



Protective effects of thymogen analogues, modified by D-alanine, in hydrazine liver damage

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Abstract

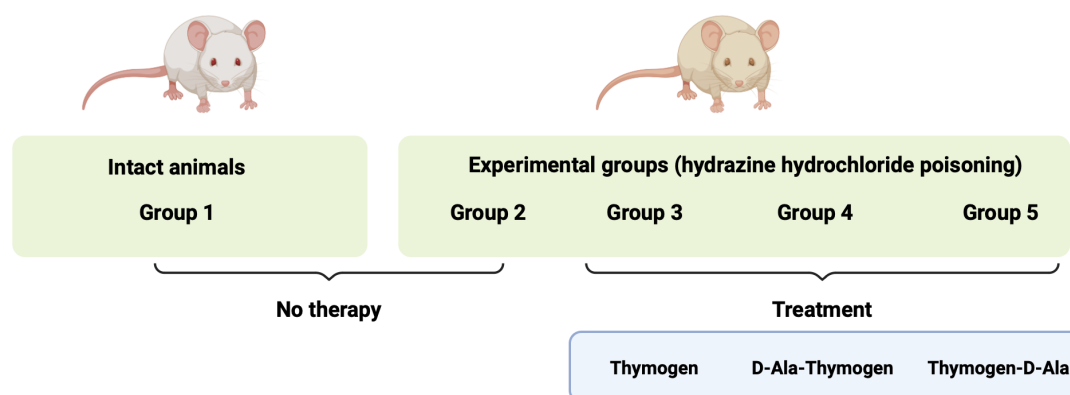
Introduction: It is known that the insertion of D-amino acids into the structure of a peptide molecule can increase its half-life. The pharmacological drug **Thymogen** was modified with **D-alanine** from the N- and C-terminus of the molecule. **The aim of the investigation** was to study the reparative and antioxidant activity of structural analogues of **thymogen** in toxic liver damage by hydrazine in rats.

Material and Methods: The investigation was conducted on 40 Wistar rats. Acute toxic liver damage was simulated with a single intraperitoneal injection of **hydrazine hydrochloride** at a dose of 50 mg/kg. **Thymogen** and its modified D-Ala analogues were administered intraperitoneally in equimolar doses for 5 days after intoxication. The animals were removed from the experiment 12 hours after the final administration of the peptides.

Results and Discussion: It has been established the reparative and antioxidant activities of **thymogen** structural analogues increased in conditions of liver damage with hydrazine. Peptides with the insertion of D-Ala reduced the level of free radical reactions more significantly in comparison with **thymogen**. All peptides comparably increased catalase activity in blood plasma and liver homogenate.

Conclusion: **Thymogen** analogues modified with D-Ala from the N- and C-terminus of the molecule can be considered as promising pharmacological substances for the development of new hepatoprotective drugs.

Graphical abstract



Keywords

thymogen, D-alanine, toxic liver damage, hydrazine, reparative activity of hepatocytes, free radical oxidation, catalase

Introduction

Currently, the established understanding of peptide regulation mechanisms of their physiological functions makes it possible to create new drugs based on regulatory peptide molecules. They are characterized by the absence of toxic and allergic side effects and high biological activity in small doses. However, the majority of natural peptide drugs have low resistance to the action of proteolysis enzymes. This fact limits their pharmacological affectivity and, as a consequence, widespread use of these preparations. It is also known that insertion of D-amino acids in the peptide molecule structures can increase their resistance to inactivation by proteolysis (Xi and Hansmann 2019; Deigin et al. 2022; Shi et al. 2022).

Liver diseases are important medical and social problems of modern society because they are mainly affect the working-age population (Balukova et al. 2018). This fact stimulates the necessity for the development of new effective hepatoprotective agents. The immunomodulator drug **Thymogen** ($\text{H}_2\text{N-L-Glu-L-Trp-COOH}$) is recommended in the complex treatment of viral hepatitis (Vyshkovsky 2022). One possible way to increase its effects was to reduce molecule biodegradation by proteases. The chemical peptide modification was applied to the **thymogen** molecule with **D-alanine (D-Ala)** which was incorporated to the N- and C-terminus of the molecule (Smakhtin et al. 2019; Chulanova et al. 2023a). Therefore, it seems relevant to find out the effects of structurally modified **thymogen** analogues in experimental models of liver damage, accompanied by the low immune reactivity of the body. Such situations most frequently occur in clinical practice (Plekhanov and Soboleva 2007).

