development.

Keywords: Mycobacterium tuberculosis, tuberculosis, drug-resistant strain, preliminary SAR study

Introduction

Tuberculosis (TB) is an airborne infectious disease caused mainly by Mycobacterium tuberculosis (Mt). According to the World Health Organization (WHO), 10 million people have been affected by the disease, with an estimated 1.2 million deaths in 2019.1,2 The recommended treatment includes two months of isoniazid (INH), rifampicin (RIF), ethambutol (ETH) and pyrazinamide (PZA), followed by four more months of INH and RIF.3,4 Although it has a cure rate of up to 95%, the regime suffers with an increasing number of individuals infected with drug-resistant strains.3 In these cases, the treatment can be extended and second-line drugs are needed, which are, in general, more expensive and toxic.4 Furthermore, the low adherence of patients to the regime treatment, adverse effects, toxicity events and difficulty of co-administration with some antiretroviral drugs have limited the use of this pharmacologic strategy.1

Within this context, new therapeutic alternatives are needed, if possible, acting via innovative mechanisms to overcome concerns of resistance to clinically available drugs. An example of this constant need for new anti-TB drugs is the reported emergence of Mt-resistant strains against the recently approved drugs bedaquiline and delamanid.5

In an attempt to collaborate with the research of new drugs for TB treatment, our research group has developed strategies for obtaining drug candidates capable of inhibiting the growth of drug-susceptible and drug-resistant Mt strains.6,8 Recently, a series of 2-quinoline-based compounds was synthesized.8 These compounds presented potent and selective activity against drug-susceptible and drug-resistant strains, with minimal inhibitory concentration (MIC) as low as 0.3 µM (1, Figure 1). Our findings inferred that antitubercular activity elicited by

*e-mail: pablo.machado@pucrs.br
synthesized molecules was carried out by targeting the cytochrome bc1 complex. Additionally, the compounds showed improved metabolic properties compared with their counterparts, and were able to inhibit intracellular Mtb growth. In another study, our research group demonstrated the antimycobacterial activity of 2-[(1H-benzo[d]imidazol-2-yl)thio]acetamide. The hybrids formed from the synthesis of acetamide-containing compounds provided structures with moderate activity. The top hit compound exhibited MIC value of 16.5 µM, opening up the possibility of new structural modifications aiming toward its optimization. It is noteworthy that 2-mercapto-1H-benzo[d]imidazole is also present as a scaffold of lanzoprazole sulfide, the active metabolite from the lanzoprazole drug. Lanzoprazole sulfide has presented significant activity against intracellular and in-broth cultures of Mtb, with half maximal inhibitory concentrations (IC50) of 0.59 and 0.46 µM, respectively. The structural requirements for the antimycobacterial activity of 2-quinoline-based compounds, along with the activity exhibited by 1H-benzo[d]imidazole-containing molecules, prompted us to evaluate if the presence of benzyl substituents attached to a 5-methoxy-1H-benzo[d]imidazole (3) scaffold could provide new compounds with superior activity (Figure 1). It is important to point out that the methoxy group has been described as a pharmacophoric group of 2-quinoline-based structures.

![Figure 1](attachment:fig1.png)

**Figure 1.** Design of new 2-(benzylthio)-1H-benzo[d]imidazoles 3, using molecular hybridization and molecular simplification approaches from 2-quinoline-based compound 1 (INCT-TB765) and 2-(1H-benzo[d]imidazol-2-yl)thio]acetamide 2 (INCT-TB808).

Therefore, in an attempt to obtain active compounds against drug-susceptible and, mainly, drug-resistant Mtb strains, a new series of 2-(benzylthio)-5-methoxy-1H-benzo[d]imidazoles was synthesized. The molecules were designed using classical molecular hybridization along with a molecular simplification approach. First, the preliminary structural requirements for potency of synthesized structures (structure activity relationship, SAR) were evaluated using MIC values. Subsequently, the most active structures against M. tuberculosis H37Rv were tested against a panel of well-characterized multidrug-resistant strains, while the viability of HepG2 and Vero cells was used as an indicator of the toxicity and selectivity of the compounds.

**Experimental**

**Chemistry**

The progress of the reaction was monitored using thin-layer chromatography (TLC) with Silicycle TLC Silica gel 60 F254 (Quebec, Canada). The melting points were measured using a Microquímica MQAPF-302 apparatus (Palhoça, Brazil). 1H and 13C nuclear magnetic resonance (NMR) spectra were acquired on a Bruker Avance III HD spectrometer (Fällanden, Switzerland). Chemical shifts (δ) were expressed in parts per million (ppm) relative to dimethyl sulfoxide (DMSO-d6), which was used as the solvent, and to tetramethylsilane (TMS), which was used as an internal standard. High-resolution mass spectra (HRMS) were obtained for all compounds on an LTQ Orbitrap Discovery mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The analyses were performed through the direct infusion of the sample in MeOH/MeCN (1:1) with 0.1% formic acid (flow rate 10 mL min-1), in a positive-ion or negative-ion mode using electrospray ionization (ESI). For elemental composition, calculations were performed using the specific tool included in the Qual Browser module of Xcalibur software (Thermo Fisher Scientific, Bremen, Germany). The compounds’ purities, solubility assays and plasma stabilities were determined using a Dionex Ultimate 3000 UHPLC chromatograph (Germering, Germany). The liquid chromatography conditions were as follows: RP column, 5 mm Nucleodur C-18ec (250 × 4.6 mm); flow rate, 1.5 mL min-1; UV detection, 254 nm; 100% water (0.1% acetic acid) to 90% acetonitrile/methanol (1:1, v/v) from 7 to 15 min (15-30 min), and subsequently returned to 100% water (0.1% acetic acid) in 5 min (30-35 min) and maintained for an additional 10 min (35-45 min). All the evaluated compounds were ≥ 90% pure. Commercially available reactants and solvents were obtained from commercial suppliers and were used without additional purification. Ethanol, chloroform, diethyl ether, hexane, ethyl acetate, hydrochloric acid, petroleum ether, and acetone were obtained from Química Moderna (Barueri, Brazil). Dimethylformamide (DMF), acetic acid (glacial), acetonitrile (high performance liquid chromatography (HPLC) grade), and methanol (HPLC grade) were obtained
from Merck KGaA (Darmstadt, Germany). Potassium carbonate was purchased from Acros Organics B.V.B.A. (Geel, Belgium). Finally, resazurin, benzyl halides, imidazoles, DMSO (anhydrous, DMSO for molecular biology), and DMSO-d$_6$ were obtained from Sigma-Aldrich Corporation (Saint Louis, USA). Additionally, NaCl, KCl, Na$_2$HPO$_4$, and KH$_2$PO$_4$ used for phosphate-buffered saline (PBS) preparation were also obtained from Sigma-Aldrich Corporation (Saint Louis, USA).

