Xymedone conjugate with *para*-aminobenzoic acid. Estimation of hepatoprotective properties*

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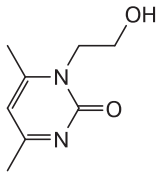
A salt-like conjugate of xymedone with *para*-aminobenzoic acid in a series of pyrimidine derivatives was synthesized, and its hepatoprotective properties were studied. The compound studied exhibits a cytoprotective effect at a concentration of 25 μmol L^{-1} enhancing the viability of normal human hepatocyte cells of the *Chang Liver* line by 2.1 times against the background of the impact of the D-galactosamine toxicant, which was shown by experiments *in vitro*. The cytotoxicity of the compound (IC_{50}) is 20.7 mmol L^{-1}. The data indicating the hepatoprotective effect most pronounced at the early stages of therapy were obtained in experiments *in vivo* performed according to the therapeutic scheme on the model of CCl_4-induced toxic hepatitis. The ability of the conjugate to exert reparative and protective effects was found, since the surface area of destructive—degenerative and necrotic injuries revealed in hematoxylin- and eosin-stained sections on the third day of intraperitoneal administration of the studied compound in a dose of 0.7 mg kg^{-1} decreased by 1.5 times. The areas of detection of lipid inclusions in frozen sections stained with Sudan black decreased by four times on the third day at a dose of 1.7 mg kg^{-1}, whereas the decrease was 3.2 times on the seventh day at a dose of 0.7 mg kg^{-1} compared to the control group of animals administrated with saline. According to the biochemical parameters, positive effects on the secretory and synthetic functions of the liver, bilirubin metabolism, and metabolism of iron and magnesium were observed during the treatment of animals with the conjugate.

Key words: pyrimidine, conjugates, hepatoprotectors, toxic hepatic injury, hepatocytes.

Hepatic diseases caused by the toxicant action become more and more significant taking into account an increase in man-caused load in the modern world and the frequent administration of various drugs by the population.[1] According to the World Health Organization data, about 2 billion people with diverse hepatic diseases were detected in 2015. Therefore, the investigation and search for potential hepatoptotectors is one of the most important tasks of the modern world.

Xymedone (1,2-dihydro-4,6-dimethyl-1-(2-hydroxyethyl)pyrimidin-2-one) (**1**) is an original domestic drug prepared in 1966 at the A. E. Arbuzov Institute of Organic and Physical Chemistry. Xymedone has a wide range of action and exerts membrane-stabilizing, regenerative, and immunostimulating, and other effects.[2,3] We showed that xymedone and its derivatives possess hepatoprotective, neuroprotective, and other properties.[4—8]



Xymedone (**1**)

Investigations on the preparation of pharmaceutical co-crystals and conjugates are being conducted presently, which makes it possible to improve bioaccessibility of drug molecules[9,10] due to the acceleration of drug dissolution

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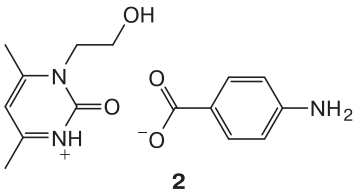
and appearance of additional biological properties.[11—13] In addition, the preparation of conjugates is used as a promising approach to the development of methods for target drug delivery to biotargets, including liver cells.[14] Therefore, for the last several years we can carrying out the works on the synthesis and study of biological properties of salt-like conjugates of compound **1** with biogenic acids representing co-crystals in the solid (undissolved) state.

para-Aminobenzoic acid was chosen for the synthesis of the conjugate due to its proliferative, anticoagulation, antioxidant, and other properties.[15—17] The conjugate was prepared in order to improve bioaccessibility and to impart new biological properties to the already known drug xymedone.[18] According to its chemical structure, conjugate **2** formed by compound **1** and *para*-aminobenzoic acid is a co-crystal in the molar ratio *para*-aminobenzoic acid : **1** = 1 : 1.



2

The ability to stimulate the regeneration of the nervous tissue of marrow upon contusion trauma was shown[19] for conjugate **2**. According to our earlier results[5,7] on the primary estimation of the hepatoprotective properties of compound **2**, this compound has the hepatoprotective properties manifested as a decrease in the activity of alanine aminotransferase (ALT) and the bilirubin concentration on the model of rats with toxic CCl_4-induced hepatitis for the prophylactic administration scheme.

In order to obtain full scientific data on the hepatoprotective properties of compound **2**, we carried out the in-depth study of its efficiency in the case of toxic CCl_4-induced hepatitis for the therapeutical administration scheme.

The purpose of this work is to investigate the hepatoprotective properties of the xymedone conjugate with *para*-aminobenzoic acid (**2**) in studies *in vitro* on the *Chang Liver* cell line and on rats *in vivo* during therapy of the toxic injury of the liver.

Results and Discussion

Cytotoxicity and cytoprotective effect of conjugate 2 in experiments *in vitro*. A series of experiments on the normal human hepatocyte *Chang Liver* cell line made it possible to determine the half-maximum inhibition dose (IC_{50}) of compound **2**, being 20.7 mmol L^{-1}, or 6.3 g L^{-1}, for the cells of this line. The cytoprotective effect of compound **2** against the induction of injuries of the *Chang Liver* cell line by D-galactosamine (D-GLA) in a concentration of 150 mmol L^{-1} was also studied. The results of the studies are presented in Fig. 1.

