Synthesis and antituberculosis activity of N'-(2-(5-((theophylline-7'-yl) methyl)-4-4H-1,2,4-triazole-3-thio)acetyl)isonicotinohydrazides

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The paper shows the results of clinical, pathological and histological studies of tuberculosis inflammation and non-specific changes in guinea pigs organs in the experimental model of tuberculosis during the comparative isoniazid and GKP-305 (N'-(2-((theophylline-7'-yl)methyl)-4-ethyl-1,2,4-triazole-3-thio)acetyl)-isonicotinohydrazide) treatment. The optimum location for the GKP-305 injection is found.

The aim of the study was to study the tuberculostatic activity of GKP-305 in vivo experiment and to evaluate its possible application in the treatment of experimental tuberculosis infection caused by Mycobacterium bovis (M. bovis).

Materials and methods. We used first time synthesized N'-(2-(5-((theophylline-7'-yl)methyl)-4-ethyl-1,2,4-triazole-3-thio)acetyl)isonicotinohydrazide. 18 small guinea pigs with an average weight of 250 g were used for the experiment. Six groups of 3 animals were formed in each. The test substances were administered as follows: the 1st group – isoniazid at a dose of 10 mg/kg of animal weight per os; the 2nd group is isoniazid at a dose of 10 mg/kg of animal weight sub cuten; the 3rd group – GKP-305 at a dose of 10 mg/kg of animal weight per os; the 4th group – GKP-305 at a dose of 10 mg/kg of animal weight sub cuten; the 5-th and 6-th groups are control. The duration of treatment was 90 days. Infection of animals was carried out by subcutaneous administration of M. bovis 100 passage at a dose of 0.01 mg wet weight in a volume of 0.5 cm³ physiological saline solution of sodium chloride. When performing the autopsy, macroscopic tuberculosis lesions were assessed in conventional units (c. u.) for each individual Cavia porcellus. For histological examination, the lymph nodes, pieces of spleen, liver, lungs, as well as the kidney, were taken from each mumps in regional guinea pigs and placed in 10 % formalin solution. Pathoanatomical dissection was performed by the method of complete evisceration according to G. V. Shor. Pathohistological studies were performed by staining with hematoxylin and eosin. The study of blood biochemical parameters was carried out with the help of the photometers.

Results. Positive results were obtained using the agent GKP-305 as only 1 % solution used internally affects tuberculostatically.

Conclusions. It has been established that subcutaneous administration of GKP-305 at a dose of 10 mg/kg of animal weight leads to the absence of specific and nonspecific manifestations of inflammation in the lungs, liver, kidneys and spleen.

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Primary resistance appears when a person gets infected with a drug-resistant strain of tuberculosis. The man who has no drug resistance during the treatment may develop secondary (acquired) resistance. It may occur because of improper treatment or failure to maintain prescribed regime accurately or taking substandard medicine. Resistant tuberculosis is a serious public health problem in many developing countries [1,2]. The treatment of the tuberculosis takes longer and requires more expensive drugs. Multidrug resistant tuberculosis (MDR-TB) is TB, which does not affect by the two most effective drugs: rifampicin and isoniazid.

The problem of side effects of xenobiotics and countering its toxic manifestation is extremely important. The problem of treatment of tuberculosis patients has significant scientific and social importance in the global epidemic of this disease in the world, including Ukraine [4,7,8]. Antibacterial drugs, paints, acids and other chemicals are factors of Mycobacterium tuberculosis variability, inducing pigments in cultures of microorganisms, the reduction of sticks with the bacterial wall defect (L-shaped), the accelerated formation of granular forms, acid resistance loss [5,6]. The work presents the urgent issues of comparative effectiveness of isoniazid and GKP-305 treatment of TB patients in a laboratory model (guinea pigs infected with pathogenic strains of Mycobacterium tuberculosis).

**Purpose of the work**

To study the modelling process and the features of tuberculosis in guinea pigs for the use of its results in further medical experiments and research practice.