General procedure for the synthesis of compounds 6

To a round-bottom flask was added the respective imidazole (0.5 mmol), the respective benzyl halide (1.2 equiv), potassium carbonate (1 equiv) and DMF (5 mL). The mixture was stirred for 2-6 h until complete consumption of imidazole, followed by TLC. Then, the reaction was diluted in chloroform (20 mL) and washed with water (3 x 20 mL). The organic layer was dried over sodium sulfate and the solvent evaporated under reduced pressure. The compounds were purified using column chromatography.

2-((Benzylthio)-5-methoxy-1H-benzo[d]imidazole (6a)

Purified by column chromatography eluting mixtures of hexanes and ethyl acetate (9:1 followed by 8:2) to obtain the title compound as a white solid in 67% yield; mp 126-129 °C; t$_g$ 15.2 min; ¹H NMR (400 MHz, DMSO-d$_6$) δ 12.42 (bs, 1H, NH), 7.49-7.18 (m, 6H, 5 Ph-H and 1 Im-H*), 6.97 (bs, 1H, Im-H), 6.75 (dd, 1H, J 8.7, 2.6 Hz, Im-H), 4.53 (d, 2H, J 4.4 Hz, S-CH$_2$), 3.76 (d, 3H, O-CH$_3$); HRMS (Fourier transform mass spectrometry (FTMS) + pESI) m/z, calcd. for C$_{15}$H$_{14}$N$_2$S [M + H]$^+$: 271.0900, found: 271.0887.*Im-H: imidazole hydrogens.

2-((2-Fluorobenzyl)thio)-5-methoxy-1H-benzo[d]imidazole (6b)

Purified by column chromatography eluting mixtures of hexanes and ethyl acetate (9:1 followed by 8:2) to obtain the title compound as a white solid in 80% yield; mp 94-97 °C; t$_g$ 15.7 min; ¹H NMR (400 MHz, DMSO-d$_6$) δ 12.44 (bs, 1H, NH), 7.41-7.22 (m, 3H, 2 Ph-H and 1 Im-H), 7.07 (ddd, 1H, J 10.3, 8.3, 2.7 Hz, Ph-H), 6.97 (bs, 1H, Im-H), 6.75 (dd, 1H, J 8.7, 2.4 Hz, Im-H), 4.53 (s, 2H, S-CH$_2$), 3.76 (s, 3H, O-CH$_3$); HRMS (FTMS + pESI) m/z, calcd. for C$_{15}$H$_{13}$FN$_2$S [M + H]$^+$: 289.0805, found: 289.0790.

2-((4-Fluorobenzyl)thio)-5-methoxy-1H-benzo[d]imidazole (6d)

Purified by column chromatography eluting mixtures of hexanes and ethyl acetate (9:1 followed by 8:2) to obtain the title compound as a yellow solid in 70% yield; mp 133-135 °C; t$_g$ 15.5 min; ¹H NMR (400 MHz, DMSO-d$_6$) δ 12.44 (bs, 1H, NH), 7.51-7.37 (m, 3H, 2 Ph-H and 1 Im-H), 7.16-7.06 (m, 2H, Ph-H), 6.88 (bs, 1H, Im-H), 6.75 (dd, 1H, J 8.7, 2.4 Hz, Im-H), 4.51 (s, 2H, S-CH$_2$), 3.75 (s, 3H, O-CH$_3$); HRMS (FTMS + pESI) m/z, calcd. for C$_{15}$H$_{13}$FN$_2$SO [M + H]$^+$: 307.0805, found: 307.0694.

2-((3,4-Difluorobenzyl)thio)-5-methoxy-1H-benzo[d]imidazole (6e)

Purified by column chromatography eluting mixtures of hexanes and ethyl acetate (9:1 followed by 7:3) to obtain the title compound as a white solid in 70% yield; mp 136-139 °C; t$_g$ 16.1 min; ¹H NMR (400 MHz, DMSO-d$_6$) δ 12.45 (bs, 1H, NH), 7.54-7.44 (m, 1H, Im-H), 7.42-7.20 (m, 3H, Ph-H), 6.98 (bs, 1H, Im-H), 6.75 (dd, 1H, J 8.7, 2.4 Hz, Im-H), 4.50 (s, 2H, S-CH$_2$), 3.75 (s, 3H, O-CH$_3$); HRMS (FTMS + pESI) m/z, calcd. for C$_{15}$H$_{13}$FN$_2$OSO [M + H]$^+$: 307.0711, found: 307.0694.

2-((2-Chlorobenzyl)thio)-5-methoxy-1H-benzo[d]imidazole (6f)

Purified by column chromatography eluting mixtures of hexanes and ethyl acetate (9:1 followed by 8:2) to obtain the title compound as a yellow oil in 90% yield; t$_R$ 16.3 min; ¹H NMR (400 MHz, DMSO-d$_6$) δ 12.44 (bs, 1H, NH), 7.51-7.37 (m, 3H, 2 Ph-H and 1 Im-H), 7.07 (ddd, 1H, J 10.3, 8.3, 4.5 Hz, Ph-H), 6.88 (bs, 1H, Im-H), 6.75 (dd, 1H, J 8.7, 2.4 Hz, Im-H), 4.61 (s, 2H, S-CH$_2$), 3.77 (s, 3H, O-CH$_3$); HRMS (FTMS + pESI) m/z, calcd. for C$_{15}$H$_{13}$ClFN$_2$ [M + H]$^+$: 305.0510, found: 305.0493.

