Received: 10 July 2020 | Revised: 8 September 2020 | Accepted: 20 October 2020

DOI: 10.1002/jbt.22660

RESEARCH ARTICLE

JOURNAL OF BIOCHEMICAL AND MOLECULAR TOXICOLOGY WILEY

Conjugate of pyrimidine derivative, the drug xymedon with succinic acid protects liver cells

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Abstract

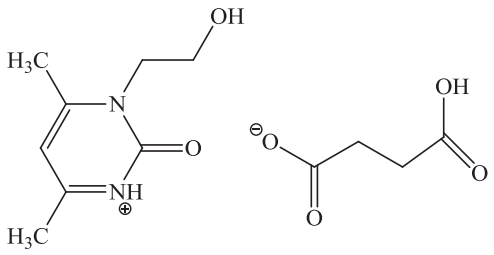
The aim of the study was to investigate the hepatoprotective properties of the conjugate of the xymedon drug substance with succinic acid (**1a**). The study presents an in vitro comparative evaluation of the cytotoxicity and cytoprotective properties of **1a** and succinic acid on a cell line of normal human hepatocytes *Chang Liver*, and in vivo investigation of the ability of **1a** to restore liver from the toxic damage caused by CCl_4 in Wistar rats. It was shown that the cytotoxicity of **1a** was 19.9 ± 0.8 mmol/L, and that of succinic acid was 14.1 ± 0.2 mmol/L. Against the background of D-galactosamine exposure, the cytoprotective effect of **1a** was found to be superior to that of succinic acid. It was shown that **1a** caused a significant reduction in necrotic and steatosis changes in the liver and restoration of biochemical markers of cytolysis, as well as bilirubin metabolism and synthetic liver function.

KEYWORDS

conjugate, hepatocytes, hepatoprotective properties, pyrimidine, succinic acid

1 | INTRODUCTION

The liver is a vital organ, which is responsible for the metabolism and detoxification of various endogenous and exogenous substances. In cases of prolonged exposure or exposure to amounts of toxic substances, which overwhelm the organ's detoxifying capacity, various pathological changes, such as cirrhosis, necrosis, fibrosis, steatosis, and so forth appear in the liver.[1] Studies aimed at accelerating the metabolism of toxins and eliminating them from the body, as well as maintaining as many healthy liver cells as possible while also stimulating their renewal, are highly relevant. The object of this study was the conjugate of the already known drug xymedon with succinic acid (hereinafter, compound **1a**). The structural formula of **1a** is presented in Formula (1).



1a

Formula (1)

Xymedon (1,2-dihydro-4,6-dimethyl-1-(2-hydroxyethyl)-pyrimidin-2-one) is an original Russian drug, a tissue regeneration stimulator, created at the A. E. Arbuzov Institute of Organic and Physical Chemistry in 1966. This drug has a wide spectrum of action, including membrane-stabilizing, regenerative, immunostimulating, antioxidant,

and so on.[2,3] The authors of this paper previously have shown that xymedon and its derivatives possessed hepatoprotective, antioxidant, neuroprotective, and other properties.[4–8]

Succinic acid and its derivatives are involved in cell metabolism, namely in the tricarboxylic acid cycle.[9] It was shown that succinate was a ligand for the GPR91 receptor.[10] This receptor in the liver is located on stellate cells. At the same time, succinic acid, when activating this receptor, is able to activate reparative processes in the liver. However, an excess of succinic acid can lead to the development of fibrosis.[11]

In the work by Vyshtakalyuk et al.,[4] the data on an initial assessment of the hepatoprotective properties of xymedon conjugates with biogenic acids were presented. As a result of the study, it was shown that among the seven compounds studied, **1a** was one of the substances that caused a significant decrease in the areas of destructive changes in liver tissue (steatosis and necrosis) in the case of toxic damage caused by CCl_4. The present study aims to investigate the efficacy of **1a** in the treatment of CCl_4-induced toxic hepatitis in rats and its cytoprotective effect against the background of exposure to the toxicant D-galactosamine on a cell line of normal human hepatocytes *Chang Liver*.

2 | MATERIALS AND METHODS

2.1 | Synthesis of xymedon conjugate with succinic acid

Xymedon (1,2-dihydro-4,6-dimethyl-1-(2-hydroxyethyl)-pyrimidin-2-one) was synthesized using the earlier described procedure[12] from 1,2-dihydro-4,6-dimethylpyrimidin-2-one and 2-chloroethanol. The studied conjugate (compound **1a**) was synthesized according to the described method.[4]

2.2 | Determination of cytotoxicity and cytoprotective properties in vitro

The tests were conducted on the human hepatocyte cell line *Chang Liver* obtained from the Russian collection of cell cultures of D. I. Ivanovskii Institute of Virology (Moscow). The cells were cultivated in an IGLA MEM medium with an added 10% fetal bovine serum, 1% essential amino acids, and gentamicin.[13] Cells were isolated using a mixture of trypsin and Versene in a ratio of 1:3. The cell-rich fluid was prepared at a concentration of 10^5 cells/ml. The fluid was put by dropper into a 96-well plate at 200 μl per well and incubated for 24 h.

To study cytotoxicity, the studied compounds were introduced into the growth medium. To study the hepatoprotective properties in vitro, according to References [14,15] a toxicant, D-galactosamine (D-GLA), was put in along with the research subjects at a concentration of 80 mmol/L. Cells that were grown without adding compound **1a** and D-GLA to the growth medium during cultivation were used as a reference control of the intact group. Only D-GLA (80 mmol/L) was introduced into the growth medium of the control group of cells (D-GLA control). The experiment was performed three times.

To determine the number of live and dead cells, a complete growth medium with fluorescent stains was prepared at a rate of 198 μl of complete growth medium + 2 μl of DAPI (4′,6-diamidino-2-phenylindole) or Hoechst 33342 (concentration: 1 mg/ml) + 0.5 μl of propidium iodide per well. Then, the culture fluid was replaced with the prepared growth medium with stains and incubated for 45 min. After the incubation, the dead and live cells were analyzed using a Cytell Cell Imaging System by GE Healthcare in accordance with the standard protocol.

2.3 | The study of hepatoprotective activity in vivo

The tests were conducted on adult male Wistar rats obtained from the Research and Production Enterprise Laboratory Animal Farm based at the Branch of Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences (Pushchino). The animals were kept in accordance with References [14,16] in standard conditions in a vivarium with 12-h daylight and free access to food and water. The animals were fed with complete feed made according to specification (protein, 22%; fiber, 4% max.; fat, 5% max.; ash, 9% max.; humidity, 13.5% max.; caloric value, 295 kcal/100 g). All animal experiments and protocols were approved by the Local Ethics Committee of Kazan Federal University (Protocol No. 4, dated: May 18, 2017).

