BIOLOGICAL EVALUATION OF NEW 2-PHENETHYLBENZOYL THIOUREA DERIVATIVES AS ANTITUBERCULOSIS AGENTS

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Abstract

In the search to find new therapeutically solutions for the tuberculosis treatment, we designed and synthesized several thiourea derivatives. We evaluated the effect on various strains of Mycobacterium tuberculosis and the acute toxicity on mice. We also performed an in silico molecular docking study in order to analyse possible interactions with certain bacterial molecular targets. All tested compounds showed a good tuberculostatic effect, no acute toxicity in mice and optimal predicted protein-ligand complex conformations, being promising lead molecules for the development of future thiourea derivatives.

Keywords: tuberculostatic, isoxyl analogues, bacterial targets, in silico, molecular docking, protein-ligand conformations

Introduction

Tuberculosis and the proliferation of multidrug-resistant tuberculosis strains represent a major public health concern, making the development of new effective antituberculous drugs a high priority [13]. Mycolic acids are key components of the mycobacterial cell wall and very important for the bacteria growth, survival and pathogenicity. Mycolic acids biosynthesis is therefore the focus of the investigation for the development of new antituberculosis agents [12]. Several antitubercular drugs, such as isoniazid, ethionamide and isoxyl inhibit the synthesis of the bacterial protective layer of mycolic acid [2, 11]. Thioacetazone and its newer and more potent derivative, SRI-224, contain like isoxyl the same pharmacophore, the thiourea moiety and all inhibit the synthesis of mycolic acid [4]. Based on our team expertise on developing thiourea derivatives with antimicrobial activity against pathogenic bacteria [5, 6, 9] and using the structural pattern of the isoxyl molecule [3], we designed and synthesized several potential antituberculosis phenethylbenzoyl thiourea derivatives as inhibitors of the mycolic acid synthesis [7, 8]. The phenethyl moiety was used as a bioisoster of the isopentyl group and the halogens were added to provide a better lipophilicity and to enhance the antimicrobial potential. In Figure 1 is presented the design of the new compounds (1-3) based on the structures of isoxyl and SRI-224. The objective of this research is the antituberculosis study of the newly synthesized compounds in order to develop efficient remedies for the treatment of multidrug-resistant tuberculosis.
Materials and Methods

Antituberculosis evaluation

This study was focused on the antituberculosis evaluation of some new N-(2-phenethylbenzoyl)-thiourea derivatives. The details of the synthesis methods are described in previous papers [7, 8]. The compounds were screened for their in vitro antimycobacterial activity against Mycobacterium tuberculosis strain H37Rv and clinical isolated strains obtained from patients with pulmonary tuberculosis. The strains isolated from clinical samples were multi-drug resistant and were not genetically identified. Test compounds were dissolved in dimethyl sulfoxide to make stock solutions that were diluted with sterile water to obtain solutions of 30 µg/mL, 10 µg/mL, 7 µg/mL and 4 µg/mL. The antimycobacterial activity was estimated using two different media, one being the conventional Löwenstein-Jensen solid medium, and the other one liquid medium represented by BACTEC™ MGIT™ Para TB Medium, a patented medium used in the BACTEC™ MGIT™ 960 Mycobacterial Detection System, a mycobacterium testing instrument produced by BD Diagnostic Systems (USA). Both media were inoculated with 0.2 mL suspension containing $10^5$ CFU/mL bacteria [9]. The mycobacteria were exposed for 1, 4 and 24 hours to each concentration of the N-(2-phenethylbenzoyl)-thiourea derivatives and afterwards the suspension was centrifuged and the supernatant removed. The results were measured after 2 months of incubation at 37°C and are presented as (+) if no bacterial growth was detected and as (-) if the compounds had no significant effect of the mycobacteria growth. As control, a 0.1% dimethyl sulfoxide solution was used.

Acute oral toxicity

Acute oral toxicity was evaluated on mice, using the “up & down” method, in accordance with European Guidelines regarding the ethics of the experimental research on animals. These guidelines specify that chemicals with presumed low toxicity can be tested using a limit test dose of 2000 mg/kg [1]. Mice weighing 31 ± 5 g were provided by the rodent farm of “Carol Davila” University of Medicine and Pharmacy, Bucharest, Romania. The animals were housed in Plexiglas cages with sawdust litter. Water and food were supplied ad libitum. The temperature and relative humidity were continuously monitored using an electronic hygrothermometer. The temperature was between 21 - 23°C and the relative humidity was generally maintained at 40 - 60%. The lighting schedule was 12 hours light/dark cycle. Prior to administration, animals fasted for 12 hours. Three groups of five mice received the compounds in a dose of 2000 mg/kg b.w. per os. The control group received distilled water per os, in the same volume as the treated groups.