2-((3-Chlorobenzyl)thio)-5-methoxy-1H-benzo[d]imidazole (6g)

Purified by column chromatography eluting mixtures of hexanes and ethyl acetate (9:1 followed by 8:2) to obtain the title compound as a white solid in 74% yield; mp 132-135 °C; t$_g$ 16.4 min; ¹H NMR (400 MHz,
DMSO-d$_6$ $\delta$ 12.46 (bs, 1H, NH), 7.51 (d, 1H, J 2.1 Hz, Im-H), 7.42-7.24 (m, 4H, Ph-H), 6.96 (bs, 1H, Im-H), 6.75 (dd, 1H, J 8.7, 2.5 Hz, Im-H), 4.52 (s, 2H, S-CH$_2$), 3.75 (s, 3H, O-CH$_3$); HRMS (FTMS + pESI) $m/z$, calcd. for C$_{15}$H$_{13}$ClN$_2$OS [M + H]$^+$: 305.0510, found: 305.0494.

2-((4-Chlorobenzyl)thio)-5-methoxy-1H-benzo[d]imidazole (6h)$^{11}$

Purified by column chromatography eluting mixtures of hexanes and ethyl acetate (8:2 followed by 7:3) to obtain the title compound as a white solid in 95% yield; mp 131-134 °C; $\tau$$_d$ 16.3 min; $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ 12.44 (bs, 1H, NH), 7.48-7.41 (m, 2H, 1 Im-H and 1 Ph-H), 7.38-7.30 (m, 3H, Ph-H), 6.95 (bs, 1H, Im-H), 6.75 (dd, 1H, J 8.7, 2.4 Hz, Im-H), 4.52 (s, 2H, S-CH$_2$), 3.76 (s, 3H, O-CH$_3$); HRMS (FTMS + pESI) $m/z$, calcd. for C$_{15}$H$_{13}$ClN$_2$OS [M + H]$^+$: 305.0510, found: 305.0491.

2-((3,4-Dichlorobenzyl)thio)-5-methoxy-1H-benzo[d]imidazole (6i)$^{11}$

Purified by column chromatography eluting mixtures of hexanes and ethyl acetate (8:2 followed by 7:3) to obtain the title compound as a white solid in 72% yield; mp 140-143 °C; $\tau$$_d$ 17.2 min; $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ 12.43 (bs, 1H, NH), 7.71 (d, 1H, J 2.0 Hz, Ph-H), 7.54 (d, 1H, J 8.2 Hz, Im-H), 7.41 (dd, 1H, J 8.3, 2.1 Hz, Ph-H), 7.35 (bs, 1H, Ph-H), 6.97 (bs, 1H, Im-H), 6.75 (dd, 1H, J 8.7, 2.4 Hz, Im-H), 4.51 (s, 2H, S-CH$_2$), 3.76 (s, 3H, O-CH$_3$); HRMS (FTMS + pESI) $m/z$, calcd. for C$_{15}$H$_{13}$ClN$_3$O$_3$S [M + H]$^+$: 339.0120, found: 339.0101.

2-((4-Bromobenzyl)thio)-5-methoxy-1H-benzo[d]imidazole (6j)$^{11}$

Purified by column chromatography eluting mixtures of hexanes and ethyl acetate (8:2 followed by 7:3) to obtain the title compound as a white solid in 68% yield; mp 128-131 °C; $\tau$$_d$ 17.2 min; $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ 12.33 (bs, 1H, NH), 8.03 (dt, 1H, J 8.2, 1.2 Hz, Ph-H), 7.74 (dd, 1H, J 7.6, 1.4 Hz, Ph-H), 7.66 (td, 1H, J 7.5, 1.3 Hz, Ph-H), 7.52 (td, 1H, J 7.8, 7.3, 1.4 Hz, Im-H), 7.33 (d, 1H, J 8.2, Ph-H), 6.95 (bs, 1H, Im-H), 6.74 (dd, 1H, J 8.7, 2.5, 1.1 Hz, Im-H), 4.81 (s, 2H, S-CH$_2$), 3.76 (s, 3H, O-CH$_3$); HRMS (FTMS + pESI) $m/z$, calcd. for C$_{15}$H$_{13}$BrN$_2$OS [M + H]$^+$: 316.0750, found: 316.0734.

2-((3-Trifluoromethyl)benzyl)thio)-5-methoxy-1H-benzo[d]imidazole (6k)$^{11}$

Purified by column chromatography eluting mixtures of hexanes and ethyl acetate (8:2 followed by 7:3) to obtain the title compound as a white solid in 66% yield; mp 172-175 °C; $\tau$$_d$ 17.3 min; $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ 8.00 (d, 1H, J 2.1 Hz, Ph-H), 8.13-8.07 (m, 1H, Ph-H), 7.94 (dt, 1H, J 7.7, 1.4 Hz, Ph-H), 7.60 (t, 1H, J 8.0 Hz, Ph-H), 7.54 (d, 1H, J 8.9 Hz, Im-H), 7.10 (d, 1H, J 2.4 Hz, Im-H), 7.01 (dd, 1H, J 8.9, 2.4 Hz, Im-H), 4.96 (s, 2H, S-CH$_2$), 3.81 (s, 3H, O-CH$_3$); HRMS (FTMS + pESI) $m/z$, calcd. for C$_{15}$H$_{13}$F$_2$N$_2$OS [M + H]$^+$: 348.9984.
obtain the title compound as a yellow solid in 75% yield; mp 170-172 °C; t<sub>r</sub> 15.9 min; 1H NMR (400 MHz, DMSO-<em>d</em><sub>6</sub>) δ 12.47 (bs, 1H, NH), 8.18-8.13 (m, 2H, Ph-H), 7.73-7.67 (m, 2H, 1 Ph-H and 1 Im-H), 7.34 (d, 1H, J 8.4 Hz, Ph-H), 7.00-6.92 (m, 1H, Im-H), 6.75 (dd, 1H, J 8.7, 2.4 Hz, Im-H), 4.65 (s, 2H, S-CH<sub>2</sub>), 3.75 (s, 3H, O-CH<sub>3</sub>); HRMS (FTMS + pESI) m/z, calcd. for C<sub>15</sub>H<sub>12</sub>N<sub>4</sub>O<sub>5</sub>S [M + H]<sup>+</sup>: 313.1369, found: 313.1350.