Two experiments were conducted. The layout of the design of the experiments is presented in Table 1. The first experiment was aimed to perform a preliminary evaluation of the hepatoprotective properties of compound **1a** at three doses: low, medium, and high. The second experiment was aimed to perform an in-depth study of the long-term application of compound **1a**. The first experiment included 4-month-old male rats with body weight 300–350 g. The second experiment included male rats with body weight 400–450 g. Carbon tetrachloride was administrated orally as a 35% oil solution at a dose of 1.5 mg/kg within 5 days to model liver damage. Compound **1a** was

TABLE 1 Design of the experiment

Experiment 1				Experiment 2			
P	Doses of 1a (mg/kg)	*n*	*l*/*m*	P	Doses of 1a (mg/kg)	*n*	*l*/*m*
0	0	6	10/3	0	0	18	10/3
6	0.33		10/3	6	0.33		10/3
9	0.8		6/6	8	0.8		10/3
	2.00			12			10/3
				16			10/3
				26			9/9

Abbreviations: *l*, number of animals for the study of biochemical parameters in blood serum; *m*, number of animals for histological examination of the liver; *n*, number of animals in each group; P, control point of the experiment (day from the beginning) when the biomaterial (blood, liver) was taken.

intraperitoneally administrated at the doses of 0.33, 0.8, and 2 mg/kg, being 1/2500, 1/1000, and 1/400 of LD_{50}, respectively, within 3 days after modeling of the toxic liver damage in the first experiment and at the doses of 0.33 and 0.88 mg/kg within 21 days after modeling the toxic liver damage in the second experiment.

Intakes of blood and liver samples were conducted according to the scheme presented in Table 1.

Blood samples were taken either during an 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 a temperature of +4°C. Before analysis, the serum was kept in a refrigerator at −25°C.

The liver for investigation was taken immediately after the euthanasia of the animals. The liver samples for histological studies were fixed in 4% buffered formalin. A Sakura Tissue-Tek® VIP™ 5 Jr automated vacuum infiltration processor and a paraffin filling station MtPoint ESD-2800 were used for embedding samples in paraffin and forming blocks. Sections 4–5-μm thick were made using a Sakura Accu-Cut SRM 200 microtome and stained with hematoxylin and eosin. To detect lipids, 5–7-μm-thick frozen sections were made on a Sakura Tissue-Tek CriO3 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.

Morphometric analysis of the preparations was carried out using a Nikon H550S direct light microscope with a Nikon digital camera and NIS Basic Research software, as described in References [5,15]. On sections stained with hematoxylin and eosin, the total area of destructive–degenerative and necrotic changes in the liver tissue in square micrometers was calculated and the percentage of the damaged area relative to the entire visible area of the section was calculated by the following equation:

$$\text{Areas of damaged tissue, \%} = \frac{\text{Sum of areas of damaged tissue}}{\text{Total visible area}} \times 100\%. \quad (2)$$

On frozen sections stained with Sudan black, the detection areas of lipid inclusions relative to the entire visible area of the section were determined similarly by the following equation:

$$\text{Areas of lipids detection, \%} = \frac{\text{Sum of areas of lipids detection}}{\text{Total visible areas}} \times 100\%. \quad (3)$$

Biochemical parameters were studied using a Cobas Integra 400 automatic biochemical analyzer (Roche) and reagent kits. The following blood parameters were determined: alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), γ-glutamate transferase (GGT), total and direct bilirubin, albumin, total protein, alkaline phosphatase (ALP), serum cholinesterase (ChE), glucose, and magnesium. The sum of globulins, indirect bilirubin, and De Ritis coefficient was calculated.

2.4 | Statistical analysis

All the obtained experimental data were included in the statistical analysis. The authors calculated the mean values and standard error of the mean. Tables and figures contain mean values for each standard parameter and standard error. For each sampling, the normality of the distribution was estimated by the Kolmogorov–Smirnov test. In cases of the normal distribution, the comparison of samplings was performed by Student's *t* test. In cases of non-normal distribution, the comparison was performed by the Mann–Whitney test. For the comparison of samplings in in vitro tests on cell culture *Chang Liver*, the Mann–Whitney test was used. The calculations were made in software SPSS 13.0. The results obtained in vivo on laboratory animals were compared by Student's *t* test in software SPSS 13.0 or Origin Lab.

3 | RESULTS

3.1 | Results of the study of cytotoxicity and cytoprotective properties of 1a in vitro

We investigated the concentrations of half-maximal inhibition of cell growth (IC_{50}) for **1a** and succinic acid. It was found that **1a** had less cytotoxicity than succinic acid. Thus, the IC_{50} of **1a** on *Chang Liver* cell line was 19.9 ± 0.8 mmol/L, and that of succinic acid was 14.1 ± 0.2 mmol/L. The cytotoxicity of **1a** and succinic acid is probably due to acidification of the culture medium for the cell line.

The results of the study of the cytoprotective effect of the conjugate of xymedon with succinic acid against the background of exposure to the toxicant D-GLA are presented in Table 2. Compound

TABLE 2 Cytoprotective effect of compound **1a** and succinic acid in different concentrations: Livability of *Chang Liver* cells under exposure of tested compounds plus D-GLA in concentration: 80 mmol/L

Concentration (μmol/L)	Compound 1a	Succinic acid	Control D-GLA	Reference (no exposure)
125	28.47 ± 0.51	16.26 ± 0.86	-	-
62.5	17.59 ± 1.78	17.45 ± 1.52	-	-
31.25	43.30 ± 9.56[a]	18.10 ± 1.24	-	-
15.6	43.17 ± 12.50[a]	18.00 ± 2.19	-	-
0	-	-	10.05 ± 0.50	98.80 ± 0.20

Note: Data are presented as mean ± *SEM*.

[a]Statistically significant differences from the D-GLA control (D-GLA concentration: 80 mmol/L; Mann–Whitney test, $p < .05$).