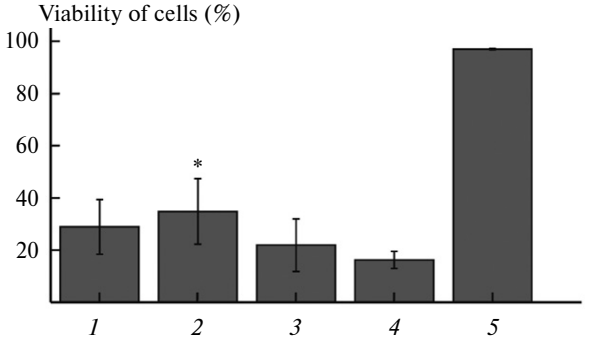


Fig. 1. Cytoprotective effect of compound **2** against the D-GLA toxic injury of the *Chang Liver* line cells: (*1—4*) 150 mmol L^{-1} D-GLA, concentration of compound **2**: 50 (*1*), 25 (*2*), and 12.5 μmol L^{-1} (*3*); (*4*) control; and (*5*) intact cells. Here and in Figs 3 and 4, arrows mark statistically significant difference from the parameters of the control group of cells (Mann—Whitney U-test, $p < 0.05$).

It follows from the obtained data that compound **2** in a concentration of 25 μmol L^{-1} exhibits the cytoprotective effect. The number of viable cells in this group increased by approximately two times compared to the control group subjected to a similar action from D-GLA.

Influence of compound 2 on the dynamics of changing the mass index of liver of rats with toxic CCl_4-induced hepatitis. The weight of the liver was measured and its mass index was determined during the experiment in the dynamics of observations for 21 days of therapy by compound **2**. The results are presented in Fig. 2.

Owing to the hepatotoxic action of CCl_4 as a result of its 5-day peroral administration, an increase in the mass

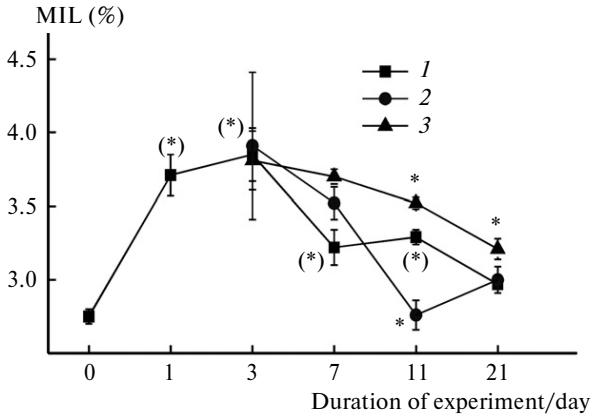


Fig. 2. Dynamics of changing the mass index of liver (MIL): *1*, control; *2* and *3*, administration of compound **2** in a dose of 0.7 and 1.7 mg kg^{-1}, respectively. Here and in Figs 5—8, arrows mark statistically significant differences with the control group ($p < 0.05$, *t*-test); asterisks in parentheses indicate the same between the control and reference (intact) groups.

index of liver (MIL) over the reference values of intact animals ("zero" day) was revealed in one day. On the seventh day and during next days after the last CCl_4 administration, the gradual reduction (decrease) of this parameter for 21 days was observed in the control group. However, the reduction of the MIL in the control group was not complete, and on the 21st day of observation the index remained enhanced exceeding the corresponding parameters of intact animals by 16.7%. These differences were statistically significant ($p < 0.05$, *t*-test).

The administration of compound **2** in a dose of 0.7 mg kg^{-1} to rats resulted in the statistically significant decrease in the liver weight over the control on the 11th day (statistically significant differences from the control by *t*-test, $p < 0.05$), but no differences with the control were observed on the 21st day (see Fig. 2).

The administration of conjugate **2** in a dose of 1.7 mg kg^{-1} did not normalize this parameter. This group exhibits an increase in the MIL on the 7th, 11th, and 21st day compared to the control group of animals administrated by a saline as a therapeutical action, and on the 11th and 21st day the increase was statistically significant ($p < 0.05$, *t*-test); *i.e.*, an increase in the dose of compound **2** decreased its hepatoprotective activity.

Influence of compound 2 on pathomorphological changes in the liver tissue against the background of CCl_4-induced injuries. The surface areas of injuries of the liver tissue were calculated during experiment in the sections stained with hematoxylin and eosin. The results are presented in Fig. 3.

It is shown that on the third day after the last administration of CCl_4 (third day of drug administration), substantial tissue injuries equal to 92.2% are detected in the hematoxylin- and eosin-stained histological sections of the liver tissue. In turn, the administration to animals of compound **2** in a dose of 0.7 mg kg^{-1} favored the protective effect, since on the third day the surface area of the injured tissue was 59.8% (statistically significant differences from control according to *t*-test, $p < 0.05$).

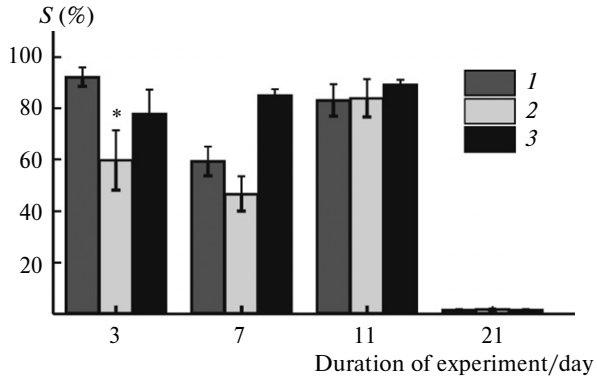


Fig. 3. Average surface areas of injuries (*S*) of the rat liver tissue after administration of compound **2** against of the background of CCl_4-induced hepatitis: *1*, control; *2* and *3*, administration of compound **2** in a dose of 0.7 and 1.7 mg kg^{-1}, respectively.