The aim of our investigation was in study the reparative and antioxidant effects of **thymogen** and its analogues, structurally modified by **D-alanine**, in case of hydrazine liver damage.

Materials and Methods

Experimental animals

The study was carried out on male Wistar rats ($n=40$) weighing 180-220 g, obtained from the vivarium of the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences. The animals were under standard vivarium conditions. The experiment was approved by the Ethical Committee of Kursk State Medical University (Minutes No. 6 of November 6, 2018) and was conducted in accordance with ARRIVE and Directive 2010/63/EU principles of European Parliament and the EU Council for the animals used for scientific purposes.

Study substances

The pharmacological drug with molecular formula $\text{H}_2\text{N-L-Glu-L-Trp-COOH}$ known as **Thymogen** (CJSC Biomedical Research and Production Company “Cytomed”, Russia) was used in the form of solution for parenteral administration. The analogues of **thymogen** had the following molecular formulas: $\text{NH}_2\text{-D-Ala-L-Glu-L-Trp-COOH}$ (D-Ala-thymogen) and $\text{NH}_2\text{-L-Glu-L-Trp-D-Ala-COOH}$ (thymogen-D-Ala). Structurally modified **thymogen** analogues were synthesized in the peptide synthesis laboratory of IQChemical LLC (St. Petersburg, Russia). The purity of the pharmacological substances used was 98.3% according to high-performance liquid chromatography. According to

instructions for **thymogen** application, a single dose for rats was 1 mcg/kg for body weight (Vyshkovsky 2022). The drug was then diluted to the required concentration with physiological solution (0.9% NaCl). The analogues were used in equimolar with **thymogen** concentrations – 1.2 µg/kg for body weight. Portions of **thymogen** analogues were weighed, and then these powders were dissolved in physiological solution. All peptides were administered intraperitoneally in a volume of 0.1 mL for 5 days after the administration of hepatotoxic poison with an interval of 24 hours.

Experimental model

Toxic liver damage was simulated with a single intraperitoneal injection of **hydrazine hydrochloride** ($\text{N}_2\text{H}_4 \cdot 2\text{HCl}$, Research Production Company Technology-SPb, purity 99.5%, GOST 22159-76) at a dose of 50 mg/kg of body mass, which was dissolved in physiological solution (0.9% NaCl) and administered in a volume of 0.1 mL. This experimental toxic agent is recommended for preclinical research of new hepatoprotectors (Mironov 2012).

Experimental design

The animals were divided into 5 groups (with 8 animals in each): 1st – intact animals without toxic agent (administration of physiological solution (0.9% NaCl) intraperitoneally); 2nd group – control with toxic agent and without peptides ($\text{N}_2\text{H}_4 \cdot 2\text{HCl}$ + 0.9% NaCl intraperitoneally); 3rd group – toxic agent and administration of **thymogen** – $\text{N}_2\text{H}_4 \cdot 2\text{HCl}$ +**thymogen**; 4th group – toxic agent and administration of D-Ala-thymogen – $\text{N}_2\text{H}_4 \cdot 2\text{HCl}$ +D-Ala-thymogen; and 5th group – toxic agent and administration of thymogen-D-Ala – $\text{N}_2\text{H}_4 \cdot 2\text{HCl}$ +thymogen-D-Ala. Rats of groups 1 and 2 were injected with physiological solution intraperitoneally in an equivalent volume (0.1 mL). Under chloral hydrate anesthesia (300 mg/kg), the animals were removed from the experiment by exsanguination and blood sampling from the right ventricle 12 hours after the final administration of **thymogen** and its structural analogues.

Research methods

For biochemical investigations, blood in a volume of 4–5 mL was taken from the right ventricle with a heparinized syringe, and the liver was isolated. The organ parts were fixed in a 10% solution of neutral formalin prepared in 0.1 M phosphate buffer. Then histological sections of the liver were prepared according to the generally accepted

method by embedding in paraffin. The sections of 7–10 µm thick were prepared, and subsequently stained with hematoxylin and eosin. To analyse the regenerative activity of the liver, the mitotic index (MI), index of binucleate hepatocytes (BNH) and the surface of hepatocyte nuclei (SHN) were determined using a Nikon ECLIPSE NI light microscope (Japan).

A liver homogenate was prepared to determine the activity of free radical reactions and the state of antioxidant protection of the affected organ. A sample of liver (600–800 mg) was placed in a 10-fold volume of ice-cold saline, homogenized and centrifuged at 3000 rpm for 10 minutes. The resulting supernatant was used for further investigations (Fomenko et al. 2017).

