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

A. S. Gotsulia[1], V. V. Zazharskyi[2], P. O. Davydenko[2]

[1]Zaporizhzhia State Medical University, Ukraine, [2]Dnipro State Agrarian and Economic University, Ukraine

Key words: tuberculosis, experimental model, treatment, 1,2,4-triazole, theophylline, isoniazid.

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E-mail: andrey.goculya@gmail.com

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-(5-((theophylline-7'-yl)methyl)-4-ethyl-4*H*-1,2,4-triazole-3-ylthio)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-4*H*-1,2,4-triazole-3-ylthio) acetyl)isonicotino-hydrazide. 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 cutem*; 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 cutem*; 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 cm^3 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.

Ключові слова: туберкульоз, експериментальна модель, лікування, 1,2,4-тріазол, теофілін, ізоніазид.

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Синтез і протитуберкульозна активність *N*'-(2-(5-((теофілін-7'-іл)метил)-4-R-4*H*-1,2,4-тріазол-3-ілтіо)ацетил)ізонікотиногідразидів

А. С. Гоцуля, В. В. Зажарський, П. О. Давиденко

Мета роботи – вивчити в експерименті *in vivo* туберкулостатичну активність ГКП-305 та оцінити можливість його застосування в лікуванні експериментальної туберкульозної інфекції, що викликана *M. bovis*.

Матеріали та методи. У дослідженнях використовували вперше синтезований *N*'-(2-(5-((теофілін-7'-іл)метил)-4-етил-4*H*-1,2,4-тріазол-3-ілтіо)-ацетил)ізонікотиногідразид. Для експерименту взяли 18 морських свинок середньою вагою 250 г. Сформували 6 груп по 3 тварини в кожній. Досліджувані речовини вводили так: 1 група – ізоніазид у дозі 10 мг/кг маси тварини *per os*; 2 – ізоніазид у дозі 10 мг/кг маси тварини *sub cutem*; 3 – ГКП-305 у дозі 10 мг/кг маси тварини *per os*; 4 група – ГКП-305 у дозі 10 мг/кг маси тварини *sub cutem*; 5 та 6 групи – контрольні. Тривалість лікування – 90 діб. Зараження тварин здійснили шляхом підшкірного введення *M. bovis* 100 пасажу в дозі 0,01 мг вологої ваги в об'ємі 0,5 $см^3$ фізіологічного розчину натрій хлориду. Під час проведення розтину тварин оцінювали макроскопічні туберкульозні ураження в умовних одиницях для кожної окремо взятої *Cavia porcellus*. Для гістологічного дослідження в кожної свинки брали реґіонарні до місця зараження лімфовузли, шматочки селезінки, печінки, легень, а також нирку та поміщали їх у 10 % розчин формаліну. Патологоанатомічний розтин виконали методом повної евісцерації за Г. В. Шором. Патогістологічні дослідження виконали, використовуючи забарвлення гематоксиліном та еозином. Вивчення біохімічних показників крові здійснили за допомогою фотометрів.

Результати. Позитивні результати отримали після застосування ГКП-305. Навіть використання 1 % розчину внутрішньо впливає туберкулостатично.

Висновки. Встановили, що підшкірне введення ГКП-305 у дозі 10 мг/кг маси тварини призводить до відсутності специфічних і неспецифічних проявів запалення в легенях, печінці, нирках, селезінці.

Ключевые слова: туберкулез, экспериментальная модель, лечение, 1,2,4-триазол, теофиллин, изониазид.

Синтез и противотуберкулезная активность *N*'-(2-(5-((теофиллин-7'-ил)метил)-4-R-4*H*-1,2,4-триазол-3-илтио)ацетил)изоникотиногидразидов

А. С. Гоцуля, В. В. Зажарский, П. А. Давиденко

Цель работы – изучить в эксперименте *in vivo* туберкулостатическую активность ГКП-305 и оценить возможность его применения в лечении экспериментальной туберкулезной инфекции, вызванной *M. bovis*.