**Material and research methods**

The strategy of the synthesis of all target products of the reaction was based on the use of theophylline as starting material. To obtain the intermediate thiol we used the esterification reaction of nucleophilic substitution, hydrazinolysis and intermolecular alkaline heterocyclization [3]. The esters of 2-(5-(theophyllin-7'-yl)methyl)-3-(theophylline-7'-yl)methyl)-4-R-1,2,4-triazole-3-ylthio]acetic acid (1-3) were obtained by two methods [3]. N'-2-(5-((theophyllin-7'-yl)methyl)-4-R-1,2,4-triazole-3-ylthio)acetyl)-isonicotinohydrazide (4–6) is obtained by interaction of methyl ester (2-(5-((theophyllin-7'-yl)methyl)-4-R-1,2,4-triazole-3-ylthio)acetic acid (R = CH₃, C₂H₅, C₃H₇) with hydrazide isonicotic acid in environment of propan-1-ol. The study of physical-chemical properties of the obtained compounds was carried out using methods listed in the State Pharmacopoeia of Ukraine. The melting point was determined using capillary method on Stanford Research Systems Melting Point Apparatus 100 (SRS, USA). The structure of the compounds was confirmed with elemental analysis on Elemental Vario EL cube (Elementar Analysensysteme, Germany), IR spectra (4000–400 cm⁻¹) were taken off the module ALPHA-T of Bruker ALPHA FT-IR spectrometer (Bruker optics, Germany). Chromatograph-mass-spectral studies were carried out on the instrument Agilent 1260 Series LC/MSD System, method of ionization – electrospray (ESI).

Research is performed in the laboratory of Histology, Immunocytochemistry and Pathomorphology of Scientific Research Center of Biosafety and Environmental Resources Control in agro-industrial complex of Dnipro State Agrarian and Economic University (DSEAU), in educational and scientific laboratory of epizootology and infection process research of tuberculosis and mycobacterioses on DSEAU animals. For the experiment, 18 guinea pigs with an average weight of 250 g were taken to form six groups of three animals each.

According to the guidelines for the diagnosis of tuberculosis of animals and poultry two guinea pigs were used for bioprocessing.

The drug was injected as follows:
- group 1: isoniazid 10 mg/kg of animal mass – a common treatment dose per orally;
- group 2: isoniazid 10 mg/kg of animal mass subcutaneously;
- group 3: GKP-305 10 mg/kg of animal mass per orally;
- group 4: GKP-305 10 mg/kg of animal mass subcutaneously.

Duration of treatment was 90 days. The control group: guinea pigs without treatment (survival test) and clinically healthy animals. The procedure of infecting animals was carried out with subcutaneous injection of M. bovis passage 100, wet weight 0.01 mg, in 0.5 cm³ volume of saline sodium chloride.

During the dissection of animals, TB macroscopic
Lesions in USD for each individual pig were estimated. Regional infected lymph nodes, pieces of spleen, liver, lung, and kidney were placed in 10 % formalin solution for histological examination of each pig.

The autopsy was performed by total evisceration method initiated by G. Shore. The material was taken immediately after examination for histopathological research carried out by hematoxylin and eosin coloration. Obtained histoagent was studied using Leica DM 1000 microscope.

In carrying out researches we used cryogenic epizootic strain *M. bovis* 100 passage, isolated from responding to PPD-tuberculin for mammal’s cow. For infecting animals we used suspension of mycobacteria 8–10 mg bacterial mass, which was removed a spatula from the surface of a dense nutrient medium and transferred to a sterile penicillin bottle with rubber stopper, which was previously weighed. Then the flacon was weight again on an analytical scale and the number of selected cultures of mycobacteria determined the difference in weight. In flack with $1\text{cm}^3$ of bacterial mass an equal amount of isotonic solution was added.

Each animal was inject with a suspension of $1\text{cm}^3$ – 1 million international units.

The study of blood chemistry values was performed using "Microlab-200" and "Vitalab Eclipse" photometers (Merck, Germany) and the software after setting reaction using “Lachema”, (Czech Republic) and “Oliveks” (Russia) diagnostic test kits (Menshikov, 1982; Alekseev, 1992; Nazarenko, 1997; Tits, 1997; Kolgarov, 1999; Marshall, 2000; Danilova, 2003).