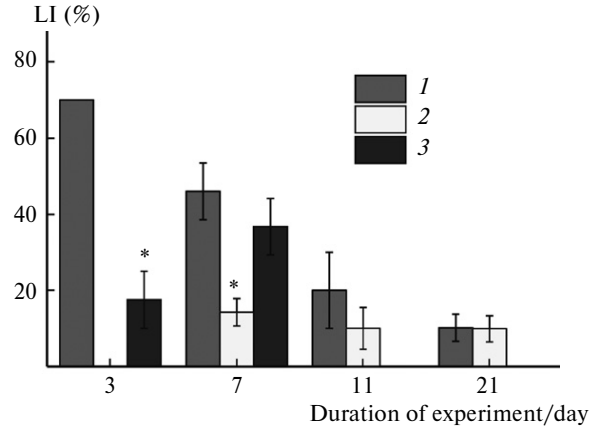


Fig. 4. Number of lipid inclusions (LI) after administration of compound **2** against of the background of CCl_4-induced injuries: *1*, control; *2* and *3*, administration of compound **2** in a dose of 0.7 and 1.7 mg kg^{-1}, respectively.

No positive effect was observed on the third day of administration when compound **2** in a dose of 1.7 mg kg^{-1} was used. No statistically significant decrease in the surface areas of the liver tissue injuries were either observed on the 7th, 11th, and 21st day using compound **2** in doses of 0.7 and 1.7 mg kg^{-1}.

Frozen sections of the tissues were prepared for some groups of animals and then were stained with Sudan black to detect lipid inclusions (LI) in hepatocytes. The results are presented in Fig. 4.

As follows from Fig. 4, on the 3rd day compound **2** (against the background of CCl_4-induced injuries) in a dose of 1.7 mg kg^{-1} favors a substantial decrease in lipid accumulation in hepatocytes (on the third day of administration the surface are of detection of hepatocytes with enhanced fatty inclusions is four times lower than that in the control group). On the seventh day, the decrease in the surface areas of lipid detection in the liver under the action of compound **2** in a dose of 0.7 mg kg^{-1} was lower by three times than that in the control group, but the decrease was not so substantial in the group to which the substance was administrated in a dose of 1.7 mg kg^{-1}. On the 11th day a decrease in the surface areas of lipid detection in the liver in the group administrated by compound **2** in a dose of 0.7 mg kg^{-1} was halved compared to the control group, but the differences between these groups were insignificant and on the 21st day the parameters in the experimental and control group were the same.

A comparison of the results obtained by analysis of the hematoxylin- and eosin-stained sections with the results of analysis of the frozen sections stained by Sudan black shows that compound **2** favors a decrease in lipid accumulation in hepatocytes to a higher extent than a decrease in necrotic injuries. The positive effect was more pronounced at the early stages of toxic injury of the liver by CCl_4 (on the 3rd and 11th day).

Influence of compound 2 on the biochemical parameters of the rat blood against the background of CCl_4-induced liver injury. One day after the last administration of CCl_4, *i.e.*, after the induction of toxic injury of the liver, the rats demonstrated pronounced changes in the biochemical parameters of blood characterizing cytolysis of hepatocytes (increase in ALT by 8.2 times, increase in aspartate aminotransferase (AST) by 4.8 times, decrease in the De Ritis ratio by two times) and dysfunction of the synthetic function of the liver (decrease in the concentration of total protein by 20%, that of albumin by 25%, that of globulins by 11%, that of glucose by 32%, and that of cholesterol by 3.75 and the activity of choline esterase by two times, as well as a decrease in the content of iron by 1.7 times and that of magnesium by two times (parameters characterizing the metabolism of mineral substances, regulating important functions in the organism, and composing specific proteins)). All changes in the biochemical markers indicated above differed statistically significantly from the zero day of experiment, *i.e.*, from the reference values of intact animals ($p < 0.05$, *t*-test). For the control group administrated with a saline instead of the treatment, the reduction to the normal level of some biochemical parameters was observed to the 21st day of therapy (26th day of experiment): cytolysis markers (ALT, AST) with an increase in the De Ritis ratio to 2.8±0.3 and some markers of the synthetic function (globulins, glucose). At the same time, the main part of markers of the synthetic function of liver (total protein, albumin, serum iron, cholesterol, choline esterase) in the control group remained decreased at the end of observation. No increase in the cholestasis markers (alkaline phosphatase, γ-glutamyl transferase (GGT), total bilirubin) was observed after CCl_4 administration. Beginning from the third day of therapy (eighth day of experiment), in the control group, on the contrary, a decrease in alkaline phosphatase, GGT, and total bilirubin was revealed, which is related to the dysfunction of the functional activity of liver cells. The level of GGT activity on the 21st day of therapy increased to 3.0 IU L^{-1} (difference from the level of intact animals is insignificant). The normalization of the level of total bilirubin was observed in the control group on the 7th day of therapy (12th day of experiment), but the concentration of the direct bilirubin fraction decreased significantly and the concentration of the toxic fraction of indirect bilirubin increased, indicating the bilirubin metabolism dysfunction, in particular, the dysfunction of the ability of hepatocytes to utilize and conjugate indirect bilirubin. Thus, no development of cholestasis was observed in this experiment in the control group of animals, but the excretory dysfunction of hepatocytes related to the inactivation of indirect bilirubin was revealed.

No substantial influence of compound **2** was revealed on a change in the markers of hepatocyte cytolysis compared to the control group in the corresponding days of experiment (Fig. 5). The De Ritis ratio was significantly lower ($p < 0.05$, *t*-test) than that in the control) only on the third day of experiment in the group administrated by compound **2** in a dose of 1.7 mg kg^{-1} (see Fig. 5, *c*).

As follows from the results of studying the markers of cholestasis liver injuries, compound **2** (in doses of 0.7 and 1.7 mg kg^{-1}) exerted no effect on the dynamics of changing the activities of GGT and alkaline phosphatase but resulted in the normalization of the bilirubin metabolism, whose parameters are presented in Fig. 6.