2-((3,5-Dinitrobenzyl)thio)-5-methoxy-1H-benz[d]imidazole (6p)

Purified by column chromatography eluting mixtures of hexanes and ethyl acetate (8:2 followed by 7:3) to obtain the title compound as a yellow solid in 75% yield; mp 170-172 °C; t<sub>r</sub> 14.3 min; 1H NMR (400 MHz, DMSO-<em>d</em><sub>6</sub>) δ 12.43 (bs, 1H, NH), 7.89-7.82 (m, 2H, Ph-H), 7.70 (d, 2H, J 8.1 Hz, Ph-H), 7.34 (d, 1H, J 8.8 Hz, Im-H), 6.97 (s, 1H, Im-H), 6.75 (dd, 1H, J 8.7, 2.4 Hz, Im-H), 4.63 (s, 2H, S-CH<sub>2</sub>), 3.76 (s, 3H, O-CH<sub>3</sub>); HRMS (FTMS + pESI) m/z, calcd. for C<sub>16</sub>H<sub>16</sub>N<sub>2</sub>O<sub>5</sub>S<sub>2</sub> [M + H]<sup>+</sup>: 349.0675, found: 349.0657.

2-((3,4-Difluorobenzyl)thio)-1H-benz[d]imidazole (6t)

Purified by column chromatography eluting mixtures of hexanes and ethyl acetate (9:1 followed by 8:2) to obtain the title compound as a brown solid in 22% yield; mp 108-110 °C (lit. 127-130 °C); t<sub>r</sub> 16.3 min; 1H NMR (400 MHz, DMSO-<em>d</em><sub>6</sub>) δ 12.60 (bs, 1H, NH), 7.53 (ddd, 1H, J 11.8, 7.9, 2.1 Hz, Ph-H), 7.48-7.42 (m, 2H, Im-H), 7.39-7.27 (m, 2H, Ph-H), 7.20-7.04 (m, 2H, Im-H), 4.54 (s, 2H, S-CH<sub>2</sub>); HRMS (FTMS + pESI) m/z, calcd. for C<sub>16</sub>H<sub>16</sub>F<sub>2</sub>N<sub>2</sub>S [M + H]<sup>+</sup>: 277.0606, found: 277.0590.

2-((2-Chlorobenzyl)thio)-1H-benz[d]imidazole (6u)

Purified by column chromatography eluting mixtures of hexanes and ethyl acetate (9:1 followed by 8:2) to obtain the title compound as a yellow solid in 35% yield; mp 157-160 °C (lit. 160-164 °C); t<sub>r</sub> 16.5 min; 1H NMR (400 MHz, DMSO-<em>d</em><sub>6</sub>) δ 12.62 (bs, 1H, NH), 7.62-7.43 (m, 4H, Ph-H), 7.33-7.21 (m, 2H, Im-H), 7.13 (dd, 2H, J 5.9, 3.0 Hz, Im-H), 4.65 (s, 2H, S-CH<sub>2</sub>); HRMS (FTMS + pESI) m/z, calcd. for C<sub>16</sub>H<sub>16</sub>ClN<sub>2</sub>S [M + H]<sup>+</sup>: 275.0404, found: 275.0389.

2-((3-Chlorobenzyl)thio)-1H-benz[d]imidazole (6v)

Purified by column chromatography eluting mixtures of hexanes and ethyl acetate (9:1 followed by 8:2) to obtain the title compound as a light yellow solid in 35% yield; mp 151-144 °C (lit. 145-147 °C); t<sub>r</sub> 16.6 min; 1H NMR (400 MHz, DMSO-<em>d</em><sub>6</sub>) δ 12.60 (bs, 1H, NH), 7.53 (d, 1H, J 2.0 Hz, Ph-H), 7.50-7.38 (m, 3H, Ph-H), 7.36-7.26 (m, 2H, Im-H), 7.17-7.09 (m, 2H, Im-H), 4.56 (s, 2H, S-CH<sub>2</sub>); HRMS (FTMS + pESI) m/z, calcd. for C<sub>16</sub>H<sub>16</sub>ClN<sub>2</sub>S [M + H]<sup>+</sup>: 275.0404, found: 275.0388.

2-((4-Chlorobenzyl)thio)-1H-benz[d]imidazole (6w)

Purified by column chromatography eluting mixtures of hexanes and ethyl acetate (9:1 followed by 8:2) to obtain the title compound as a brown solid in 80% yield; mp 176-179 °C (lit. 180-181 °C); t<sub>r</sub> 16.6 min; 1H NMR (400 MHz, DMSO-<em>d</em><sub>6</sub>) δ 12.59 (bs, 1H, NH), 7.57-7.43 (m, 3H, Ph-H), 7.39-7.33 (m, 3H, 1 Ph-H and 2 Im-H), 7.12 (dt, 2H, J 7.2, 3.6 Hz, Im-H), 4.56 (s, 2H, S-CH<sub>2</sub>); HRMS...
Design, Synthesis and Antitubercular Activity of 2-(Benzylthio)-1H-benzo[d]imidazoles


2-(4-Bromobenzyl)thio)-1H-benzo[d]imidazole (6x)

Purified by column chromatography eluting mixtures of hexanes and ethyl acetate (8:2 followed by 7:3) to obtain the title compound as a white solid in 76% yield; mp 195-198 °C (lit. 198-201 °C); 1H NMR (400 MHz, DMSO-d6) δ 12.55 (bs, 1H, NH), 7.60-7.32 (m, 6H, 4 Ph-H and 2 Im-H), 7.23-7.01 (m, 2H, Im-H), 4.54 (s, 2H, S-CH2); HRMS (FTMS + pESI) m/z, calcd. for C14H11BrN2S [M + H]+: 331.0482, found: 331.0496.