The state of the body's pro- and antioxidant systems was assessed by the concentration of malonic dialdehyde (MDA) and catalase activity in blood plasma and liver homogenate. The level of MDA was determined by reaction with thiobarbituric acid on a PE-5300VI spectrophotometer (Russia). Catalase activity was determined on the same device by reaction with 3% solution of hydrogen peroxide.

Statistical analysis

The obtained results were processed using STATISTICA 13.3 (TIBCO Software Inc., USA). The data in this article are presented as median (Me) and interquartile range (Q1; Q3). The significance of the differences in the obtained data was determined using the nonparametric Mann-Whitney test (comparison of intact and control groups of animals) and the Kruskal-Wallis test. Differences were considered statistically significant at $p < 0.05$.

Results and Discussion

It was found that **hydrazine hydrochloride** caused a significant decrease in catalase activity, an increase in the concentration of MDA, a decrease in SHN and a compensatory increase in BNH. Under conditions of intoxication with hepatotropic poison, the peptide injections increased the reparative regeneration of hepatocytes, helped to reduce the activity of free radical reactions and stimulated the antioxidant system. Histological examination of liver sections revealed significant differences between groups of animals injected with **thymogen** and its modified analogues. The administration of D-Ala-thymogen resulted in an 8-time MI increase ($p < 0.001$), thymogen-D-Ala – by 6.2 times ($p < 0.001$). Administration of **thymogen** did not increase MI under these conditions (Table 1).

Table 1. Reparative effects of **thymogen** and its structural analogues in acute toxic liver damage by hydrazine (Me [1Q; 3Q])

Experimental groups (n = 40)	MI, (%)	BNH, (%)	SHN, µm ²
Intact group (n = 8)	3.09 (2.71; 3.60)	11.33 (10.68; 12.24)	40.64 (39.32; 41.80)
$\text{N}_2\text{H}_4 \cdot 2\text{HCl}$ -control (n = 8)	2.80 (1.80; 3.70)	16.45 (15.40; 17.30)***	38.40 (37.40; 38.90)***
$\text{N}_2\text{H}_4 \cdot 2\text{HCl}$ + thymogen (n = 8)	2.30 (1.80; 2.70)	13.10 (12.20; 14.30)+++	39.60 (38.60; 41.40)+++
$\text{N}_2\text{H}_4 \cdot 2\text{HCl}$ +D-Ala-thymogen (n = 8)	22.50 (20.90; 23.40)+++	24.30 (23.50; 25.20)+++	63.60 (62.30; 68.60)+++
$\text{N}_2\text{H}_4 \cdot 2\text{HCl}$ +thymogen-D-Ala (n = 8)	17.31 (16.40; 17.80)+++	16.30 (15.70; 17.60)	73.60 (71.90; 78.90)+++

Note: *** – $p < 0.001$ compared to the intact group; +++ – $p < 0.001$ compared to the control group.

SHN increased most significantly after the use of structural analogues of **thymogen**. A statistically significant increase in BNH ($p < 0.001$) was observed only after the injection of analogue modified with D-Ala from the N-terminus of the molecule.

Also, **thymogen** analogues had a pronounced effect on MDA concentration in blood plasma. The most pronounced effect in blood plasma was found in the group injected with thymogen-D-Ala ($p < 0.01$), in the homogenate – with D-Ala-thymogen ($p < 0.01$). In addition, all peptides comparably increased catalase activity in blood plasma and liver homogenate (Table 2).

It is known that the hepatoprotective effect of **thymogen** may be realized through the correction of immune function (Okovityi and Gaivoronskaia 2002). That **thymogen** is considered to be an immunoregulatory peptide. When **thymogen** interacts with target cells, an intracellular regulatory cascade mechanism is triggered, accelerating cell proliferation and differentiation (Uspensky et al. 2023). It has been established that it enhances the transmembrane exchange of Ca^{2+} ions in lymphocytes, as a result of which the intracellular balance of cAMP and cGMP changes and the activity of protein kinases increases (Khavinson et al. 2013). These changes can stimulate cellular metabolism in lymphocytes and production of cytokines, which can act as growth factors and enhance regeneration of affected tissues. Perhaps the same mechanism may be realized in liver pathology as well, as this organ includes different immune cells, which can stimulate hepatocytes repair.