The following parameters were observed for 14 days: lethality, body weight determined every other day, motor behaviour, external stimulus reactions, palpebral ptosis.

Research was carried out in accordance with the Directive 2010/63/UE, regarding the protection of animals used for experimental and other scientific purposes. All experimental procedures were approved by the Ethical Committee of the Faculty of Pharmacy, Bucharest, Romania.

Statistical analysis

Results were analysed using GraphPad Prism 5 for Windows (GraphPad Software - San Diego, California, SUA, www.graphpad.com).

Data were presented as mean values of 5 animals per group ± SD (standard deviation). The type of distribution in the groups was established with the Kolmogorov-Smirnov test. T Student test was used for assessing significance between treated groups vs. control group.

The results were considered statistically significant when p < 0.05.

Molecular docking

In silico studies were performed in order to analyse possible interactions between 2-phenethylbenzoyl thiourea derivatives and potential molecular targets which are essential for M. tuberculosis viability.
Three-dimensional crystal structure files of InhA (PDB ID: 4DRE), MabA (PDB ID: 1UZN) and PanK (PDB ID: 3AF3) were retrieved from RCSB Protein Data Bank (https://www.rcsb.org/pdb/home/home.do) and the complexed experimental ligands, water molecules and hetero atoms were removed using UCSF Chimera 1.11.2 software (University of California, San Francisco, CA, USA). All polar hydrogens were added to the proteins and Gasteiger partial charges were computed using AutoDock Tools 1.5.6 graphical user interface (Scripps Research Institute, San Diego, CA, USA) [14].

The compounds were prepared with Open Babel 2.3.2 [15] in order to generate 3D structures, adding polar hydrogen atoms, and converting the files in the adequate format required for the docking process, while AutoDock Tools was used to calculate Gasteiger charges. The experimental ligand structures which served as control compounds for the present docking study were retrieved from the PDB files of the target proteins (1,4-dihydronicotinamide adenine dinucleotide for InhA, nicotinamide adenine dinucleotide phosphate for MabA, phosphomethylphosphonic acid guanylate ester and pantothenoic acid for PanK).

In order to perform the molecular docking experiment, the active binding sites of the proteins were identified by analysing the crystal structures of protein targets in complexes with the experimental ligands [16-18] and the grid box was set by including the active site residues. Grid parameters were calculated using AutoGrid 4.0 and the flexible ligand docking process was employed using AutoDock Vina 1.1.2 (Scripps Research Institute, San Diego, CA, USA) [19], a docking platform which generates nine ligand poses and displays the predicted free binding energy (ΔG) and root-mean-square-deviation (RMSD) for each conformation. A lower relative free binding energy indicates a higher binding affinity to the protein.

Both programs have been run using the free AutoDock Tools graphical user interface.

The most optimal protein-ligand complex conformation was chosen using Discovery Studio® Visualizer (Accelrys Software Inc., San Diego, CA, USA), a software that can be used to visualize various protein-ligand interactions and to build two-dimensional interaction diagrams.

**Results and Discussion**

In our previous research we obtained N-(2-phenethylbenzoyl)-N-(3,5-dichlorophenyl)-thiourea (1), N-(2-phenethylbenzoyl)-N-(3,4,5-trifluorophenyl)-thiourea (2) and N-(2-phenethylbenzoyl)-N-(2,4,6-trifluorophenyl)-thiourea (3), compounds that were analysed in vitro on *Mycobacterium tuberculosis* species in order to develop new antituberculosis agents.

![Chemical structure of the new compounds (1-3)](image)

The results of the experiments on solid medium are presented in Table I and they indicate a tuberculostatic effect for all tested compounds in concentrations of 10 µg/mL and higher. The effect was independent of the contact time.