The data in Fig. 6 show a sharp decrease in total bilirubin and the fractions of direct and indirect bilirubin in the control group on the third day of treatment with a saline, which is an indicator of pathological dysfunctions in the organism. The level of total bilirubin in the control group normalized on the 7th day of therapy (12th day of experiment), but the concentration of direct bilirubin remained significantly decreased and that of indirect bilirubin was increased over the normal parameters. This change in the ratio of bilirubin fractions is an indicator of the excretory dysfunction of hepatocytes, or the ability to absorb and bind indirect bilirubin and isolate direct bilirubin. The full reduction of the bilirubin metabolism parameters in the control group was observed on the 11th day of therapy (16th day of experiment).

Unlike the control group, the decrease in the total bilirubin concentration due the administration of compound **2** on the third day of therapy was less pronounced (at a dose of 0.7 mg kg^{-1} the differences with the control were statistically significant at $p < 0.05$). At the same time, upon the administration of compound **2** in a dose of 0.7 mg kg^{-1}, total bilirubin on the 7th day was lower than the control: 1.7±0.3 and 2.5±0.1 mmol L^{-1}, respectively ($p < 0.05$) (see Fig. 6, *a*). According to the ratio of the concentrations of direct (see Fig. 6, *a*) and indirect bilirubin (see Fig. 6, *b*), the excretory function of hepatocytes when using compound **2** in a dose of 0.7 mg kg^{-1} improved significantly compared to the control group: on the 7th day of therapy, when the maximum changed were detected in the control group, upon the administration of compound **2** in a dose of 0.7 mg kg^{-1}, the concentrations of both bilirubin fractions were nearly the same as the normal level (zero day of experiment) but differed statistically significantly ($p < 0.05$) from the corresponding control parameters (see Fig. 6, *a*, *b*). No differences from the control group was observed for the levels of direct and indirect bilirubin on the 11th and 21st day. When compound **2** was administrated in a dose of 1.7 mg kg^{-1}, no effect of the substance on the bilirubin metabolism and no improvement of the secretory function of liver were revealed.

As said above, the induction of the toxic injury of liver resulted in a decrease in the concentration of total protein and its fractions (albumin and globulins) and a decrease in the activity of serum choline esterase. During the 21-day observation of the therapeutical effect of the drugs, the levels of total protein and albumin and activity of choline

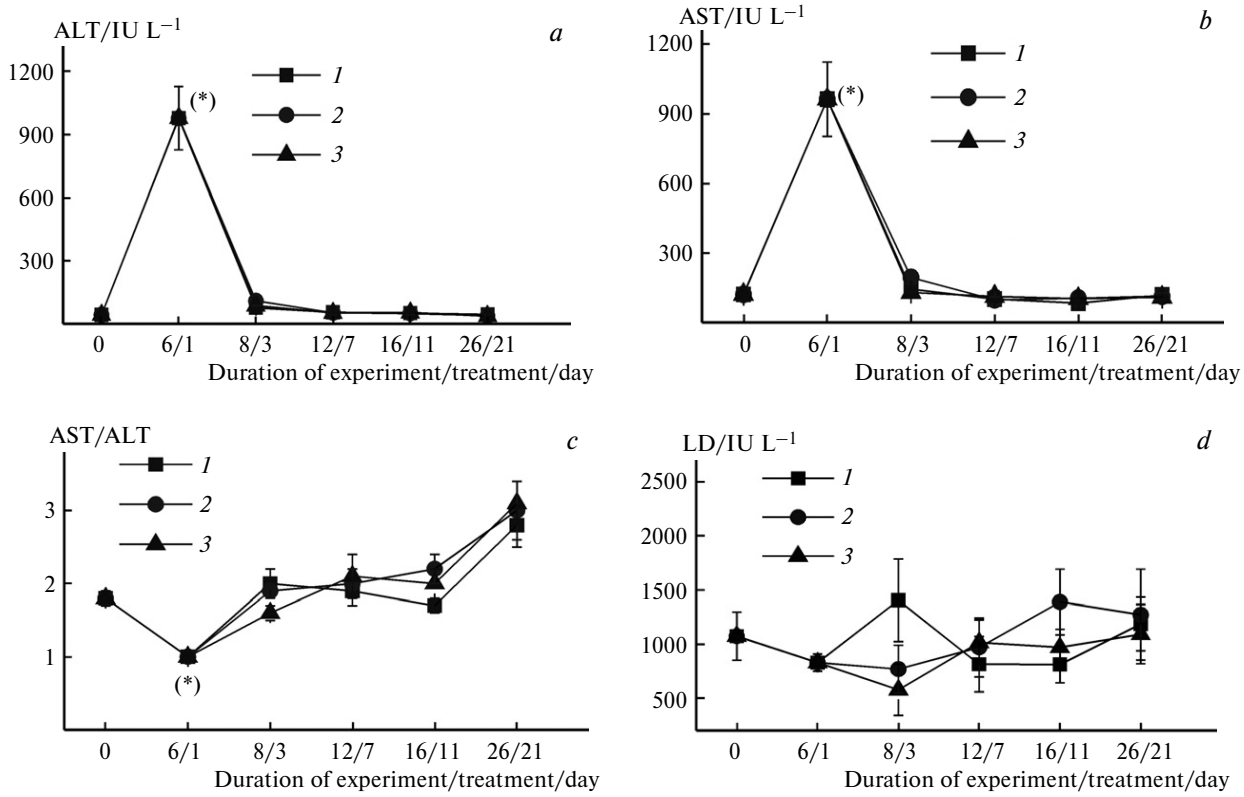


Fig. 5. Influence of compound **2** on the dynamics of changing biochemical cytolysis markers in rats with toxic CCl_4-induced hepatitis: activity of ALT (*a*) and AST (*b*) enzymes, De Ritis ratio (AST/ALT ratio) (*c*), and activity of lactate dehydrogenase (LD) enzyme (*d*); *1*, control; *2* and *3*, administration of compound **2** in a dose of 0.7 and 1.7 mg kg^{-1}, respectively.

esterase remained decreased in the control group (Fig. 7, *a*, *b*, *d*), whereas the concentration of globulins reduced on the 11th day (Fig. 7, *c*). The application of compound **2** normalized the protein metabolism primarily due to an increase in the globulin fraction (see Fig. 7, *a*, *c*). The dynamics of changing the albumin concentration and choline esterase activity in the group of animals administrated by compound **2** differed insignificantly from that of the control group (see Fig. 7, *b*, *d*).