2-(4-Fluorobenzyl)thio)-1H-benzo[d]imidazole (6y)

Purified by column chromatography eluting mixtures of hexanes and ethyl acetate (8:2 followed by 7:3) to obtain the title compound as a white solid in 80% yield; mp 146-149 °C (lit. 148-149.5 °C); 1H NMR (400 MHz, DMSO-d6) δ 12.60 (s, 1H, NH), 7.85 (d, 1H, J 2.0 Hz, Ph-H), 7.81-7.73 (m, 1H, Ph-H), 7.64-7.48 (m, 3H, 2 Ph-H and 1 Im-H), 7.43-7.29 (m, 1H, Im-H), 7.19-7.07 (m, 2H, Im-H), 4.65 (s, 2H, S-CH2); HRMS (FTMS + pESI) m/z, calcd. for C15H11F3N2S [M + H]+: 309.0678, found: 309.0668.

2-(3-(3,5-Dinitrobenzyl)thio)-1H-benzo[d]imidazole (6z)

Purified by column chromatography eluting mixtures of hexanes and ethyl acetate (8:2 followed by 7:3) to obtain the title compound as a yellow solid in 68% yield; mp 151-154 °C (lit. 155-158 °C); 1H NMR (400 MHz, DMSO-d6) δ 12.60 (s, 1H, NH), 8.83 (d, 2H, J 2.1 Hz, Ph-H), 8.66 (t, 1H, J 2.2 Hz, Ph-H), 7.43 (s, 2H, Im-H), 7.15-7.07 (m, 2H, Im-H), 4.79 (s, 2H, S-CH2); HRMS (FTMS + pESI) m/z, calcd. for C16H10N6O4S [M + H]+: 331.0496, found: 331.0482.

Susceptibility testing against M. tuberculosis

Compounds 6p and 6z were further tested by REMA, as described above, for their inhibitory potential against three multidrug-resistant clinical isolates of M. tuberculosis.15 The clinical isolates (named PT2, PT12 and PT20) were obtained from patients in the Lisbon Health Region, Lisbon, Portugal.15 INH and RIF were used as control drugs to demonstrate the MDR phenotype of these isolates.

Cytotoxicity investigation

Cellular viability determination after incubation with the test compounds was performed using two different methods: the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method and neutral red uptake assay.16,17 First, Vero and HepG2 cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% inactivated fetal bovine serum, 1% antibiotic (gentamicin) and 0.01% antifungal (amphotericin B). Cells were seeded at 4 × 10^3 (HepG2) or 2 × 10^4 cells per well (Vero) in a 96-well microtiter plate, and incubated for 24 h. Test compounds were diluted in three different concentrations (1, 5 and 20 mM) using 2% DMSO, and were incubated with the cell lines for 72 h at 37 °C under 5% CO2.18 For the MTT assay, the cultures were incubated with MTT reagent (5 mg mL^-1) for 4 h. The absorbance was measured using excitation and emission wavelengths of 570 and 655 nm, respectively (SpectraMax M2e, Molecular Devices, San Jose, USA). The precipitated purple formazan crystals were directly proportional to the number of live cells with active mitochondria. For the neutral red assay, after 72 h of incubation with the compounds, the cells were washed with PBS before the addition of 200 mL of neutral red dye solution (25 mg mL^-1, Sigma, Saint Louis, USA) prepared in serum-free medium. The plate was incubated for an additional 3 h at 37 °C under 5% CO2. After incubation, cells were washed with PBS, followed by incubation with...
100 mL of a desorb solution (CH₃COOH/EtOH/H₂O, 1:50:49) for 30 min, with gentle shaking to extract neutral red dye from the viable cells. The absorbance was measured at 562 nm using a microtiter plate reader. The percentage of cell viability for the treated groups was reported by considering the control wells (2% DMSO) as 100% of cell viability: cell viability (%) = (absorbance of treated wells/ absorbance of control wells) × 100. Statistical analysis was performed using one-way analysis of variance using GraphPad Prism 5.0 software.¹⁹

**Solubility assay and plasma stability**

The solubility tests were performed according to a previously published protocol,²⁰ with slight modifications. Accordingly, 1 mL of a prepared solution (1× PBS, pH 7.4 or 0.1 mol L⁻¹ HCl, pH 1.0) was added to 1 mg of compound (in triplicate). The final solutions were vortexed (1 min) and the resulting suspensions were shaken for 4 h at 25 °C. Then, the solutions were centrifuged (13000 rpm for 15 min at 25 °C) obtaining a pellet and the remaining solutions were quantified by liquid chromatography (as per the conditions described in the Experimental section) using single-point calibration of a known concentration of the compounds in DMSO.

The plasma stability was also conducted according to the literature.²¹ Compounds 6p and 6z were dissolved separately in DMSO to a concentration of 800 μM. The stock samples were prepared in triplicate. Subsequently, commercially-obtained mice plasma (Equitech-Bio, Inc, Kerrville, USA) was diluted with PBS 1× (pH 7.4) to obtain a stock solution (1:1). Afterwards, 5 μL of each stock sample were diluted with 195 μL of stock solution, obtaining an appropriate assay condition (20 μM of sample concentration, 50% of plasma concentration in buffer, and 2.5% of DMSO).²¹ Afterward, the solutions were vortexed (1 min) and incubated at 37 °C in a shaker for 3 h. At the end of the incubation, acetonitrile (600 μL) was added, and the samples were centrifuged at 13000 rpm for 15 min (4 °C). The supernatants obtained were analyzed using liquid chromatography.

**Results and Discussion**

The synthesis of the designed compounds was performed in one single and easy-to-perform synthetic step. The desired molecules were obtained using a second-order nucleophilic substitution reaction (Sn2) between 2-mercapto-5-methoxy-1H-benzo[d]imidazole 4a and different benzyl halides (5) (Scheme 1). The reaction was performed in the presence of potassium carbonate as a base and DMF as the solvent, at room temperature for 2-6 h (Scheme 1). The products 6 were isolated with yields varying from 22 to 95% and purities over 90%, as determined by HPLC analysis. Spectroscopic (Supplementary Information section) and spectrometric data were found to be in total agreement with the proposed structures.