<table>
<thead>
<tr>
<th>compound</th>
<th>H37Rv strain (µg/mL)</th>
<th>clinical isolated strain (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
<td>7</td>
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<tr>
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<tr>
<td>2</td>
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<tr>
<td>3</td>
<td>-</td>
<td>-</td>
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<tr>
<td>control</td>
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The tuberculocidal effect of the new compounds was determined using liquid medium for the bacteria growth and the results are presented in Table II. None of the compounds presented any bactericidal effect at the tested concentrations.
The analysis of the results revealed for all compounds a tuberculostatic effects with a minimum inhibitory concentration of 10 µg/mL on both standard and clinical isolated strains of *Mycobacterium tuberculosis*. The position and the nature of the halogens seem to have little importance on the tuberculostatic effect. The acute toxicity research showed no lethal effect of the three compounds at the dose of 2000 mg/kg b.w. We concluded that *per os*, the lethal dose is probably much higher and for bioethical reasons, we did not conduct our research at higher doses.

The body weight was determined every other day for fourteen days. The evolution of body weight was similar between treated mice and control group. The initial and final median values are presented in Table III and the body weight variation in the experiment is showed in Figure 3.

### Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>H37Rv strain (µg/mL)</th>
<th>clinical isolated strain (µg/mL)</th>
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<tr>
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<td>control</td>
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</table>

### Table II

Bactericidal effect assay of the new thiourea derivatives

Table III

<table>
<thead>
<tr>
<th>Body weight (g)</th>
<th>Group</th>
<th>Control</th>
<th>Compound 1</th>
<th>Compound 2</th>
<th>Compound 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial (i) ± SD</td>
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<td>33.8 ± 0.37</td>
<td>34.6 ± 0.34</td>
<td>33.5 ± 0.41</td>
<td>34.2 ± 0.39</td>
</tr>
<tr>
<td>Final (f) ± SD</td>
<td></td>
<td>35.7 ± 0.45</td>
<td>36.4 ± 0.45</td>
<td>35.6 ± 0.32</td>
<td>36.1 ± 0.45</td>
</tr>
<tr>
<td>Δ (f-i)</td>
<td></td>
<td>+1.9</td>
<td>+1.8</td>
<td>+2.1</td>
<td>+1.9</td>
</tr>
<tr>
<td>t Student</td>
<td></td>
<td>p &gt; 0.05</td>
<td>p &gt; 0.05</td>
<td>p &gt; 0.05</td>
<td>p &gt; 0.05</td>
</tr>
</tbody>
</table>

**Figure 3.** Evolution of body weight after acute administration

There was no difference between treated mice and control group regarding all the other monitored parameters. The motor behaviour was similar; the response to the external auditory and tactile stimuli was present. No animal manifested palpebral ptosis.

**Molecular docking**

An *in silico* experiment was conducted in an attempt to correlate the tuberculostatic activity of phenethylbenzoyl thiourea compounds with possible competitive inhibitory effects on several bacterial molecular targets. For this purpose, we applied a computational docking algorithm in order to predict the relative binding affinities and interactions between our ligands and *M. tuberculosis* enoyl acyl carrier protein reductase (InhA), β-ketoacyl acyl carrier protein reductase (MabA), two enzymes which participate in the mycolic acid biosynthetic pathway [20, 21], and pantothenate kinase (PanK), respectively, which is essential for coenzyme A biosynthesis [22]. Predicted relative free binding energy of the most optimal pose for each docked ligand are shown in Table IV, based on which it can be estimated the inhibitory constant (Ki). Hydrogen bond interactions and the identified active site residues are shown in Table V. The residues that form hydrogen bonds with both control ligands and phenethylbenzoyl thiourea compounds are bolded.
Judging by the simulated ligand-protein interactions, the structure-activity relationship of docked phenethylbenzoyl thiourea derivatives can be attributed to the ability of 2-phenethylbenzoyl thiourea moiety to act as a pharmacophore, thus enabling hydrogen bonding with the protein through the sulphur and nitrogen atoms that constitute the thiourea substructure, while the phenethylbenzoyl radical binds into the active site pocket through van der Waals, hydrophobic and various types of \(\pi\) interactions and by accepting hydrogen bonds at the oxygen atom. Moreover, the fluorine substituents on the phenyl radical fulfil the role of accessory subunits, hence increasing the potency of the compounds by participating in halogen and hydrogen bonding with the target protein.