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Материалы и методы. В исследованиях использовали впервые синтезированный *N'*-(2-(5-((теофиллин-7'-ил)метил)-4-этил-4*H*-1,2,4-триазол-3-ил-тио)ацетил)изоникотиногидразид. Для эксперимента взяли 18 морских свинок средним весом 250 г. Сформировали 6 групп по 3 животных в каждой. Исследуемые вещества вводили следующим образом: 1 группа – изониазид в дозе 10 мг/кг массы животного *per os*; 2 – изониазид в дозе 10 мг/кг массы животного *sub cutem*; 3 – ГКП-305 в дозе 10 мг/кг массы животного *per os*; 4 группа – ГКП-305 в дозе 10 мг/кг массы животного *sub cutem*; 5 и 6 группы – контрольные. Продолжительность лечения составляла 90 суток. Заражение животных проводили путем подкожного введения *M. bovis* 100 пассажа в дозе 0,01 мг влажного веса в объеме 0,5 см^3 физиологического раствора натрий хлорида. При проведении вскрытия животных оценивали макроскопические туберкулезные поражения в условных единицах (у. е.) для каждой отдельно взятой свинки. Для гистологического исследования у каждой *Cavia porcellus* принимали регионарные к месту заражения лимфоузлы, кусочки селезенки, печени, легких, а также почку и помещали их в 10 % раствор формалина. Патологоанатомическое вскрытие проводили методом полной эвисцерации по Г. В. Шору. Патогистологические исследования проводили с использованием окраски гематоксилином и эозином. Изучение биохимических показателей крови проводили с помощью фотометров.

Результаты. Положительные результаты получены после использования ГКП-305. Даже использование 1 % раствора внутренне действует туберкулостатически.

Выводы. Установлено, что подкожное введение ГКП-305 в дозе 10 мг/кг массы животного приводит к отсутствию специфических и неспецифических проявлений воспаления в легких, печени, почках и селезенке.

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 affected 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 pigment 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-((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-4*H*-1,2,4-triazol-3-ylthio) acetyl)-isonicotinohydrazide (4–6) is obtained by interaction of methyl ester (2-(5-((theophyllin-7-yl)methyl)-4-R-4*H*-1,2,4-triazole-3-ylthio) acetic acid (R = CH_3, C_2H_5, C_6H_5) with hydrazide isonicotinic 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). Chromato-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 (DSAEU), in educational and scientific laboratory of epizootology and infection process research of tuberculosis and mycobacterioses on DSAEU 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 cm^3 volume of saline sodium chloride.