Experimental data was processed by the software package for statistical analysis of Excel 2003 (Microsoft corp.) with integrated data analysis software add-in AtteStat. Data with continuous distribution represented as average and error average, and discretely distributed data-in the form of median and interquartile scale. The reliability of the differences between the experimental groups was assessed by the Student’s t-test (for continuously distributed data and data with a normal distribution) and Wilcoxon signed-rank test (for discretely distributed data), considering the differences reliable at $P < 0.05$.

**Results**

For analysis, we used *N*-2-(5-((theophylline-7’-yl)methyl)-4-R-4H-1,2,4-triazole-3-thio)acetylisonicotinohydrazides (4-6), which were synthesized at the department of toxicological and inorganic chemistry of Zaporizhzhia State Medical University. The structure of the labeled compounds is shown in figure 1. In the IR-spectrum of these compounds there are characteristic absorption bands of valence

**Table 1.** Characterization data of synthesized compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Melting point, °C</th>
<th>Yield, %</th>
<th>$^1$H NMR (400 MHz, DMSO-$d_6$), δ ppm</th>
<th>Elemental analysis: calculated, % [found], %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C</td>
</tr>
<tr>
<td>4</td>
<td>121-123</td>
<td>69</td>
<td>1.65 (t, 3H, CH$_3$), 3.15 (s, 3H, CH$_3$), 3.35 (s, 3H, CH$_3$), 3.85 (s, 2H, CH$_2$CH$_3$), 5.75 (s, 2H, CH$_2$), 7.50 (dd, 2H, Py), 8.32 (s, 1H, CH), 8.55 (dd, 2H, Py), 9.20 (s, 1H, NH), 8.65 (s, 1H, NH)</td>
<td>48.08</td>
</tr>
<tr>
<td>5</td>
<td>103-105</td>
<td>78</td>
<td>2.88 (s, 3H, CH$_3$), 3.05 (s, 3H, CH$_3$), 3.65 (s, 3H, CH$_3$), 3.78 (s, 2H, CH$_2$), 5.75 (s, 2H, CH$_2$), 7.55 (dd, 2H, Py), 7.93 (s, 1H, CH), 8.33 (s, 1H, NH), 8.65 (dd, 2H, Py), 9.05 (s, 1H, NH)</td>
<td>47.10</td>
</tr>
<tr>
<td>6</td>
<td>113-115</td>
<td>73</td>
<td>2.95 (s, 3H, CH$_3$), 3.12 (s, 3H, CH$_3$), 3.73 (s, 2H, CH$_3$), 5.00 (s, 2H, CH$_2$), 7.37-7.60 (m, 3H, Ph), 7.85 (dd, 2H, Py), 8.32-8.40 (m, 2H, Ph, 1H, NH), 9.03 (s, 1H, NH), 9.15 (dd, 2H, Py)</td>
<td>52.74</td>
</tr>
</tbody>
</table>

Fig. 1. The scheme of synthesis of *N*-2-(5-((theophylline-7’-yl)methyl)-4-R-4H-1,2,4-triazole-3-thio)acetylisonicotinohydrazides.
fluctuations of the NH-group of medium intensity at 3410–3335 cm⁻¹, carbonyl NHC=O-group at 1690–1675 cm⁻¹ and at 1655–1635 cm⁻¹, C=N-bond in the cycle at 1640–1610 cm⁻¹ and the C-C and C-N bonds of the pyridine ring at 1540–1435 cm⁻¹.

In the ¹H NMR spectra of compounds 4–6, the signaling of methyl groups appears in the form of singlets at 2.88–3.15 and at 3.65-3.85 ppm, in the form of a triplet at 1.65 ppm, signals of methylene groups – in the form of singlet at 3.77–3.85 and at 5.00–5.75 ppm, protons of the pyridine ring in the form of two doublets at 7.50–9.15 ppm. The NH signal is within the range of 8.32–9.65 ppm in the form of a singlet (Table 1).