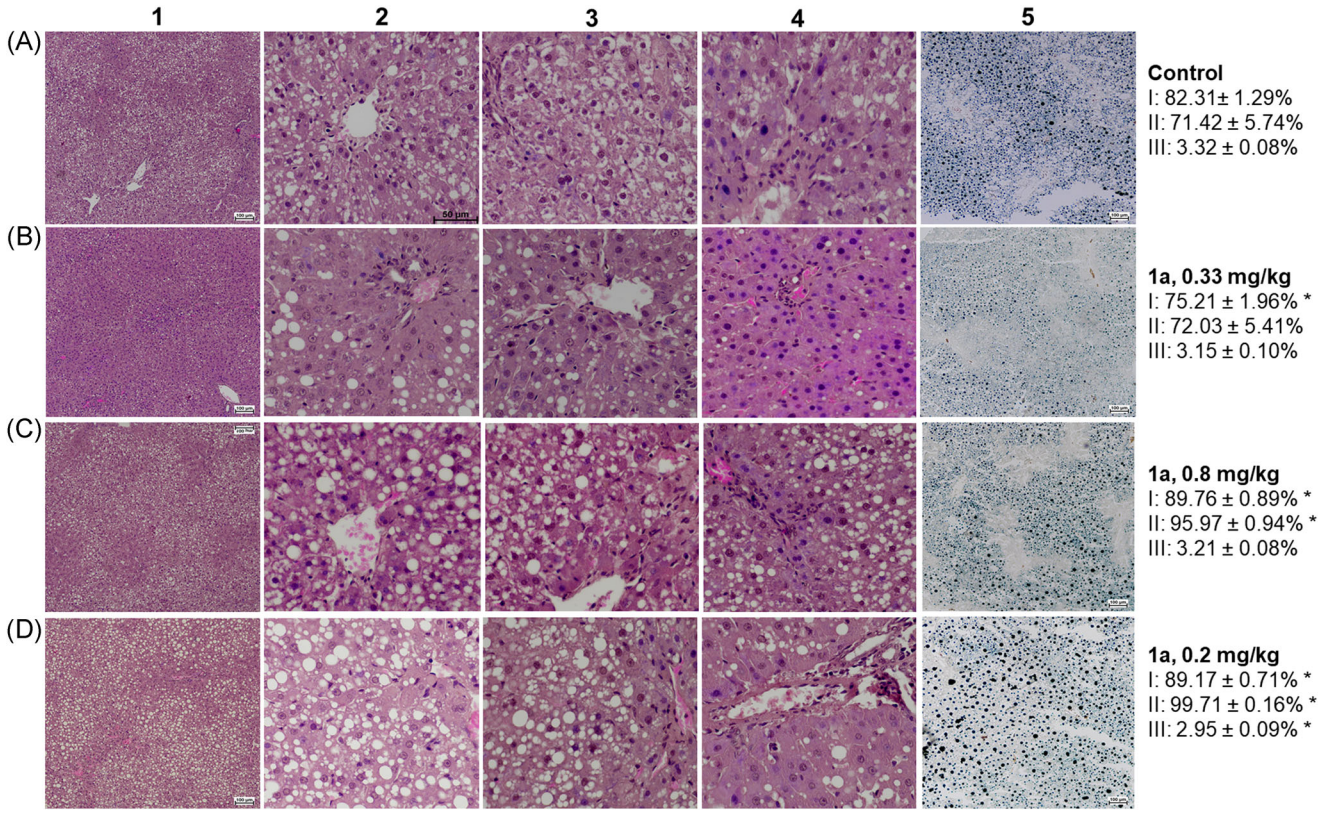


FIGURE 1 Pathomorphological changes in the liver in experimental groups on the ninth day of experiment: (A) Control group; (B) group with the administration of **1a** at a dose of 0.33 mg/kg; (C) same, at a dose of 0.8 mg/kg; (D) same, at a dose of 2 mg/kg. Columns designated by numbers 1–5: (1) general view of the liver section (zoom ×150, hematoxylin–eosin); (2, 3) areas in the center of the lobule (two different sections, zoom ×600, hematoxylin–eosin); (4) areas in the periphery of the lobule (zoom ×600, hematoxylin–eosin); (5) lipid detection in the liver (zoom ×150, Sudan black). I - sum of areas of damaged tissue; II - sum of areas of lipids detection; III - the mass coefficient of liver. *Difference of the parameters with the control group is statistically significant, *t* test, $p < .05$

1a, as can be seen from Table 2, has a more pronounced cytoprotective effect than succinic acid.

The data obtained indicate that **1a**, at a concentration of 25 μmol/L, contributes to a significant (*U* test, $p < .05$) increase in the number of viable cells compared with the control group. Similar results showing an increase in cell viability under D-GLA exposure were obtained for the conjugate of xymedon with *para*-aminobenzoic acid.[15]

3.2 | Preliminary evaluation of hepatoprotective properties of compound 1a at low, medium, and high doses

In the first experiment, it was shown that after a 5-day course of administration of a toxicant (CCl_4), on Day 6, there were signs of destructive–degenerative changes in hepatic cells, as it is shown in Figure S1. The mass coefficient of the liver was 4.00 ± 0.12% at the norm for rats 2.7%–3%. A general picture of structural–morphological disturbances in the liver after the administration of CCl_4 is shown in Figure S1A. The most evident changes were observed in the center of the lobule (Figure S1B). Among destructive–degenerative alterations in the condition of hepatocytes in the center of the lobule were necrosis, balloon and hydropic degeneration, decrease in the size of nuclei, their dislocation to the periphery, and karyorrhexis. In the area of triad and periphery of the lobule, destructive alterations of the cells were less expressed and included macrovesicular and microvesicular liver steatosis, a sharp narrowing of intercellular space, disturbances of the structural tissue organization, basophilic aggregations in hepatocytes that is a sign of the appearance of the damaged and aggregated ribonucleic acids (Figure S1C). By the results of the count, the general area of the affected hepatocytes on Day 6 of the trial was 95.03 ± 0.88%. The major part of the hepatic parenchyma (99.30 ± 0.67%) exerts a positive reaction on the immunohistochemical identification of lipids during Sudan black staining (Figure S1D), that is, pathological accumulation of lipids is observed in hepatocytes.

Three days after the last administration of CCl_4 (Day 9 of the experiment), in the control group, a decrease in the mass coefficient of the liver to 3.32 ± 0.08% was observed. At the same time, structural–morphological disturbances became less expressed than on Day 6 or a day after the last administration of the toxicant (Figure 1A). On Day 9, in the control group, the affected area was 82.31 ± 1.29%, and the area of lipid detection was 71.42 ± 5.74%. In the center of the lobule, there were cells in the condition of microvesicular and macrovesicular hepatic dystrophy, focal or total

TABLE 3 Biochemical blood markers of liver damages in rats in the 1st experiment

Parameters	0th day (reference values)	Control, 6th day	Control, 9th day	1a (0.33 mg/kg), 9th day	1a (0.8 mg/kg), 9th day	1a (2.00 mg/kg), 9th day
ALT (U/L)	70.7 ± 12.2	46.1 ± 13.5	110.4 ± 5.9**	95.4 ± 6.4	71.6 ± 4.0*	68.6 ± 1.9*
AST (U/L)	98.8 ± 4.7	81.7 ± 26.1	197.4 ± 11.4**	163.6 ± 13.2	142.0 ± 6.5*	98.6 ± 17.8*
De Ritis ratio	1.60 ± 0.15	1.79 ± 0.20	1.80 ± 0.11	1.74 ± 0.19	1.99 ± 0.06	1.43 ± 0.25
γGT (U/L)	4.30 ± 1.00	7.30 ± 0.75**	6.00 ± 0.55	5.60 ± 0.51	6.20 ± 0.73	5.00 ± 1.05
LDH (U/L)	1415 ± 304	5399 ± 1205**	5085 ± 703**	2592 ± 836*	3018 ± 735	1153 ± 134*
ALP (U/L)	216.4 ± 14.1	359.2 ± 13.2**	219.0 ± 12.5	189.2 ± 5.9	194.0 ± 7.4	206.8 ± 6.1
Total bilirubin (μmol/L)	2.18 ± 0.18	5.37 ± 0.42**	3.03 ± 0.12**	2.30 ± 0.28	2.05 ± 0.13*	1.90 ± 0.17*
Direct bilirubin (μmol/L)	0.84 ± 0.19	5.52 ± 0.54**	1.65 ± 0.43	1.14 ± 0.12	1.18 ± 0.13	1.34 ± 0.28
Indirect bilirubin (μmol/L)	1.40 ± 0.21	0.16 ± 0.11**	1.38 ± 0.40	1.16 ± 0.17	0.87 ± 0.19	0.56 ± 0.12
Total protein (g/L)	64.70 ± 2.86	54.95 ± 1.99**	51.80 ± 0.73**	53.20 ± 0.70*	48.20 ± 1.66	46.80 ± 3.01
Albumen (g/L)	46.20 ± 2.42	47.00 ± 1.87	41.40 ± 1.03	41.60 ± 1.12	36.60 ± 1.33*	33.40 ± 1.50*
Globulin (g/L)	18.50 ± 1.96	7.95 ± 1.17**	10.40 ± 0.75**	11.60 ± 1.40	11.60 ± 1.03	13.40 ± 2.18*
Cholesterol (g/L)	2.15 ± 0.10	0.59 ± 0.09	2.43 ± 0.09	2.35 ± 0.08	1.68 ± 0.12	1.14 ± 0.09

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; γGT, γ-glutamate transferase.