The observation of the dynamics in the control group showed that the glucose level normalized to the third day of therapy (Fig. 8, *a*) and the levels of cholesterol, serum iron, and magnesium remained decreased (Fig. 8, *b*—*d*). No statistically significant differences with the control group were revealed on the corresponding days of measurements for the glucose level in the groups of animals administrated by compound **2** (see Fig. 8, *a*).

Upon the administration of compound **2** in doses of 0.7 and 1.7 mg kg^{-1}, the cholesterol concentration (see Fig. 8, *b*) increased over the control ($p < 0.05$, *t*-test) but remained lower than the reference values, which indicates the normalization of the lipid metabolism and synthesis of bile acids. The cholesterol level in the experimental groups also decreased and was at the level of the control group on the seventh day and further days of observation.

After tetrachloromethane administration, hypoferremia was observed and enhanced within 21 days of observation (see Fig. 8, *c*), which implies the metabolism dysfunction of hemin proteins in the liver, since in the organism iron exists only in the state bound to proteins. The iron content in the blood serum increased to the normal level on the third day of the administration of compound **2** in doses of 0.7 and 1.7 mg kg^{-1} and on the 11th day in a dose of 0.7 mg kg^{-1}, and the differences with the control group of animals were statistically significant ($p < 0.05$, *t*-test). Upon the administration of compound **2** in a dose of 1.7 mg kg^{-1} beginning from the 7th day and in a dose of 0.7 mg kg^{-1} on the 7th and 21st day of therapy, no differences in the iron level with the control group were detected. This parameter was decreased compared to the normative intact values.

Magnesium plays an important role in the metabolism of nucleic acids and nucleotides in cells. Magnesium activates DNA polymerase, and RNA polymerase, polynucleotidase, ribonuclease, deoxyribonuclease, and a series of other enzymes of nucleic metabolism. The toxic effect of CCl_4 resulted in a decrease in the magnesium content in the blood serum (see Fig. 8, *d*). The decrease makes progress within 21 days of observation, which can be a reason for serious dysfunctions of nucleic acid metabolism in cells and, as a consequence, for the dysfunction

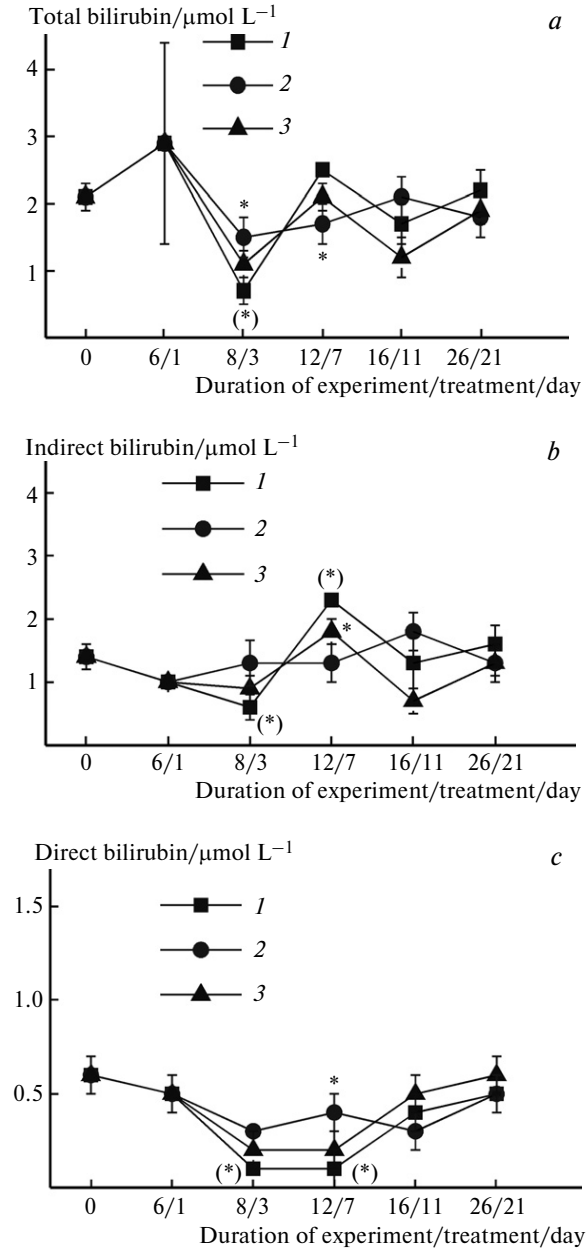


Fig. 6. Influence of compound **2** on the bilirubin metabolism in rats with toxic CCl_4-induced hepatitis: level of total bilirubin (*a*), level of indirect bilirubin (*b*), and level of direct bilirubin (*c*); *1*, control; *2* and *3*, administration of compound **2** in a dose of 0.7 and 1.7 mg kg^{-1}, respectively.

of protein synthesis. When the animals were treated with compound **2**, the magnesium level in the blood serum in both doses significantly increased compared to the control on the third day and on the 11th day of administration of a dose of 1.7 mg kg^{-1} and on the 11th day and on the 21st day for a dose of 0.7 mg kg^{-1}. However, no complete reduction of the magnesium level was observed under the action of compound **2**, and this parameter in the experimental groups remained lower than the normative reference values.