As a result of infecting laboratory animals with Mycobacterium pathogenic strains guinea pigs of a control group visually demonstrated ulcer at the site of M. bovis culture injection.

Lungs, liver, kidneys and spleen were marked with significant specific inflammatory process and the evolving of Besnier-Boeck-Schaumann syndrome with granulomas in polynuclear cells. Caseous-necrotic and degenerative changes were observed.

There were no pathological changes in clinically healthy animals.

The specific inflammation centers consist mostly of epithelioid and lymphoid cells, including single giant polynuclear Langhans–Pirogov cells. In addition to this, there can be seen histiocytes and plasma cells with eccentrically placed nuclei, single mononuclear macrophages.

In liver specimen there were degenerative changes of hepatocytes, specific inflammation centers with caseous necrosis. Lymphoid and epithelioid infiltrates and giant multinuclear macrophages were found at the periphery of them. Severe degenerative changes of epithelial direct tubules. A significant TB progression in kidneys is indicated by giant multinuclear Langhans–Pirogov cells. Spleen tissue contains numerous inflammation lesions in the form of caseous necrosis.

At the periphery of lesions there are large multinuclear macrophages in Langhans-Pirogov cells, indicating severe specific inflammation. Pathological changes in animal organs infected with 100 passage M. bovis are put in the Table 2.

The data shows that animal organisms infected with M. bovis passage 100 (control group) underwent featured pathological changes – lungs demonstrated primary symptoms of pneumonia with granuloma necrosis in the center, perifocal inflammation and tubercles. Liver
modification revealed fatty degeneration, diffuse and nodular histolympohcytic infiltrates, unspecific vasculitides. Spleen tissue contained numerous foci of tuberculosis inflammation and caseous necrosis. At the periphery of lesions large multiamchaphore Pirogov–Langhans cells are found, indicating specific inflammation. Spleen was featured with tubercle (miliary tuberculosis) large foci changes, splenomegalia tuberculosis, amyloidosis, lesions in lymph nodes and kidneys. Thus, the lymph nodes are rich with the giant elongated cells of Pirogov–Langhans type and epithelial cells, typical for infectious granulomas. Kidneys showed globocellular cell infiltration, connective tissue growth zone capsules, vascular sclerosis, hyalinosis, granular and fatty degeneration tubules. Changes are characterized with nodular histolympohcytic infiltrates, glomerular infiltration and capillary epithelial necrosis.

When GKP-305 agent was used subcutaneously, it showed greater tuberculosis effect compared with isoniazid, featured with the absence of pathological changes in lungs, liver, spleen, lymph nodes and kidneys (Fig. 2).

### Discussion

In our opinion, this method results in the use of isoniazid intoxication sick animals, although discovered tuberculous effect in relation to control group (infected animals). So isoniazid treatment significantly reduced the intensity of tuberculosis lesions, but not completely eliminated, which was confirmed by the presence of small foci of tuberculous lesions in the lungs, lymph nodes and spleen. The use of isoniazid subcutaneously on animals infected M. bovis 100 passage led to permanent tuberculostatic exposure: lung, spleen, lymph nodes were found as pathologic changes characteristic to tuberculosis lesions, although found in liver fatty degeneration of hepatocytes and kidney dystrophy protein winding tubules. Positive results were obtained using the agent GKP-305 as only 1 % solution used internally affects tuberculostatically, except for liver and kidneys with imperceptible fatty hepatocyte and convoluted tubules degeneration.

When GKP-305 agent was used subcutaneously, it showed greater tuberculosis effect compared with isoniazid, featured with the absence of pathological changes in lungs, liver, spleen, lymph nodes and kidneys (Fig. 2).

### Conclusion

The examples illustrate the results of a comparative analysis of isoniazid and GKP-305 treatment based on clinical, pathological and histological studies of tuberculosis inflammations symptoms and nonspecific changes in the organs of guinea pigs with the usage of experimental model of tuberculosis. It is found that subcutaneous injection of GKP-305 at a dose of 10 mg/kg of animal mass causes the absence of specific and non-specific symptoms of inflammation in lungs, liver, kidneys and spleen.
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