*Differences with control are significant at $p < .05$

**Differences between the values in control CCl_4 group and reference values are significant at $p < .05$.

necrosis, and pathological basophil aggregations. A similar picture was also observed on the periphery of the lobule. In general, in the control group, the structural organization of hepatic tissue was disturbed; the cells were enlarged and deformed.

In the group of animals that were administered compound **1a** at a dose of 0.33 mg/kg, the mass coefficient of the liver was slightly lower than in the control group and was equal to 3.15 ± 0.10%. In this group, pathomorphological signs of the liver damage were significantly less expressed in comparison with the control group: the affected area was 75.21 ± 1.96% (difference from the control group was statistically significant, $p < .05$) and the area of lipid detection was 72.03 ± 5.41%. Among the pathological changes, microvesicular and macrovesicular liver steatosis prevailed. The number of cells with signs of necrosis was significantly lower. In the peripheral area of the lobule, the signs of steatosis and necrosis were weakly expressed; hepatocytes were smaller and had a well-expressed basophil mesh structure that was a sign of an increasing number of RNA and the development of endoplasmic reticulum, that is, activation of the synthetic function of the cells (Figure 1B). Among these cells, there are binuclear hepatocytes, which indicate the activation of cells mitosis.

In the group that received conjugate **1a** at the doses of 0.8 and 2 mg/kg, the mass coefficient of the liver was 3.21 ± 0.08 and 2.95 ± 0.09%, respectively. When **1a** was administered at a dose of 2 mg/kg, a decrease in the mass coefficient was statistically significant ($p < .05$) in comparison with the control. Although in groups that received **1a** at medium and high doses (0.8 and 2 mg/kg), the areas of the detection of the affected area of the liver and lipids reduced less on Day 6 in comparison with the control group. Unlike the control group, in groups that received compound **1a**, the signs of necrosis, enlargement, and deformation of hepatocytes were less expressed, and the signs of structural organization in the hepatic tissue remained. Among the signs of pathomorphological disturbances in these groups, reversible alterations (microvesicular and macrovesicular steatosis) prevailed (Figure 1C,D).

After a 5-day course of an administration of CCl_4, on Day 6 of the experiment, the authors registered changes in the biochemical blood parameters that confirmed the development of toxic damage of the liver in rats. Among them, the authors observed an increase in the total and direct bilirubin, γGT, LDH, ALP by 2.5, 6.6, 1.7, 3.8, and 1.7 times, respectively, and a decrease in total protein, cholesterol, direct bilirubin, and globulins by 1.2, 3.6, 2.3, 8.75, and 2.3 times, respectively (Table 3). All the revealed changes in the biochemical parameters of blood were statistically significant in comparison with normal (reference) baseline values. On Day 9, in the control group, the authors observed the following statistically significant changes in the biochemical blood parameters: an increase in ALT, ACT, total bilirubin, LDH, and cholesterol by 1.55, 2, 1.4, 3.6, and 1.13 times, respectively, and a decrease in total protein and globulins by 1.25 and 1.78 times, respectively (Table 3).

The exposure to compound **1a** led to the following positive changes in the biochemical blood parameters (markers of liver damage). The level of the activity of enzymes of ALT, ACT, LDH, the levels of total bilirubin and cholesterol decreased by 13.6%, 17.1%, 49.02%, 24.1%, and 3.3%; 35.1%, 28.1%, 40.7%, 32.3%, and 30.9%; 37.9%, 50.0%, 77.3%, 37.3%, and 53.1% under the influence of

compound **1a** at the doses of 0.33, 0.8, and 2 mg/kg, respectively. The level of globulins was statistically significant (by 28.8%) under the influence of compound **1a** at the dose of 2 mg/kg. Under the influence of compound **1a** at the doses of 0.33 and 0.8 mg/kg, an increase in the level of globulins was statistically insignificant (11.5%; Table 3).

3.3 | An in-depth study of the hepatoprotective properties of the long-term administration of compound 1a at low and medium doses

An in-depth study of the hepatoprotective properties of the long-term administration of compound **1a** at only low and medium doses was conducted because of the inexpediency of the application of the compound at the maximum dose.

Under the influence of carbon tetrachloride, in the second experiment, an increase in liver mass occurs due to increased blood supply, stagnant biliary processes, a significant accumulation of lipids in hepatocytes, and so forth. The mass coefficient of the liver allows assessing the general state of the organ. As can be seen from the graph (Figure 2A), a 5-day oral administration of carbon tetrachloride causes an increase in the mass coefficient of the liver in comparison with the reference values (Day 0).

By the end of the experiment, both in the control group and in those groups of animals that were administered compound **1a**, the liver mass coefficient was restored. On the 8th, 12th, and 16th day of the experiment, in the group of rats that were injected with substance **1a** at a dose of 0.33 mg/kg, a decrease in the liver mass coefficient was observed compared with the control group of animals by 9.35%, 7.14%, and 4.56%, respectively. The revealed differences with the control were not statistically significant; however, in the control group, the liver mass coefficient until the end of the experiment was significantly higher than the initial (reference) values, and in the group administered **1a** at a dose of 0.33 mg/kg, the differences with the reference values were already not significant from the third day of administration. At a dose of 0.8 mg/kg, compound **1a** had the worst effect on the mass coefficient of the liver, considering that on the 8th and 12th day of the experiment, there was a tendency to increase the indicator compared with the control (Figure 2A). Thus, compound **1a** had a positive effect on the mass coefficient of the liver only with the introduction of a low dose of 0.33 mg/kg.