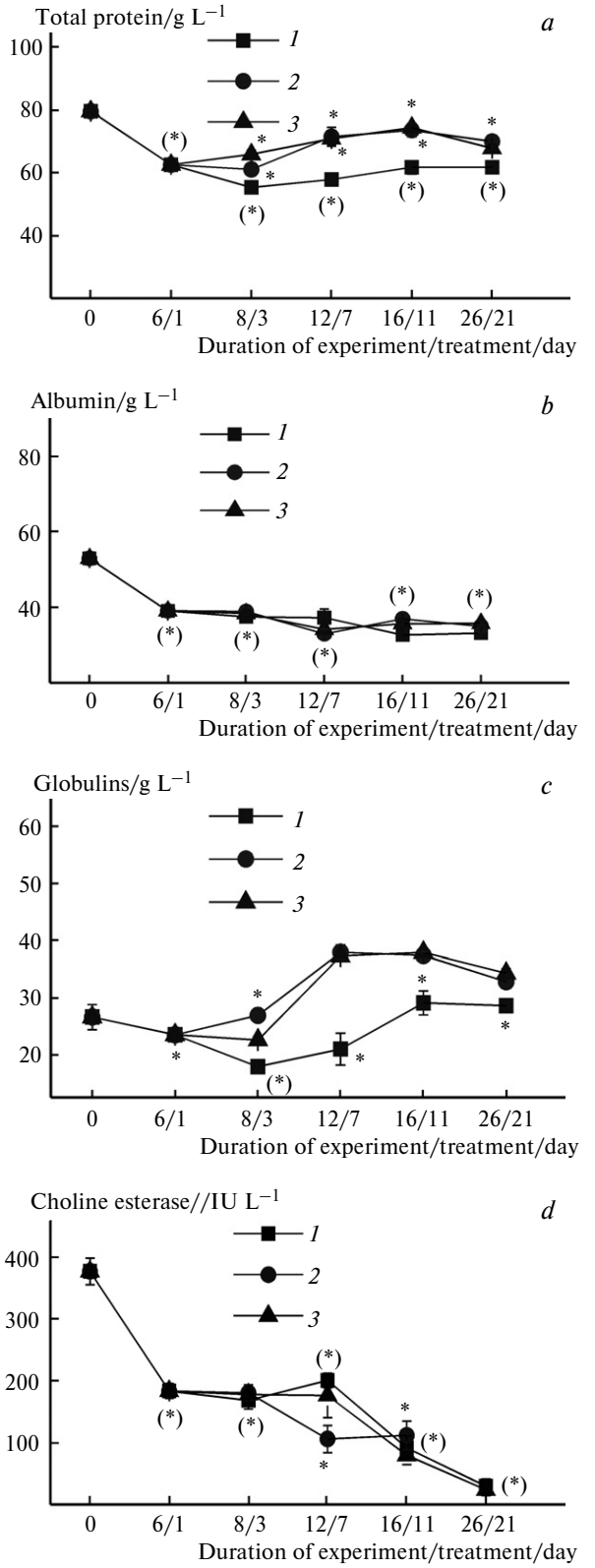


Fig. 7. Influence of compound **2** on the protein metabolism parameters and synthetic function of liver in rats with CCl_4-induced hepatitis: level of total protein (*a*), level of albumin (*b*), level of globulins (*c*), and activity of serum choline esterase enzyme (*d*); *1*, control; *2* and *3*, administration of compound **2** in a dose of 0.7 and 1.7 mg kg^{-1}, respectively.