Regarding computed interactions between the docked ligands and InhA, compounds 2 and 3 showed identical binding energies (-9.9 Kcal/mol) and calculated inhibitory constants of nanomolar order of magnitude. Both ligands form stronger interactions through fluorine atoms (Figure 5, Figure 6), while compound 1 participates in weaker alkyl and \(\pi\)-alkyl interactions through chlorine atoms (Figure 4), thus having a lower binding affinity. The phenethylbenzoyl subunits stabilize the protein-ligand complex by forming alkyl, \(\pi\)-alkyl, \(\pi\)-\(\pi\) and \(\pi\)-\(\sigma\) interactions. All 3 compounds and 1,4-dihydronicotinamide adenine dinucleotide (NADH) form hydrogen bonds with SER94. Ligands 2 and 3 also bind to GLY14, a residue which is implicated in the interaction of InhA and the experimental ligand, as shown in the retrieved crystal structure. However, compound 3 shows an unfavourable donor-donor interaction.
Figure 4.
(A) 3D conformation of docked InhA-compound 1 complex. (B) 2D diagram of InhA-compound 1 predicted binding interactions.

Figure 5.
(A) 3D conformation of docked InhA-compound 2 complex. (B) 2D diagram of InhA-compound 2 predicted binding interactions.

Figure 6.
(A) 3D conformation of docked InhA-compound 3 complex. (B) 2D diagram of InhA-compound 3 predicted binding interactions.
Figure 7.
(A) 3D conformation of docked MabA-compound 1 complex. (B) 2D diagram of MabA-compound 1 predicted binding interactions.

Figure 8.
(A) 3D conformation of docked MabA-compound 2 complex. (B) 2D diagram of MabA-compound 2 predicted binding interactions.

Figure 9.
(A) 3D conformation of docked MabA-compound 3 complex. (B) 2D diagram of MabA-compound 3 predicted binding interactions.
Compound 2 exhibited the highest binding affinity to MabA ($\Delta G = -8.3$ Kcal/mol), due to its ability to form 7 hydrogen bonds, one carbon-hydrogen bond and 2 fluorne bonds, although it is also implicated in an unfavourable positive-positive interaction (Figure 8). Hydrogen binding residues GLY22, ARG25, ARG47, VAL62, GLY90 coincide with predicted NADP (adenine dinucleotide phosphate) interactions, while ARG25 and VAL62 are also found as binding residues in the crystal structure of the experimental protein-ligand complex. Since compound 2 showed a lower free binding energy than compound 3, in this case it can be stated that the difference in the substitution positions of the phenyl radical influenced the binding affinity to the protein. Furthermore, compound 1 showed a slightly lower free binding energy of the protein-ligand complex than compound 3, yet it also suffers from an unfavourable positive-positive interaction (Figure 7). Interactions between compound 3 and MabA are displayed in Figure 9.

Predicted protein-ligand interactions revealed compound 3 as the most potent PanK inhibitor ($\Delta G = -8.4$ Kcal/mol). The docked complex showed the formation of 4 hydrogen bonds, 1 carbon hydrogen bond and 1 fluorne bond (Figure 12). Hydrogen bond interactions with residues TYR235, ARG238, ASN277 were found to participate in the binding of pantothenate and phosphomethylphosphonic acid guanylate ester (GMPPCP) to PanK. TYR235 is also present as an interacting residue in the PanK-pantothente retrieved crystal structure. Protein-ligand interactions between PanK and compounds 1 and 2 are presented in Figure 10 and Figure 11, respectively.

![Figure 10](image1.png)

**Figure 10.**
(A) 3D conformation of docked PanK-compound 1 complex. (B) 2D diagram of PanK-compound 1 predicted binding interactions.

![Figure 11](image2.png)

**Figure 11.**
(A) 3D conformation of docked PanK-compound 2 complex. (B) 2D diagram of PanK-compound 2 predicted binding interactions.
The 2-phenethylbenzoyl thiourea derivatives showed the highest predicted inhibitory potency on InhA. Overall, protein-ligand computed interactions highlighted that compound 2 might be the better candidate as an efficient and potent tuberculostatic agent.

Conclusions

In this study we tested several N-(2-phenethylbenzoyl)-thiourea derivatives on both standard and clinical isolated strains of *Mycobacterium tuberculosis* and the minimum inhibitory concentrations were determined. The compounds showed no sign of toxicity in acute experiment. The experimental data demonstrated that the designed compounds are promising compounds for the development of novel agents for the treatment of tuberculosis. Furthermore, the molecular docking study showed promising data regarding predicted protein-ligand interactions between the tested compounds and three essential bacterial molecular targets, suggesting competitive inhibition of InhA, MabA and PanK as possible bacteriostatic mechanisms. However, further *in vitro* studies are needed to confirm the obtained *in silico* results.

Acknowledgement

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