It was shown that the size of area of damaged liver tissue when using compound **1a** at a dose of 0.8 mg/kg on the eighth day of the experiment (third day of administration) was decreased by 23% compared with the control group of animals on the same day. The differences are statistically significant (*t* test, $p < .05$). The use of a low dose of **1a** (0.33 mg/kg) did not significantly reduce the area of damage. A subsequent study of this indicator on the 12th and 26th day of the experiment indicates that **1a** does not have a positive effect on the reduction of necrotic lesions compared with the control group. Thus, the presented conjugate has a positive effect and

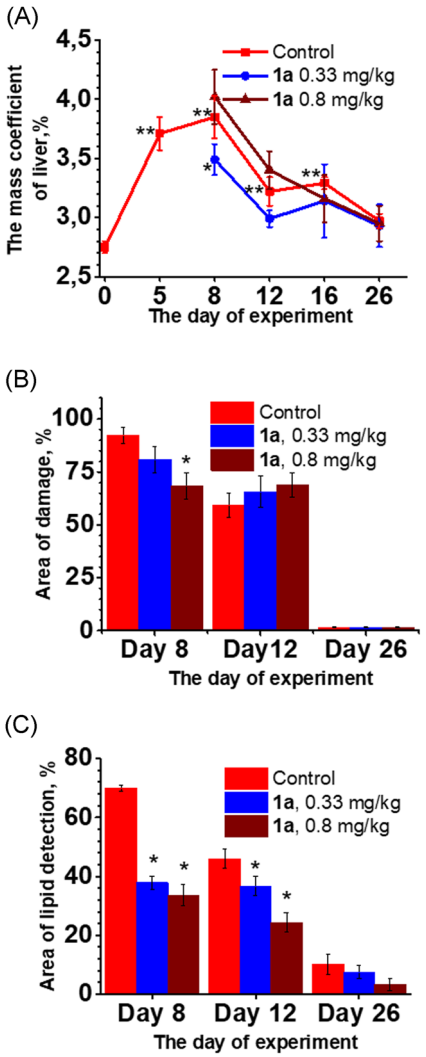


FIGURE 2 Parameters of pathomorphological changes in the liver in the second experiment: (A) the mass coefficient; (B) areas of damage by steatosis, necrosis to rat liver tissue; (C) the detection regions of lipid inclusions on sections of the liver. *Differences with the control group are statistically significant, *t* test, $p < .05$; **differences between the control group and the reference values are statistically significant, *t* test, $p < .05$

reduces the amount of damage only in the early stages of treatment only (Figure 2B).

We calculated the amount of lipid inclusions on frozen sections stained with Sudan black. The results are presented in Figure 2C. A 5-day administration of carbon tetrachloride promotes the accumulation of lipids in hepatocytes and the manifestation of large- and small-droplet fatty degeneration (Figure 2C). The administration of compound **1a** within 3 days after the course of the introduction of carbon tetrachloride, at doses of 0.8 and 0.33 mg/kg, causes a statistically significant (*t* test, $p < .05$) decrease in lipid inclusions in liver cells detected by Sudan black, in comparison with the control group of animals (36% and 32%, respectively; Figure 2C). On the 12th day of the experiment (the seventh day of administration of **1a**), a statistically significant decrease in lipid inclusions was also observed in

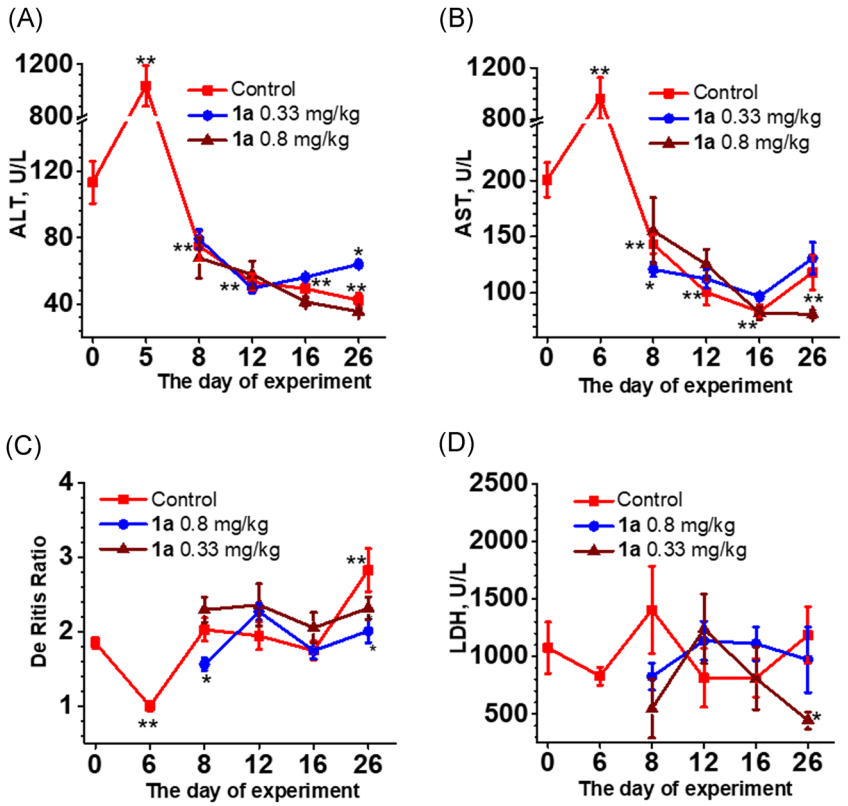


FIGURE 3 The effect of compound **1a** on the dynamics of changes in biochemical markers of cytolysis in rats with toxic CCl_4-induced hepatitis: (A) alanine aminotransferase (ALT), (B) aspartate aminotransferase (AST); (C) De Ritis ratio; (D) lactate dehydrogenase (LDH). *Differences with the control group are statistically significant, *t* test, $p < .05$; **differences between the control group and the reference values are statistically significant, *t* test, $p < .05$

comparison with the control group. Compound **1a** had the most significant effect on the reduction of lipid inclusions or fatty degeneration of hepatocytes (steatosis) at a dose of 0.8 mg/kg, which led to a decrease in lipid inclusions by approximately 30% in comparison with the control group (statistically significant differences from the control, *t* test, $p < .05$). At a dose of 0.33 mg/kg on the eighth day, **1a** had a lesser effect than at a dose of 0.8 mg/kg as a decrease in the number of lipid inclusions was observed by approximately 20%. However, the revealed differences from the control group were statistically significant (*t* test, $p < .05$). On the 26th day of the experiment, the amount of lipids evened out to similar levels for all three groups (Figure 2C).

Thus, considering the obtained data on the amount of fat inclusions in hepatocytes, it can be concluded that compound **1a** helps to accelerate lipid metabolism and reduce its pathological accumulation in cells, that is, contributes to the development of steatosis. The most visible effect, in comparison with the control group of animals, was noted on the eighth day of the experiment with the introduction of **1a** at a dose of 0.8 mg/kg. That is, this compound is most effective in the early stages of therapy and helps to reduce both necrotic and steatosis damage to liver tissue.

In the current study, after a course of administration of a toxicant (sixth day of the experiment), a sharp increase in the activity of transaminases in the blood serum was observed (Figure 3A,B). This was evident because hepatocyte damage and the release of these enzymes into the peripheral blood occurred.[17–19] The ratio of AST to ALT (De Ritis coefficient) is diagnostically important for determining the localization of lesions. ALT (Figure 3A) is found to a greater extent in the liver and is a specific enzyme for the diagnosis of diseases associated with hepatocyte cytolysis. AST (Figure 3B) is found in many body tissues—in the liver, heart muscle, skeletal muscle, kidneys, brain, pancreas, lungs, white blood cells, and red blood cells.[20] Thus, according to the de Ritis coefficient (Figure 3C), which on the sixth day decreased almost twofold, in comparison with the reference values (Day 0), one can conclude that hepatocytes were damaged to a greater extent. However, in the following days of the experiment, a substantial decrease in the number of aminotransferases was observed in all groups, and the de Ritis coefficient became comparable with the reference (Day 0) values. This situation is explained by the following: the activity of aminotransferases released from hepatocytes on the sixth day of the experiment into the blood was close to zero by the eighth day of the experiment, the period of activity of these enzymes in the blood is limited; in humans, it is 17 ± 5 h for AST, 47 ± 10 h for ALT, respectively,[21] and the synthesis of new enzymes is difficult due to massive liver tissue damage.[22] However, by the 26th day of the experiment, compound **1a** at a dose of 0.33 mg/kg promoted a statistically significant (*t* test, $p < .05$) increase in the activity of the specific hepatic ALT enzyme in comparison with the control group of animals, but without exceeding physiological standards (reference values). This has the potential to be a confirmation of the normalization of hepatocyte function.