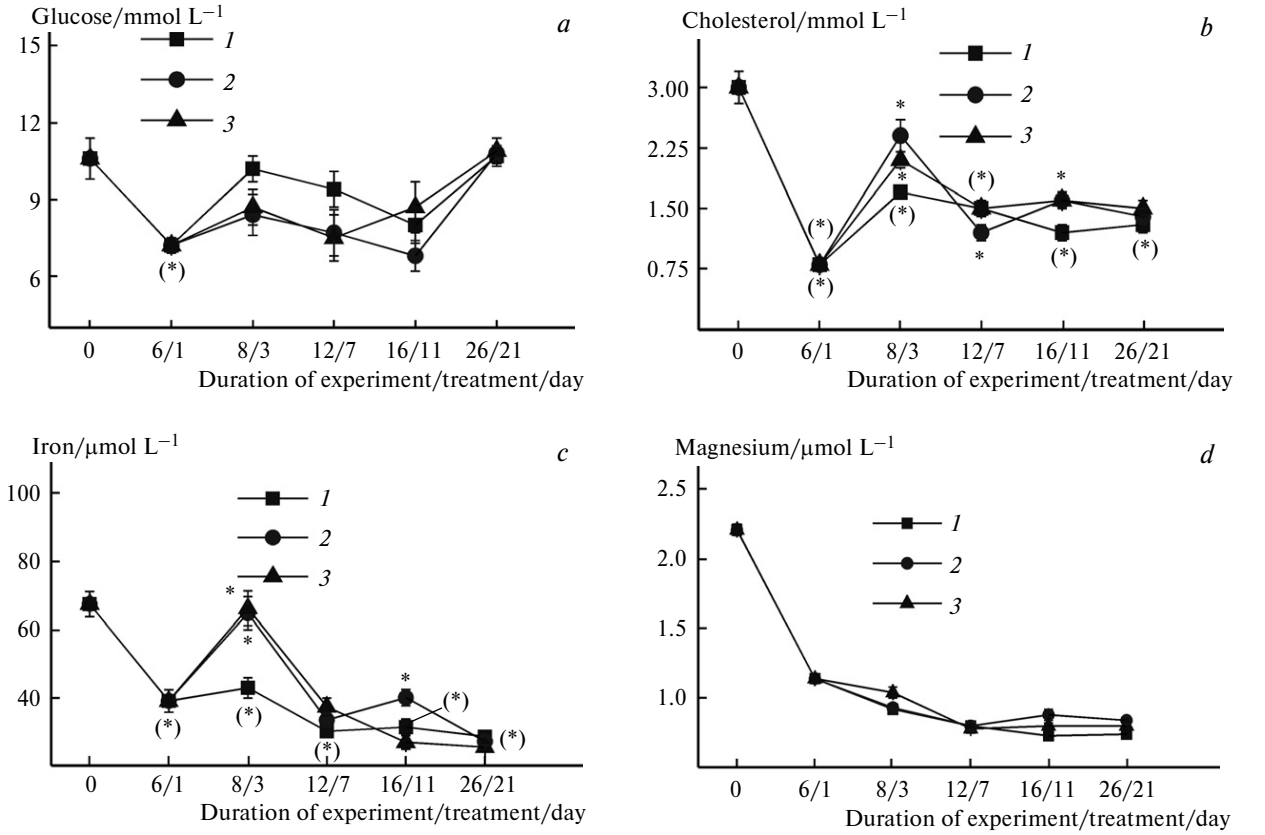


Fig. 8. Influence of compound **2** on the dynamics of changing biochemical parameters: level of glucose (*a*), level of cholesterol (*b*), level of iron (*c*), and level of magnesium for toxic CCl_4-induced hepatitis (*d*); *1*, control; *2* and *3*, administration of compound **2** in a dose of 0.7 and 1.7 mg kg^{-1}, respectively.

Thus, salt-like conjugate **2** of xymedone drug with *para*-aminobenzoic acid was synthesized. The cytotoxicity and cytoprotective properties of compound **2** were studied in experiments *in vitro* on the *Chang Liver* cell line of normal human hepatocytes. It was found that IC_{50} of the studied conjugate on the *Chang Liver* hepatocytes was 20.7 mmol L^{-1}. The studied compound in a concentration of 25 μmol L^{-1} was revealed to favor the enhancement of the viability of the *Chang Liver* cell line against the background of toxicant D-GLA compared to the control group of cells subjected to a similar action of D-GLA. The studies *in vivo* on rats revealed the manifestation of the hepatoprotective properties of compound **2**, which were most pronounced at the early stages of therapy of toxic CCl_4-induced hepatitis. We revealed the ability of compound **2** to decrease the surface area of necrotic injuries detected on the hematoxylin- and eosin-stained sections on the third day of administration and to decrease the amount of the detected infiltration of hepatocytes by lipids on the frozen sections stained with Sudan black on the third and seventh day of administration of the studied compound compared to the control group of animals, which was administrated with a saline instead of a solution of the drugs. A positive effect on the secretory and synthetic functions of the liver and on bilirubin, iron, and magnesium metabolism was observed by the biochemical parameters when treating the animals by compound **2**. Thus, the conjugate of xymedone drug with *para*-aminobenzoic acid exerts the cytoprotective *in vitro* and hepatoprotective *in vivo* effects.

Experimental

Synthesis of the xymedone conjugate with *para*-aminobenzoic acid (2). Xymedone (1,2-dihydro-4,6-dimethyl-1-(2-hydroxyethyl)pyrimidin-2-one) was synthesized using the earlier described procedure[20] from 1,2-dihydro-4,6-dimethylpyrimidin-2-one and 2-chloroethanol. The studied conjugate (compound **2**) was synthesized according to the described method[5] by the dissolution of compound **1** (5 g, 29.8 mmol) and *para*-aminobenzoic acid (4.08 g, 29.8 mmol) in methanol (50 mL). The yield of compound **2** was 9.0 g (89%), m.p. 113—114 °C. The following physicochemical characteristics (that coincide with those described earlier[5]) were determined for the synthesized conjugate. IR, ν/cm^{-1}: 3461, 3364, 2675, 1664, 1625, 1442, 1423, 1313, 1292, 1174, 843, 772. ^{1}H NMR, δ: 7.65 (d, 2 H, Ar2, J = 8.6 Hz); 6.70 (d, 2 H, Ar3, J = 8.6 Hz); 6.40 (s, 1 H, H(5)); 4.06 (t, 2 H, H(1′), J = 5.4 Hz); 3.81 (t, 2 H, H(2′), J =5.4 Hz); 2.39 (s, 3 H, H(6)); 2.26 (s, 3 H, H(4)). ^{13}C NMR, δ: 173.91 (C(4)), 171.35

(COO), 161.98 (C(6)), 156.10 (C(2)), 150.36 (C, Ar4), 130.92 (C, Ar2), 120.53 (C, Ar1), 114.40 (C, Ar3), 108.13 (C(5)), 57.86 (C(2′)), 47.66 (C(1′)), 21.79 (C(4)$\underline{C}H_3$), 19.29 (C(6)$\underline{C}H_3$). Found (%): C, 59.15; H, 6.34; N, 13.64. $C_{15}H_{19}N_3O_4$. Calculated (%): C, 59.01; H, 6.27; N, 13.76.

Determination of the cytotoxicity and cytoprotective properties *in vitro*. Experiments were carried out on the *Chang Liver* cell line of normal human hepatocytes obtained from the Collection of Cell Cultures of the D. I. Ivanovskii Research Institute of Virology (Russian Academy of Medical Sciences). The cells were cultivated in Eagle′s MEM medium with the addition of 10% bovine serum FBS, 1% essential amino acids, and gentamicin antibiotic.[21]

The cells were detached from the vial with a trypsin— versene (1 : 3) mixture, and a cellular suspension with a concentration of 10^5 cells per 1 mL was prepared, distributed dropwise by 200 μL of the suspension to a 96-well table, and incubated for 24 h. In order to study the hepatoprotective effect, D-GLA as a toxicant in a concentration of 150 mmol L^{-1} was added together with the studied compound according to the described procedure.[22] The growth medium to which compound **2** and D-GLA were not added during cell cultivation was used as a reference group of the intact cells. Only D-GLA was added to the growth medium of the control cell group. Three sequences of the experiment were conducted.