![Scheme 1](image)

It is noteworthy that proposed structural modifications were aimed at the creation of an electron density gradient of the substituents in a preliminary SAR study.

Afterwards, all synthesized 1H-benzo[d]imidazoles 6 were tested in whole-cell assays against *M. tuberculosis* H37Rv, using the first-line drug INH as a reference in accordance with previously-described protocols.¹³,¹⁴ In general, the screening revealed that most compounds evaluated showed moderate activity or were inactive at the highest doses tested. In contrast, 3,5-dinitro compounds 6p and 6z exhibited good activity, with MIC values similar to the first-line anti-TB drug INH (Table 1). From proposed substituents, one can conclude that the non-substituted compound 6a did not show antimycobacterial activity at the highest concentration tested. Positioning fluorine atoms at the 2-, 3- or 4-position of the benzene ring (6b-6d) did not produce structures capable of inhibiting the growth of mycobacteria under the evaluated conditions. Interestingly, the presence of two fluorine atoms attached at the 3,4-positions of the benzene ring resulted in a structure with modest activity against Mtb. The difluorinated compound 6e was able to inhibit mycobacterial growth, with an MIC value of 130.6 μM. The presence of chlorine atoms at the 2-, 3- or 4-position in compounds 6f-6h also produced molecules with modest and equipotent activities, with MIC values of 131.2 μM. Compared to monofluorinated compounds 6b-6d, chlorinated molecules 6f-6h have higher lipophilicity (Table 1), which may be related to the higher antimycobacterial activity observed for these structures. When two chlorine atoms were positioned at the 3,4-positions of the benzene ring (6i), the molecule was inactive against Mtb at the highest concentrations evaluated. The bioisosteric exchange between chlorine and bromine was well-tolerated, as compound 6j showed an MIC value of 114.5 μM. Likewise, the 3-trifluoromethyl group attached at position 3 provided the structure 6k, which exhibited an MIC value of 118.2 μM. Positioning
the trifluoromethyl group at the 4-position in 6l generated an inactive compound when tested at 20 µM concentration. Continuing the evaluation of electron-withdrawing chemical groups, the presence of the nitro group at the 2-, 3- or 4-position of the benzenic ring (6m-6o) produced inactive structures when tested at the highest concentrations permitted due to the molecules’ solubilities. Indeed, 1H-benzo[d]imidazoles 6m-6o presented MIC >10 µM. In contrast, the dinitro-substituted compound 6p exhibited a good capacity to inhibit the growth of M. tuberculosis H37Rv in vitro. The structure showed an MIC value of 6.9 µM, only 3-fold less potent when compared to the first-line drug INH. The use of electron-donating substituents in compounds 6q and 6r did not furnish active structures against Mtb. Furthermore, the sulfonomethyl-substituted compound 6s also did not show inhibitory activity on the bacillus growth at the highest concentration evaluated.

In the second round of evaluation of the activity of 1H-benzo[d]imidazole derivatives, the contribution of the methoxy group attached at the 5-position of the heterocyclic ring was evaluated. Compounds 6u-6z were obtained from the S-alkylation reaction of 2-mercapto-1H-benzo[d]imidazole 4b, using the substituents of the most effective 5-methoxy-based structures. In general, the absence of the methoxy group reduced the growth inhibition effectiveness against the M. tuberculosis H37Rv strain. Unlike 1H-benzo[d]imidazoles 6v and 6z, the other compounds were inactive at the highest concentrations tested. While the 3-chloro-substituted compound showed an MIC value of 145.6 µM, the molecule containing the 3,5-dinitro group showed inhibition of mycobacterial growth with an MIC of 3.8 µM. The activity shown by compound 6z was similar to INH when tested under the same experimental conditions.

It is important to mention that 1H-benzo[d]imidazoles 6u-6z have previously been evaluated against M. tuberculosis 331/88 strain.12 Despite the different experimental conditions and differences between Mtb strains, the MIC values observed were similar. However, to the best of our knowledge, the activity of compound 6z against multidrug-resistant strains has not been described. Therefore, the two most active 1H-benzo[d]imidazoles (6p

Table 1. Yields of 2-(benzylthio)-1H-benzo[d]imidazoles 6a-6z, ClogP values, and in vitro activities against M. tuberculosis H37Rv strain