LDH is an enzyme that is found in many cells of the body. LDH catalyzes the conversion of lactate to pyruvate and vice versa, as it is capable of oxidizing and reducing NAD. A high level of this enzyme may indicate pathological conditions that may be associated with damage to the liver or muscles (e.g., acute myocardial infarction).[23]

Metabolites of carbon tetrachloride, selected as an inducer of liver damage, can damage many different body tissues, such as cardiac, reproductive, lung, kidney, and so on.[24–27] As LDH is present in almost all tissues, an increase or decrease in its activity over time may depend on the cytolysis of cells of various tissues at different time intervals.

In the control group, insignificant fluctuations in LDH in the dynamics of observation were observed. At the end of the CCl_4 administration course, a slight decrease in LDH activity was observed from 1075 ± 225 to 830 ± 80 U/L, and after 3 days, that is, on the eighth day of the experiment, the level of LDH activity increased to 1405 ± 381 U/L (Figure 3D). The results, combined with a significant increase in the activity of ALT and AST enzymes, suggest a pattern of preferential injury to liver cells and mild, delayed cytolysis over nonhepatic tissue. In the subsequent periods of observation, LDH activity did not differ significantly from the initial (reference) values.

In both experimental groups, a sharp increase in LDH on the eighth day of the experiment and in the further dynamics of observation was not detected, which indicates a decrease in tissue cytolysis compared with the control group. The decrease in LDH activity on the 26th day of the experiment to 446 ± 75 U/L, which is within the physiological norm, in the group administered with **1a** at a dose of 0.8 mg/kg, can be explained by the effect of succinic acid present in compound **1a** on energy metabolism. Namely, succinic acid, being a substrate of energy metabolism in the Krebs cycle, when entering the body leads to a decrease in the breakdown of glucose, the formation of lactic acid, and, accordingly, reduces the need for the production of LDH enzyme.

Bilirubin is the end product of heme breakdown. It is formed in phagocytes from biliverdin under the action of the enzyme biliverdin reductase.[28] In the experiment, there was an increase in the concentration of total bilirubin and indirect bilirubin (Figure 4A,C) and a decrease in direct bilirubin on the 12th day of the experiment (Figure 4B), which indicates impaired hepatocyte activity, impaired bilirubin metabolism, and development of jaundice.[29] An additional stimulus for disturbing the metabolism of bilirubin is a decrease in the albumin content in blood plasma (Figure 5B), which, in turn, is synthesized, in particular, in hepatocytes.[30] Albumin plays an important role in transporting indirect bilirubin to liver cells. Unbound bilirubin is insoluble and toxic to the body. Unbound bilirubin binds to plasma albumin and is transported to hepatocytes. In liver cells, bilirubin is conjugated to glucuronic acid to form bilirubin–diglucuronide (conjugated, direct bilirubin). After this, its excretion in the biliary tract occurs.[31] Thus, a decrease in the albumin concentration (Figure 5B) contributes to the accumulation of the toxic fraction of indirect bilirubin. However, on the 16th day of the experiment, in response to the administration of compound **1a** at a dose of 0.8 mg/kg, a sharp decrease in the concentration of indirect bilirubin occurred (Figure 4B). This may be explained by an improvement in the excretory function of the liver—the speed and number of reactions capturing pigment from the blood by the liver cells increased, and the rate of excretion into the bile ducts increased. This is due to increased activity of hepatocytes undamaged by the toxicant, as according to histological data (Figure 2), the amount of damage done by this day did not decrease, and hepatocyte cytolysis markers, such as ALT, AST, and LDH remained at the level of the control group of animals (Figure 3A–D). Under the influence of compound **1a** at a dose of 0.33 mg/kg, in comparison with the control, hepatocyte excretory function did not improve.

The increased activity of γGT and alkaline phosphatase usually indicates congestive processes in the liver, impaired biliary excretion. However, in the current study, a systematic decrease in the activity of γGT and alkaline phosphatase was observed until the 16th day. By the 26th day, γGT activity increased the level of the reference values (Day 0; Figure 4D), and the level of alkaline phosphatase remains the same as on the 16th day (Figure 4E). A decrease in the activity of these two enzymes simultaneously indicates total damage to liver tissue, leading to a decrease in the production of these enzymes. Extensive hepatic necrosis is also supported by the above results obtained for AST and ALT. As γGT is an enzyme that is not specific to the liver and is found in other organs, for example, the kidneys,[32] and carbon tetrachloride also damages the kidneys,[5,27] it can be assumed that an increase in γGT activity from the 16th to 26th day in all groups, including the control, is associated with a prolonged damaging effect of CCl_4 on kidney tissue.

As follows from Figure 4E, a decrease in alkaline phosphatase activity in the dynamics of observation occurs in parallel with a decrease in magnesium levels. Recent studies have shown a direct relationship between the activity of ALP and Mg^{2+}: A decrease in the concentration of Mg^{2+} and Zn^{2+} leads to a decrease in enzyme activity.[33] ALP, in its structure, has at least two Zn^{2+} ions and one Mg^{2+}. They are necessary for enzymatic activity and regulation of enzyme conformation.[34] On the 8th and 16th day of the experiment, an increase in the concentration of magnesium was observed in the experimental groups compared with the control group (*t* test, $p < .05$; Figure S2). However, the magnesium level remained below the reference values (Day 0), that is, the compound did not lead to a complete normalization of the metabolism of magnesium.