During the dissection of animals, TB macroscopic

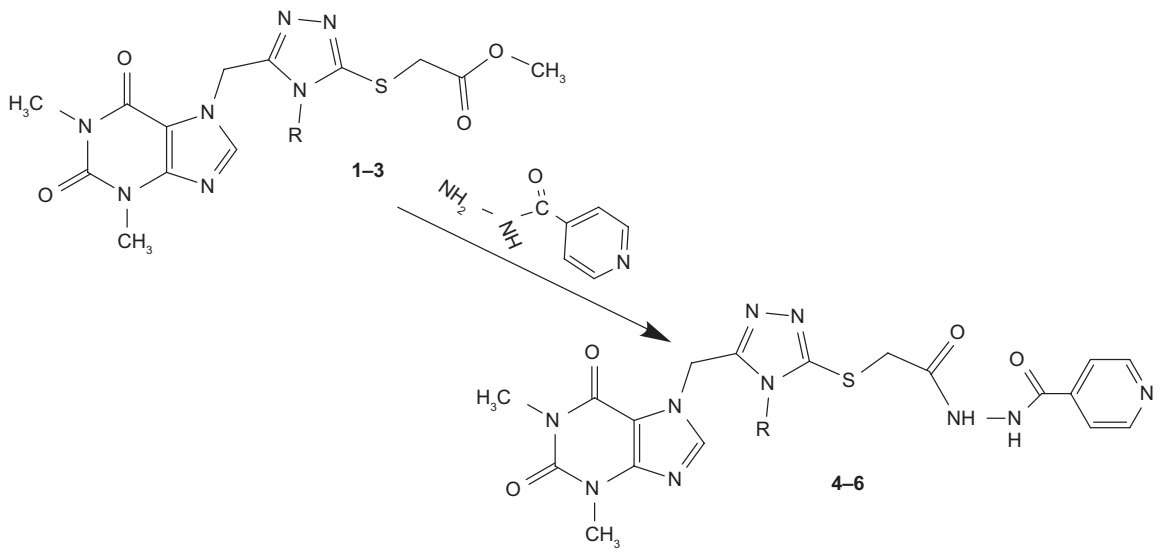

Fig. 1. The scheme of synthesis of *N*'-(2-(5-((theophylline-7'-yl)methyl)-4-R-4*H*-1,2,4-triazole-3-ylthio)acetyl)isonicotinohydrazides.

Table 1. Characterization data of synthesized compounds

Compound	Melting point, °C	Yield, %	^{1}H NMR (400 MHz, DMSO-d_6), δ ppm	Elemental analysis: calculated, % [found], %			
				C	H	N	S
4	121-123	69	1.65 (t, 3H, C*H*$_3$CH$_2$), 3.15 (s, 3H, CH$_3$), 3.35 (s, 3H, CH$_3$), 3.85 (s, 2H, CH$_3$C*H*$_2$), 5.75 (s, 2H, CH$_2$), 7.50 (dd, 2H, Py), 8.32 (s, 1H, CH), 8.65 (dd, 2H, Py), 9.20 (s, 1H, NH), 9,65 (s, 1H, NH)	48.08 [48.19]	4.46 [4.45]	28.15 [28,10]	6.42 [6,43]
5	103-105	78	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$_2$),7.65 (dd, 2H, Py), 7.93 (s, 1H, CH), 8,33(s, 1H, NH), 8.65 (dd, 2H, Py), 9.05 (s, 1H, NH)	47.10 [47.02]	4.16 [4.17]	28.91 [28,86]	6.62 [6,63]
6	113-115	73	2.95 (s, 3H, CH$_3$), 3.12 (s, 3H, CH$_3$), 3.73 (s, 2H, CH$_2$), 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)	52.74 [52.66]	4.06 [4.07]	25.63 [25,59]	5.87 [5,86]

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. The histoagent photofixation was processed with digital camera Leica DFC 295.

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 cm^3 of bacterial mass an equal amount of isotonic solution was added.

Each animal was inject with a suspension of 1cm^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 '"Olveks" (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-4*H*-1,2,4-triazole-3-ylthio)acetyl)isonicotinohydrazides (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