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>R’</th>
<th>Yield a / %</th>
<th>ClogP b</th>
<th>MIC (H37Rv) c / (µg mL -1)</th>
<th>MIC (H37Rv) c / µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>6a</td>
<td>OMe</td>
<td>H</td>
<td>67</td>
<td>4.58</td>
<td>&gt;40</td>
<td>148.0</td>
</tr>
<tr>
<td>6b</td>
<td>OMe</td>
<td>2-F</td>
<td>80</td>
<td>4.72</td>
<td>&gt;40</td>
<td>&gt;138.7</td>
</tr>
<tr>
<td>6c</td>
<td>OMe</td>
<td>3-F</td>
<td>66</td>
<td>4.72</td>
<td>&gt;40</td>
<td>138.7</td>
</tr>
<tr>
<td>6d</td>
<td>OMe</td>
<td>4-F</td>
<td>70</td>
<td>4.72</td>
<td>&gt;40</td>
<td>&gt;138.7</td>
</tr>
<tr>
<td>6e</td>
<td>OMe</td>
<td>3,4-(F)2</td>
<td>70</td>
<td>4.79</td>
<td>40</td>
<td>130.6</td>
</tr>
<tr>
<td>6f</td>
<td>OMe</td>
<td>2-Cl</td>
<td>90</td>
<td>5.29</td>
<td>40</td>
<td>131.2</td>
</tr>
<tr>
<td>6g</td>
<td>OMe</td>
<td>3-Cl</td>
<td>74</td>
<td>5.29</td>
<td>40</td>
<td>131.2</td>
</tr>
<tr>
<td>6h</td>
<td>OMe</td>
<td>4-Cl</td>
<td>95</td>
<td>5.29</td>
<td>40</td>
<td>131.2</td>
</tr>
<tr>
<td>6i</td>
<td>OMe</td>
<td>3,4-(Cl)2</td>
<td>72</td>
<td>5.88</td>
<td>&gt;40</td>
<td>&gt;117.9</td>
</tr>
<tr>
<td>6j</td>
<td>OMe</td>
<td>4-Br</td>
<td>68</td>
<td>5.44</td>
<td>40</td>
<td>114.5</td>
</tr>
<tr>
<td>6k</td>
<td>OMe</td>
<td>3-CF3</td>
<td>78</td>
<td>5.46</td>
<td>40</td>
<td>118.2</td>
</tr>
<tr>
<td>6l</td>
<td>OMe</td>
<td>4-CF3</td>
<td>76</td>
<td>5.46</td>
<td>&gt;20</td>
<td>&gt;59.1</td>
</tr>
<tr>
<td>6m</td>
<td>OMe</td>
<td>2-NO2</td>
<td>82</td>
<td>4.24</td>
<td>&gt;10</td>
<td>&gt;31.7</td>
</tr>
<tr>
<td>6n</td>
<td>OMe</td>
<td>3-NO2</td>
<td>66</td>
<td>4.32</td>
<td>&gt;40</td>
<td>&gt;126.8</td>
</tr>
<tr>
<td>6o</td>
<td>OMe</td>
<td>4-NO2</td>
<td>86</td>
<td>4.32</td>
<td>&gt;10</td>
<td>&gt;31.7</td>
</tr>
<tr>
<td>6p</td>
<td>OMe</td>
<td>3,5-(NO2)2</td>
<td>85</td>
<td>4.06</td>
<td>2.50</td>
<td>6.9</td>
</tr>
<tr>
<td>6q</td>
<td>OMe</td>
<td>4-iPr</td>
<td>81</td>
<td>6.00</td>
<td>&gt;5</td>
<td>&gt;16.0</td>
</tr>
<tr>
<td>6r</td>
<td>OMe</td>
<td>4-tBu</td>
<td>78</td>
<td>6.04</td>
<td>&gt;5</td>
<td>&gt;15.3</td>
</tr>
<tr>
<td>6s</td>
<td>OMe</td>
<td>4-SO2Me</td>
<td>83</td>
<td>2.94</td>
<td>&gt;20</td>
<td>&gt;57.4</td>
</tr>
<tr>
<td>6t</td>
<td>H</td>
<td>3,4-(F)2</td>
<td>22</td>
<td>4.49</td>
<td>&gt;40</td>
<td>&gt;144.8</td>
</tr>
<tr>
<td>6u</td>
<td>H</td>
<td>2-Cl</td>
<td>35</td>
<td>4.98</td>
<td>&gt;10</td>
<td>&gt;36.4</td>
</tr>
<tr>
<td>6v</td>
<td>H</td>
<td>3-Cl</td>
<td>59</td>
<td>4.98</td>
<td>40</td>
<td>145.6</td>
</tr>
<tr>
<td>6w</td>
<td>H</td>
<td>4-Cl</td>
<td>80</td>
<td>4.98</td>
<td>&gt;10</td>
<td>&gt;36.4</td>
</tr>
<tr>
<td>6x</td>
<td>H</td>
<td>4-Br</td>
<td>76</td>
<td>5.14</td>
<td>&gt;5</td>
<td>&gt;15.7</td>
</tr>
<tr>
<td>6y</td>
<td>H</td>
<td>3-CF3</td>
<td>80</td>
<td>5.16</td>
<td>&gt;10</td>
<td>&gt;32.4</td>
</tr>
<tr>
<td>6z</td>
<td>H</td>
<td>3,5-(NO2)2</td>
<td>68</td>
<td>3.76</td>
<td>1.25</td>
<td>3.8</td>
</tr>
<tr>
<td>INH</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–0.67</td>
<td>0.3</td>
<td>2.3</td>
</tr>
</tbody>
</table>

aIsolated yield; blogarithm of partition coefficient calculated by ChemBioDraw Ultra, version 13.0.0.3015; cminimal inhibitory concentration; dreaction performed at 40 °C for 4 h. INH: isoniazid.
and 6z) were selected for both inhibitory activity of three multidrug-resistant *M. tuberculosis* strains (PT2, PT12, and PT20) and for viability studies, using Vero and HepG2 cells (Table 2). The multidrug-resistant strains PT2, PT12, and PT20 have been described as resistant to drugs such as INH, RIF, streptomycin, ethionamide, and rifabutine. Additionally, PT12 and PT20 are also resistant to drugs such as PZA and ETH, and PT12 presents additional resistance to amikacin and capreomycin. Considering the PT2 strain, both molecules maintained similar MIC results, demonstrating that 6p and 6z possibly do not share the same drug resistance mechanism of INH, RIF, streptomycin, ethionamide, and rifabutine. Unlike compound 6p, the MIC value presented by 1H-benzo[d]imidazole 6z was 4-fold lower against the PT12 strain compared to that observed for the *M. tuberculosis* H37Rv strain (Table 2). This finding infers that structure 6z may share resistance mechanisms with one or more drugs between PZA, ETH, amikacin, and capreomycin. Interestingly, the PT20 strain showed resistance to inhibitory action promoted by compounds 6p and 6z. These molecules were 4- and 8-fold less active against PT20, respectively, when compared to MIC values against the *M. tuberculosis* H37Rv strain. These data suggest that 1H-benzo[d]imidazole 6p may share the resistance mechanism with the first-line drugs PZA and/or ETH. It is noteworthy that both molecules (6p and 6z) were active against all multidrug-resistant strains tested, with MIC values lower than those of the first-line drugs INH and RIF (Table 2). Finally, the presence of the methoxy group seems to increase the spectrum of action of compound 6p, considering the assayed strains. However, more studies are needed to clarify the resistance mechanism, action mechanism and spectrum of activity of synthesized 1H-benzo[d]imidazoles.