With extensive cirrhosis, a decrease in the synthetic function of the liver and a decrease in the concentration of whey protein and albumin are observed.[35,36] After the course of CCl_4 administration (the sixth day of the experiment), a decrease by about half in the total protein and its albumin and globulin fractions was observed (Figure 5). In the further dynamics of observation in the control group, 3 days after the administration of CCl_4 (the eighth day of the experiment), the concentration of total protein decreased by another 11.5% (Figure 5A), mainly due to a decrease in the fraction of globulins (Figure 5C), the concentration of albumin decreased by 4.1% (Figure 5B), and globulin by 23.8%. Moreover, the albumin–globulin ratio in the control group before the experiment was 1.98, on the sixth day—1.66, and on the eighth day—2.09. On the 12th day of the experiment, there was a slight increase in the concentration of total protein (57.8 ± 1.7 instead of 55.3 ± 1.9 g/L). At the same time, the level of albumin did not increase, but the level of globulins rose from 17.9 ± 1.1 to 21.0 ± 2.8 g/L on the 12th day of the experiment and to

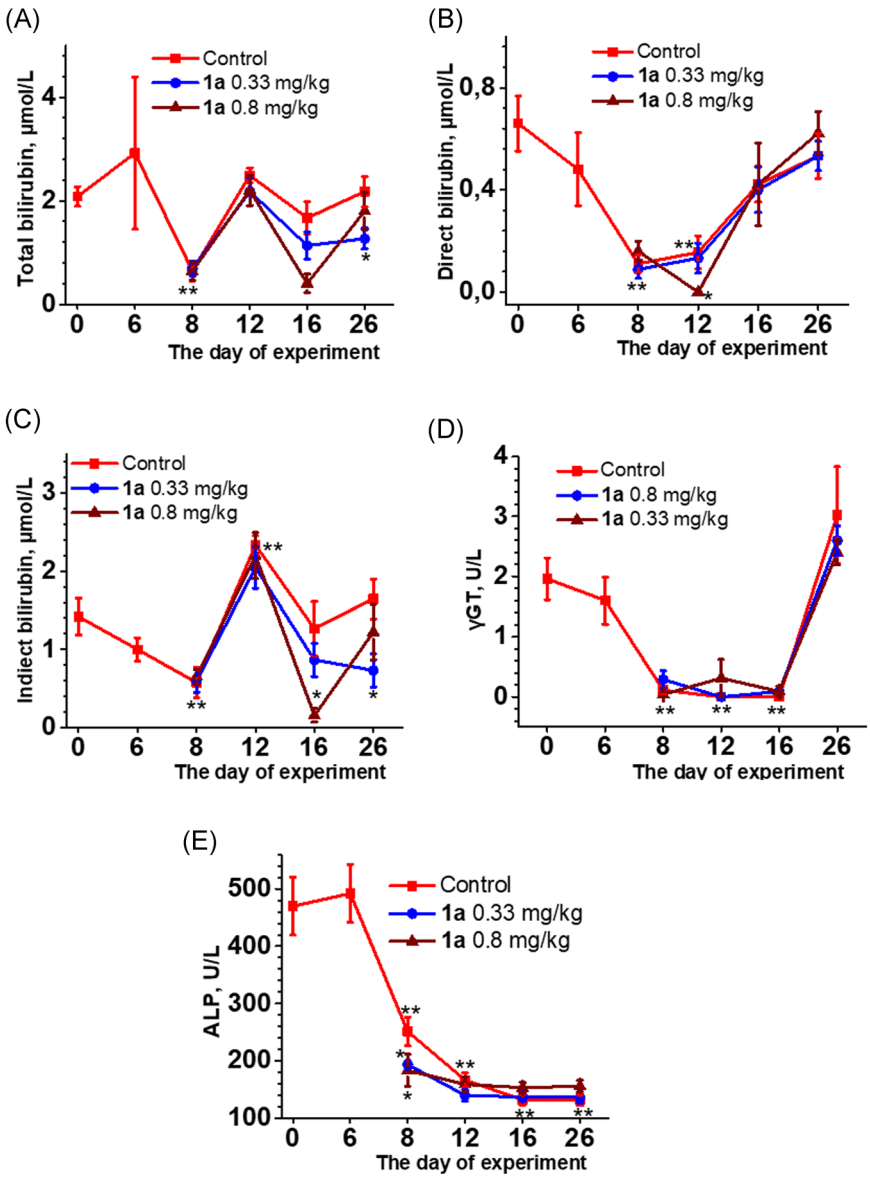


FIGURE 4 The effect of compound **1a** on bilirubin metabolism and markers of cholestasis in rats with toxic CCl_4-induced hepatitis: (A) total bilirubin; (B) direct bilirubin; (C) indirect bilirubin; (D) γ-glutamate transferase (γGT); (E) alkaline phosphatase (ALP). *Differences with the control group are statistically significant, *t* test, $p < .05$; **differences between the control group and the reference values are statistically significant, *t* test, $p < .05$

29.1 ± 7.1 and 28.6 ± 0.7 g/L on the 16th and 26th day, respectively. Moreover, the albumin/globulin ratio in the control group on the 12th, 16th, and 26th day was 1.77, 1.12, and 1.16, respectively. The study results indicate an impairment of the synthetic function of the liver in the control group.

The amount of the determined total protein in the blood serum upon the administration of compound **1a** in doses of 0.33 and 0.8 mg/kg on the days of measurements exceeded corresponding indices in the control group. The most pronounced, statistically significant (*t* test, $p < .05$) increase in protein was observed when **1a** was administered at a dose of 0.33 mg/kg on the 8th and 16th day of the experiment and at a dose of 0.8 mg/kg on the 12th day of the experiment (Figure 5A). Moreover, at an earlier stage of treatment, an increase in albumin was observed (Figure 5B), and at a later date—an increase in globulins (Figure 5C). On the 16th day, the level of globulins in the experimental groups almost reached the initial (reference) indicators (Figure 5C). Moreover, on the 12th and 16th day, the albumin–globulin ratio in the experimental groups decreased even more than in the control group and was 1.65 and 0.93, respectively, with **1a** at a dose of 0.33 mg/kg and 1.32 and 0.78 at a dose of 0.8 mg/kg.

Globulins are represented by proteins that perform many different functions. Among them are transport proteins that carry, for example, iron (ferritin, transferrin), protective (immunoglobulins), enzymes, and so forth. The results show that both in the control and experimental groups of animals that were administered **1a**, the activation of protein synthesis was aimed primarily at replenishing functional proteins (globulins), and not albumin. Moreover, under the action of **1a**, protein synthesis was enhanced, especially with the introduction of the compound at a dose of 0.33 mg/kg. Thus, compound **1a** stimulates the protein synthesis function of the liver.

ChE is a protein molecule, a serum enzyme synthesized by hepatocytes, and its level also characterizes the synthetic function of the liver.[37] In this study, compound **1a** at a dose of 0.33 mg/kg on the 12th day led to a mild increase in cholinesterase activity. At a dose of 0.8 mg/kg, a statistically significant (*t* test, $p < .05$) increase in