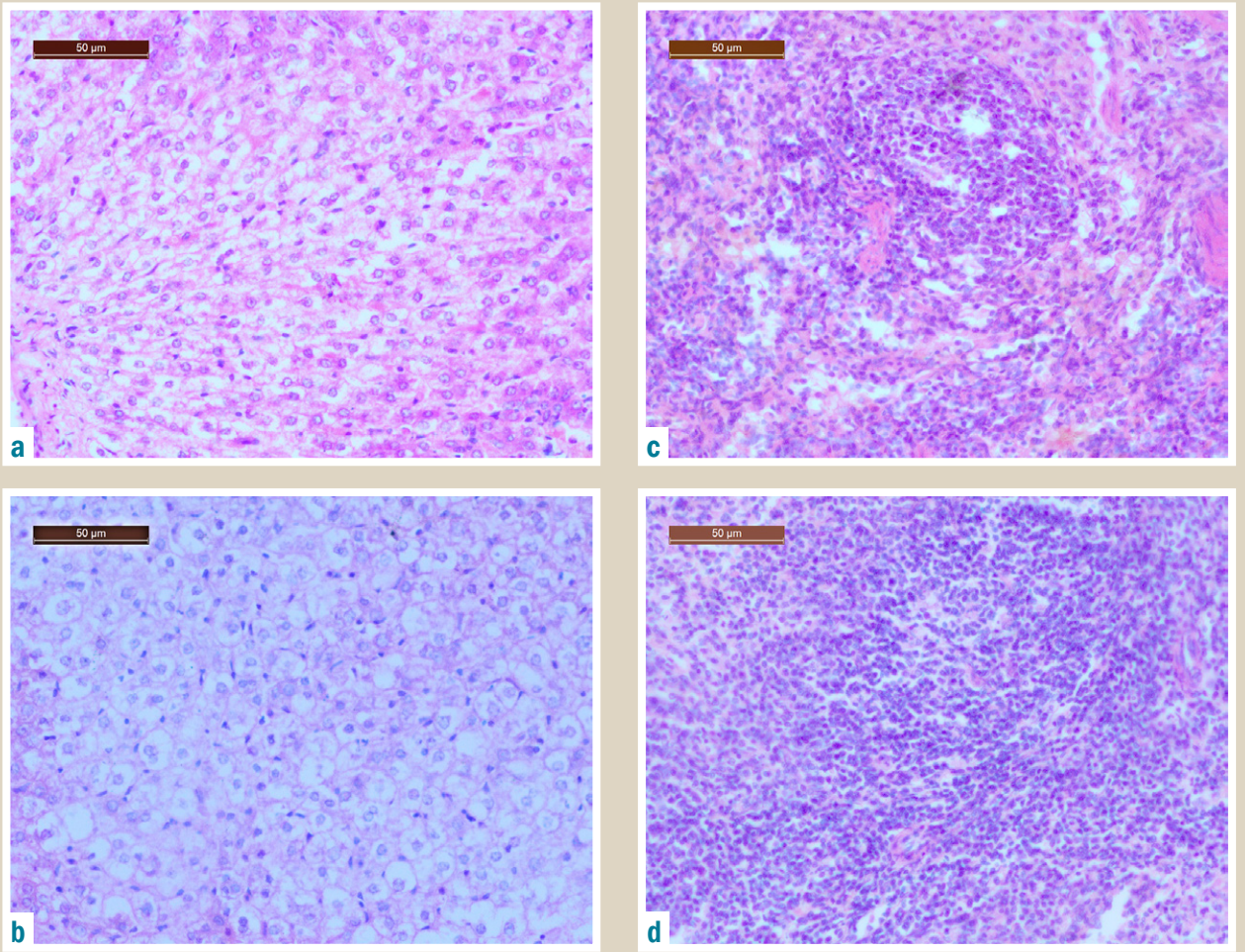

Fig. 2. **a**: dystrophic hepatocyte changes specific inflammation legions with caseous (control – infected animal); histological sections; hematoxylin-eosin, ×200; **b**: protein degeneration of hepatocytes (GKP-305 agent, internal use); histological sections; hematoxylin-eosin, ×200; **c**: zone of caseous necrosis of spleen tissue (control – infected animal); histological sections; hematoxylin-eosin, ×200; **d**: spleen of an animal (GKP-305 agent, subcutaneous use); histological sections; hematoxylin-eosin, ×200.

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 tuburcles. Liver

Table 2. Pathological changes in organs of the animals infected with *M. bovis* passage 100