In order to assess selectivity, the basic principle of pharmacology, and the cytotoxicity of nitrated derivatives 6p and 6z, the viability of the Vero and HepG2 cells in the presence of these molecules was determined. Although compounds containing nitro groups attached to aromatic systems have been associated with toxicity *per se*, exposing the HepG2 and Vero cell lineages to 6p and 6z for 72 h did not significantly affect the cell viability (Table 2). Cellular viability was determined after incubation with the test compounds at 1, 5 and 20 µM, using the MTT method and neutral red uptake assay. While MTT determines mitochondrial activity, neutral red assesses the lysosomal viability of the cells. The results suggest a possible low toxicity of the compounds to mammalian cells, and a likely high degree of selectivity for *Mtb*.

Solubility and stability have been described as important properties in early drug discovery. Thus, 1H-benzo[d]imidazoles 6p and 6z were evaluated for their solubilities in PBS (pH 7.4) and 0.1 mol L$^{-1}$ HCl (pH 1.0) (Table 3). The solubility of these compounds was markedly higher at pH 1.0 than in aqueous solution at pH 7.4. The compounds 6p and 6z exhibited solubilities of 26.9 ± 0.6 µM and 11.2 ± 1.2 µM at pH 7.4, respectively. The solubility was improved at pH 1.0, with 324 ± 39 µM for compound 6p and 339 ± 6 µM for its analogue 6z. These observed values are higher than 100 µM, which has been described as a cutoff in early drug discovery programs. In addition, the plasma stability of 3,5-dinitro compounds 6p and 6z was also determined. After incubation for 3 h at 37 °C, the evaluated molecules showed higher than 95% of their initial concentrations (Table 3). This stability is consistent with the assessment of activity of these structures in TB animal models.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solubility</th>
<th>Plasma stability* / %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS / µM</td>
<td>0.1 mol L$^{-1}$ HCl / µM</td>
</tr>
<tr>
<td>6p</td>
<td>26.9 ± 0.6</td>
<td>324 ± 39</td>
</tr>
<tr>
<td>6z</td>
<td>11.2 ± 1.2</td>
<td>339 ± 6</td>
</tr>
</tbody>
</table>

*Phosphate-buffered saline; *percentage of remaining compound after 3 h at 37 °C.

### Table 2. Selected 2-(benzythio)-1H-benzo[d]imidazoles, ClogP values, in vitro activities against *M. tuberculosis* H37Rv and multidrug-resistant *M. tuberculosis* strains, and evaluation of the viability of Vero and HepG2 cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>ClogP*</th>
<th>MIC H37Rv / (µg mL$^{-1}$)</th>
<th>MIC 6z / (µg mL$^{-1}$)</th>
<th>MIC PT2 / µM</th>
<th>MIC PT12 / µM</th>
<th>MIC PT20 / µM</th>
<th>CC_{50} Vero / (µg mL$^{-1}$)</th>
<th>CC_{50} HepG2 / µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>6p</td>
<td>4.06</td>
<td>2.5</td>
<td>6.9</td>
<td>2.5</td>
<td>6.9</td>
<td>10</td>
<td>&gt; 7.2</td>
<td>&gt; 20</td>
</tr>
<tr>
<td>6z</td>
<td>3.76</td>
<td>1.25</td>
<td>3.8</td>
<td>2.5</td>
<td>7.6</td>
<td>5</td>
<td>&gt; 6.6</td>
<td>&gt; 6.6</td>
</tr>
<tr>
<td>INH</td>
<td>-0.67</td>
<td>0.3</td>
<td>2.3</td>
<td>40</td>
<td>291.7</td>
<td>20</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RIF</td>
<td>3.71</td>
<td>0.08</td>
<td>0.1</td>
<td>40</td>
<td>291.7</td>
<td>20</td>
<td>&gt; 20</td>
<td>&gt; 20</td>
</tr>
</tbody>
</table>

*Logarithm of partition coefficient calculated by ChemBioDraw Ultra, version 13.0.0.3015.

*Bold* indicates multidrug-resistant clinical isolates of *M. tuberculosis*; *the toxicity and selectivity of the compounds was studied on HepG2 and Vero cells. The 50% cytotoxic concentration (CC_{50}) determined by MTT and neutral red assays. Vero are kidney epithelial cells from African green monkey (Cercopithecus aethiops). HepG2 are lineage of cells isolated from human hepatocyte carcinoma. INH: isoniazid; RIF: rifampicin.
Conclusions

In summary, herein the synthesis of new series of 2-(benzylthio)-1H-benzo[d]imidazoles and their in vitro antitubercular activities have been shown. The compounds were obtained in reasonable yields and high purity, through a simple and easy-to-perform, one-step synthetic approach, from accessible reactants and reagents. In addition, some compounds showed activity against wild-type Mtb, and the leading compounds showed activity against multidrug-resistant Mtb strains with no apparent cytotoxicity to mammalian cells. Finally, leading compounds have shown good aqueous solubility and high plasma stability. These results suggest that this class of compounds may provide candidates for the future development of new therapeutic alternatives for the treatment of tuberculosis.

Supplementary Information

Supplementary information (1H NMR spectra for all compounds) is available free of charge at http://jbcs.sbq.org.br as PDF file.

Acknowledgments

This work was supported by CNPq/FAPERGS/CAPES/BNDES, Brazil (grant numbers: 421703-2017-2/17-1265-8/14.2.0914.1) to C. V. B., L. A. B., and P. M. In addition, this study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil (CAPES), finance code 001. C. V. B., L. A. B., and P. M. are Research Career Awardees of the National Research Council of Brazil (CNPq). The fellowships from CNPq (R. S. R., E.C.W., B. L. A., A. S. D., and N. S.) and CAPES (M. D. S.) are also acknowledged. Finally, we are grateful to Dr Miguel Viveiros from the Universidade Nova de Lisboa (Lisbon, Portugal) for providing the clinical isolates of M. tuberculosis used in this study.

Author Contributions

RSR was responsible for investigation, conceptualization, writing original draft; ECW for investigation; BLA for investigation; MDS for investigation; ASD for investigation; NS for investigation; CVB for conceptualization, project administration; LAB for conceptualization, project administration; PM for conceptualization, project administration; writing-review and editing.

References


19. *GraphPad Prism*, version 5.00; GraphPad Software, Inc., USA, 2007.