In order to determine the number of viable cells, the full growth medium with fluorescent dyes was prepared: 198 μL of the full growth medium + 2 μL of DAPI or Hoechst 33342 (concentration 1 mg mL^{-1}) + 0.5 μL of propidium iodide. Then the cultural liquid was replaced by the prepared growth medium with the dyes, and the latter was incubated for 45 min. After incubation, viable and died cells were calculated on a Cytell Cell Imaging System cell analyzer (GE Healthcare) using the standard protocol.

Study of the hepatoprotective activity *in vivo*. Experiments were conducted on non-breed adult white rat males with the body weight 300—350 g. The animals were kept according to the published rules[23,24] under the standard vivarium conditions with the 12-h light day and unrestricted access to food and water. The animals were fed with a complex feed prepared according to the Specification (protein 22%, cellulose not higher than 4%, fat not higher than 5%, ash not higher than 9%, moisture content not higher than 13.5%, calorie content 295 cal/100 g). All studies and protocols on with work with animals were approved by the Local Ethical Committee of the Kazan (Volga Region) Federal University (Protocol No. 4 of May 18, 2017).

The scheme of experiment is shown in Fig. 9. According to the published procedure,[22] carbon tetrachloride was administrated perorally as a 35% oil solution in a dose of 1.5 mg kg^{-1} to model liver injuries. Compound **2** was intraperitoneally administrated in doses of 0.7 and 1.7 mg kg^{-1}, being 1/2500 and 1/1000 of LD_{50}, respectively, within 21 days after modeling the toxic liver injury. Intakes of blood and liver samples were conducted according to the scheme presented in Fig. 9.

Blood samples were taken either during animal′s lifetime (from the tail end), or after euthanasia. The blood serum was prepared by twice centrifugation of the blood at 3000 rpm and temperature 4 °C. Prior to analysis, the serum was kept in a refrigerator at −25 °C.

The liver for investigation was taken immediately after euthanasia to the animals. The liver samples for histological studies were fixed in 4% buffered formalin. A histological construction was made on a Sakura Tissue-Tek® VIP™ 5 Jr automated histoprocessor. The samples were embedded in paraffin, and blocks were formed. Sections 4—5 μm thick were made on a SakuraAccu-Cut SRM200 microtome and stained with hematoxylin and eosin. To detect lipids, frozen sections 5—7 μm thick were made on a Sakura Tissue-Tek $CriO_3$ cryotome and stained with Sudan black. A mixture of glycerol and 15% gelatin in a ratio of 1 : 2 was used as a mounting medium.

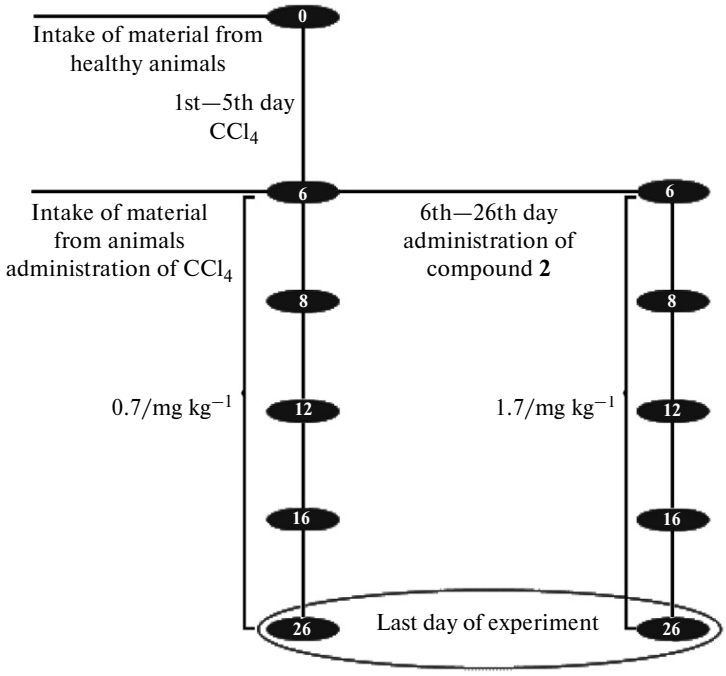


Fig. 9. Scheme of experiment. Control experimental points (duration in days from the experiment onset) are the intake of the biomaterial (blood, liver).

The morphometric analysis of the drugs was carried out on a Nikon H550S direct optical microscope with a Nikon digital camera and the NIS Basic Research software as described earlier.[6] The surface areas of destructive—degenerative and necrotic changes in the liver tissue in the hematoxylin- and eosin-stained sections were calculated in μm^2, and the fraction (in %) of the injured area relative to the whole visible surface area of the section was calculated using the equation

$$\text{Regions of liver injuries (\%)} = \frac{\text{Sum of injury surface areas}}{\text{Overall field of vision}} \cdot 100\%.$$

The surface areas of lipid inclusion detection relative to the visible surface area of the section were similarly determined on the frozen sections stained with Sudan black using the equation

$$\text{Regions of lipid detection (\%)} = \frac{\text{Sum of surface areas of lipid detection}}{\text{Overall field of vision}} \cdot 100\%.$$

NMR spectra were recorded on an Avance-500 FT-IR spectrometer (Bruker) with the working frequencies 500.13 (^{1}H) and 125.77 MHz (^{13}C) in D_2O at 30 °C using tetramethylsilane as an external standard.

The biochemical parameters were studied in blood serums on a Cobas Integra 400 automated biochemical analyzer (Roche, Switzerland) using original reagent sets and the protocol from the producer. The following parameters of the blood serum were determined: activities of ALT, AST, lactate dehydrogenase, GGT, serum choline esterase, and alkaline phosphatase and concentrations of total and direct bilirubin and albumin, total protein, glucose, iron, and magnesium. The sum of globulins, indirect bilirubin,[25] and the De Ritis ratio [26] were calculated.

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