Tests of taken animal organ samples	Animal group					
	Isoniazid (*per os*)	Isoniazid (*subcutem*)	GKP-305 (*per os*)	GKP-305 (*sub cutem*)	Control (infected animals)	Control (apparently healthy animals)
Lungs	Primary pneumonia cells – necrosis in the centers of granulomas, perifocal inflammation and tubercle in the area	no pathological changes	no pathological changes	no pathological changes	primary pneumonia cells – necrosis in the centers of granulomas, perifocal inflammation and tubercle in the area	no pathological changes
Liver	Fatty degeneration of hepatocytes	fatty degeneration of hepatocytes	protein degeneration of hepatocytes	no pathological changes	fatty infiltration and fatty degeneration diffuse and nodule histo-lymphocytic infiltrates, unspecific vasculitides	no pathological changes
Spleen	Minor caseous necrosis, splenomegaly	no pathological changes	no pathological changes	no pathological changes	tubercle (miliary) tuberculosis, macrofocal changes, splenomegaly tuberculosis, amyloidosis	
Lymphatic nodes	Inflammation foci with giant Langhans-Pirogov cells	no pathological changes	no pathological changes	no pathological changes	inflammation foci with giant cells of Pirogov-Langhans type and elongated form and epithelial cells, typical for infectious granulomas	no pathological changes
Kidneys	Fatty degeneration of convoluted tubules	protein degeneration of convoluted tubules	fatty degeneration of convoluted tubules	no pathological changes	globocellular infiltration lesions, connective tissue capsule growth zone, vascular kidney sclerosis hyalinosis, grainy and fatty tubule rebirth; changes featured with nodular histo-lymphocytic infiltrates, a sharp stagnation in kidneys and glomerular infiltration and epithelial capillar necrosis	no pathological changes

modification revealed fatty degeneration, diffuse and nodule histolymphocytic infiltrates, unspecific vasculitides. Spleen tissue contained numerous foci of tuberculosis inflammation and caseous necrosis. At the periphery of lesions large multimacrophage Pirogov–Langhans cells are found, indicating specific inflammation. Spleen was featured with tuburcle (miliary tuberculosis) large foci changes, splenomegaly 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 hystolymphocyte infiltrates, glomerular infiltration and capillar epithelial necrosis.

We conducted a comparative analysis of the impact of a 1 % solution of isoniazid and GKP-305 on the body of guinea pigs infected with *M. bovis* passage 100 by different methods of administration (subcutaneous and intra). As a result of observation for 90 days we found that for the internal use of isoniazid in lung cells of primary pneumonia – in the granulomas of necrosis phenomena, and around there perifokalne inflammation and tubercles; liver fatty degeneration of hepatocytes; small cells in the spleen caseous necrosis, splenomegaly. In the lymph nodes showed the presence of inflammatory foci of giant cells Pirogov–Langhans; kidney fatty convoluted tubules.

Discussion

In our opinion, this method results in the use of isoniazid intoxication sick animals, although discovered tuberculostatic effect in relation to the control group (infected animals). So isoniazid treatment significantly reduced the intensity of tuberculous 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 tuberculostatic 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.

Фінансування

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Information about authors:
Gotsulia A. S., PhD, Associate Professor, Department of Toxicology and Inorganic Chemistry, Zaporizhzhia State Medical University, Ukraine.
Zazharskyi V. V., PhD, Associate Professor, Department of Epizotology and Infectious Diseases, Dnipro State Agrarian and Economic University, Ukraine.
Davydenko P. O., PhD, Associate Professor, Department of Epizotology and Infectious Diseases, Dnipro State Agrarian and Economic University, Ukraine.

Відомості про авторів:
Гоцуля А. С., канд. фарм. наук, доцент каф. токсикологічної та неорганічної хімії, Запорізький державний медичний університет, Україна.
Зажарський В. В., канд. ветерин. наук, доцент каф. епізотології та інфекційних хвороб, Дніпровський державний аграрно-економічний університет, Україна.
Давиденко П. О., канд. ветерин. наук, доцент каф. епізотології та інфекційних хвороб, Дніпровський державний аграрно-економічний університет, Україна.

Сведения об авторах:
Гоцуля А. С., канд. фарм. наук, доцент каф. токсикологической и неорганической химии, Запорожский государственный медицинский университет, Украина.
Зажарский В. В., канд. ветерин. наук, доцент каф. эпизотологии и инфекционных болезней, Днепровский государственный аграрно-экономический университет, Украина.
Давиденко П. А., канд. ветерин. наук, доцент каф. эпизотологии и инфекционных болезней, Днепровский государственный аграрно-экономический университет, Украина.

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