Direct action of **thymogen** on hepatocytes cannot be excluded either. Partly it is proved by the characteristics of **thymogen** pharmacokinetics. Thus, the investigation of **thymogen** distribution in the body using a radioactive label found that the highest levels of its absorption were in the liver, thymus, plasma, adrenal glands, kidneys, and lymph nodes (Khavinson et al. 2017). Maybe due to the high accumulation of **thymogen** in the liver, its reparative effects are realized through the same mechanisms at the levels of lymphocytes and hepatocytes.

Previously, an increase in the pharmacological activity of **thymogen** analogues, modified with D-Ala from the N- and C-terminus of the molecule, was detected in skin wounds (Smakhtin et al. 2019) and liver damage with

carbon tetrachloride (Chulanova et al. 2023a; Chulanova et al. 2023b). Increased pharmacological activity of new **thymogen** analogues may be due to the prolongation of their action caused by less inactivation. **Thymogen** can also normalize the balance of matrix metalloproteinases during skin damage (Kudriavtceva et al. 2019; Petlenko et al. 2019). It is known that imbalance of these enzymes is associated with the severity and duration of liver diseases (Koroy et al. 2023). Probably, the reparative effect of structurally modified analogues with the addition of D-Ala from the N- and C-terminus of the peptide is realized through the mechanisms similar to those of the original drug.

In addition, D-Ala is able to maintain mitochondrial membrane potential (Iwata et al. 2022; Shi et al. 2022), which can reduce the production of free radicals that disrupt the integrity of the cell membrane and organelles.

In a previous investigation with structurally modified D-Ala **thymogen** analogues in carbon tetrachloride hepatopathy, we showed that the thymogen-D-Ala peptide had a more perspective pharmacological activity (Chulanova et al. 2023a; Chulanova et al. 2023b). Comparison with the results of these investigations suggests that the analogue with the incorporation of D-Ala from the peptide C-terminus exhibits high biological activity in experimental models of liver damage with different levels of immune reactivity.

Conclusion

Thus, modification of **thymogen** with D-alanine helps to increase the reparative and antioxidant effects in case of liver damage induced by hydrazine hydrochloride. The results of this investigation can be used for further studies and development of drugs based on modified **thymogen** analogues to increase the regenerative activity of hepatocytes and, probably, other damaged tissues.

Conflict of interest

The authors declare no conflict of interests.

Data availability

All of the data that support the findings of this study are available in the main text.

Table 2. Antioxidant effects of **thymogen** and its chemically modified analogues with D-alanine in toxic liver damage by hydrazine (Me [1Q; 3Q])

Parameter	Intact group (n = 8)	$\text{N}_2\text{H}_4 \cdot 2\text{HCl}$ - control (n = 8)	$\text{N}_2\text{H}_4 \cdot 2\text{HCl}$ + thymogen (n = 8)	$\text{N}_2\text{H}_4 \cdot 2\text{HCl}$ + D-Ala-thymogen (n = 8)	$\text{N}_2\text{H}_4 \cdot 2\text{HCl}$ + thymogen-D-Ala (n = 8)
Activity of free radical reactions					
MDA in blood plasma, $\mu\text{mol/liter}$	3.68 (3.25; 3.97)	5.16 (3.84; 6.01)	3.14 (2.83; 4.02) ⁺	3.58 (2.85; 4.28) ⁺	2.52 (2.00; 3.38) ⁺⁺
MDA in liver homogenate, $\mu\text{mol/liter}$	3.77 (2.59; 4.32)	6.50 (5.25; 7.78) ^{**}	4.21 (3.62; 5.73)	3.19 (2.53; 4.16) ⁺⁺	3.78 (3.41; 5.18) ⁺
Catalase activity					
Catalase in blood plasma, mcat/liter	11.29 (9.24; 12.89)	7.60 (6.73; 9.32) [*]	11.77 (10.07; 12.68) ⁺⁺	10.99 (9.96; 13.84) ⁺	9.72 (8.75; 12.66) ⁺
Catalase in liver homogenate, mcat/liter	6.61 (5.07; 7.53)	4.96 (4.20; 5.44) [*]	6.43 (4.77; 7.20) ⁺	6.72 (5.25; 7.10) ⁺	6.08 (5.17; 7.57) ⁺

Note: * – $p < 0.05$, ** – $p < 0.01$ compared to the intact group; + – $p < 0.05$, ++ – $p < 0.01$ compared to the control group.

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