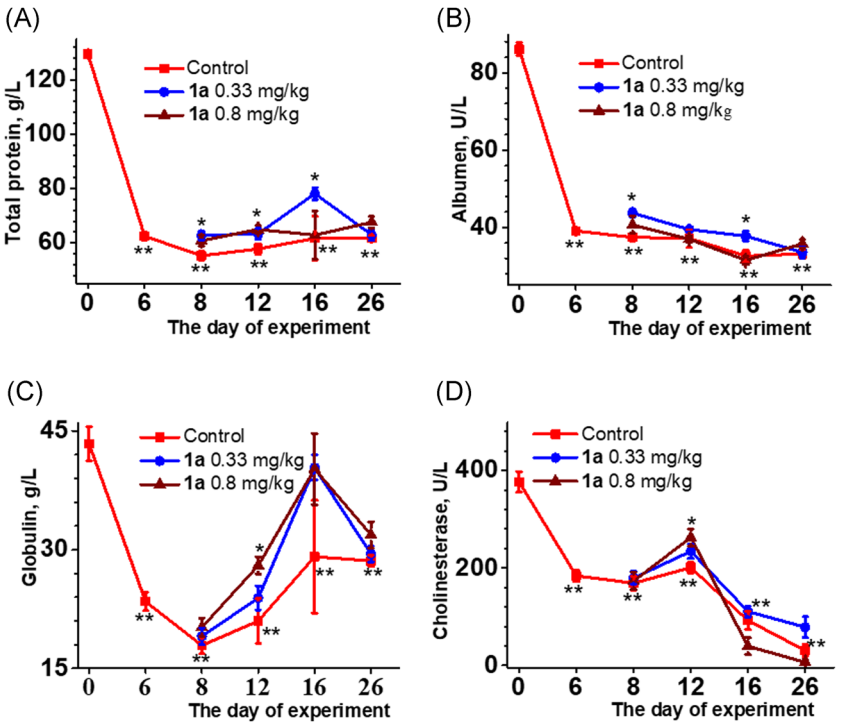


FIGURE 5 Effect of compound **1a** on the dynamics of changes in the biochemical markers of protein synthetic liver function in rats with toxic CCl_4-induced hepatitis: (A) total protein; (B) albumen; (C) globulin; (D) activity of serum cholinesterase. *Differences with the control group are statistically significant, *t* test, $p < .05$; **differences between the control group and the reference values are statistically significant, *t* test, $p < .05$

the activity of this enzyme in serum blood relative to the control group was observed (Figure 5D).

The cholesterol level (Figure S3) showed a 3.75-fold decrease under the influence of CCl_4. As 50% of cholesterol is synthesized in liver cells, the results also indicate a violation of the synthetic function of the liver associated with lipid metabolism. During the period of animal observation, the cholesterol level in the control group did not recover to the reference values. When compound **1a** was administered at a dose of 0.33 mg/kg, a statistically significant (*t* test, $p < .05$) increase in the serum cholesterol concentration was observed on the 6th, 16th, and 26th day of the experiment. However, the cholesterol level with the introduction of substance **1a** at a dose of 0.33 mg/kg did not reach the initial values. At a dose of 0.8 mg/kg, the substance had no effect on the cholesterol level in the blood serum.

Compound **1a** had no significant effect on indicators such as glucose (Figure S4) and serum iron (Figure S5).

Under the action of CCl_4, a 1.6-fold decrease in pancreatic enzyme lipase activity was observed. The lipase activity decreased even more on the 12th day of the experiment and did not fully recover until the end of the observation (Figure S6). On the 12th day, under the influence of compound **1a** in both doses, a significant increase in lipase activity was observed (*t* test, $p < .05$). On the 16th and 26th day of the experiment, when **1a** was administered at a dose of 0.33 mg/kg, the activity of the lipase enzyme was almost at the level of intact animals (Day 0), and was significantly higher in comparison with the control group (*t* test, $p < .05$; Figure S6). In the late stages of administration (16th and 26th day) at a dose of 0.8 mg/kg, compound **1a** had no effect on lipase activity. Thus, compound **1a** contributes to the restoration of pancreatic functions, with the greatest effect at a dose of 0.33 mg/kg.

4 | DISCUSSION

The search for innovative therapies for hepatic diseases has been studied over the past decades.[38] The present study evaluated the protective effects of **1a** compound, which is the conjugate of the pyrimidine derivative xymedon with succinic acid on hepatocytes of *Chang Liver* cell line under toxic exposure of D-galactosamine and on destructive–degenerative changes in the liver, such as steatosis, hepatocyte necrosis in laboratory animals, induced by the toxic influence of CCl_4.

A comparative assessment of the cytotoxicity (IC_{50}) of compound **1a** and succinic acid was carried out. It was shown that the IC_{50} of the studied conjugate (19.9 ± 0.8 mmol/L) on the cell line was less than that of succinic acid (14.1 ± 0.2 mmol/L). Moreover, a more pronounced cytoprotective effect of **1a** was revealed against the background of the cell damage caused by D-galactosamine, as compared with succinic acid. The data obtained indicate an improvement in protective properties as a result of the conjugation of xymedon with succinic acid. Because the damaging effect of D-galactosamine on the liver cells is associated with inhibition of the synthesis of RNA, protein, glycogen, glycoprotein, and glycolipid membrane components,[39] we suggest that the test substance has a protective effect on these mechanisms.

The results of the first experiment showed that in groups that were administered compound **1a** at medium and high doses, the majority of the biochemical parameters normalized, that is, the markers of cytolysis (ALT, ACT, LDH), bilirubin metabolism (total bilirubin), and cholesterol decreased, and the marker of the synthetic functions (globulins) increased. The minimal dose of compound **1a** exerted the most expressed effect on the structural–morphological organization of the liver. It can be suggested that based on the

obtained histological data that compound **1a** in the minimal dose exerts a stimulating effect on the regenerative–reparative processes in the hepatic tissue.

According to the results of the histological evaluation of liver tissue in the second experiment, it was found that **1a** is more effective against destructive–degenerative changes in the liver in the early stages of therapy. On the third day after modeling liver damage, it helped to reduce the necrotic damage and steatosis to liver tissue. In the later stages, **1a** was more effective against fatty degeneration of hepatocytes, and on the 12th and 26th day of therapy, it helped to reduce lipid accumulation in liver cells. A positive effect of **1a** on a number of biochemical parameters of the blood was noted. The study revealed the ability of **1a**, especially at a dose of 0.8 mg/kg, to effect on the metabolism of bilirubin. This effect was manifested in a decrease in total and unconjugated bilirubin. This indicates the normalization of the liver's ability to conjugate bilirubin to form bilirubin–diglucuronide (conjugated, direct bilirubin).[31]

In addition, a positive effect of **1a** on metabolism can be noted. So, by the amount of albumin, globulin, total protein, and ChE activity, the restoration of protein synthetic liver function was noted, and by the amount of cholesterol—normalization of lipid metabolism. A particularly pronounced effect was revealed in the case of intraperitoneal administration of the conjugate at a dose of 0.33 mg/kg. In addition, with the introduction of **1a**, especially at a dose of 0.33 mg/kg, a positive effect on the functional state of the pancreas was revealed, as under its influence, there was a normalization of lipase activity, reduced as a result of the toxic effects of CCl_4.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Data collection: Andrey A. Parfenov, Lilya F. Gumarova, Leyla R. Khasanshina, Grigory P. Belyaev, Darya A. Kondrashina, and Nail G. Nazarov. *Synthesis of test compound*: Irina V. Galyametdinova and Vyacheslav E. Semenov. *Literature analysis*: Andrey A. Parfenov, Nail G. Nazarov, and Vladimir E. Zobov. *Data analysis*: Alexandra B. Vyshtakalyuk, Andrey A. Parfenov, and Vladimir V. Zobov. *Design*: Alexandra B. Vyshtakalyuk, Vyacheslav E. Semenov, and Andrey A. Parfenov.

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How to cite this article: Vyshtakalyuk AB, Parfenov AA, Gumarova LF, et al. Conjugate of pyrimidine derivative, the drug xymedon with succinic acid protects liver cells. *J Biochem Mol Toxicol*. 2020;e22660. https://doi.org/10.1002/jbt.22660