BioNanoScience (2022) 12:901–911
https://doi.org/10.1007/s12668-022-01010-z

Antiapoptotic Effect of Pyrimidine-Derived Drug Xymedon and Its Conjugate with *L*-Ascorbic Acid on Chang Liver Cells Under Apoptosis Induced by d-Galactosamine

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Accepted: 3 July 2022 / Published online: 9 July 2022

Abstract
The present study aims to evaluate the effect of the pyrimidine-derived drug Xymedon and its conjugate with L-ascorbic acid, which exerts hepatoprotective activity on the apoptosis of normal human hepatocytes of the *Chang Liver* cell line, which has been damaged by the toxic effect of *d*-galactosamine (*d*-GLA). The results of the study allowed the authors to evaluate the contribution of the mechanism of apoptosis in the manifestation of hepatoprotective effect of pyrimidine-derived drug Xymedon and its conjugate with L-ascorbic acid and identify their influence on the key pathways of apoptosis. All experiments were performed on the *Chang Liver* cell line. The cytoprotective and antiapoptotic effect of the tested compounds was studied against the background of *d*-GLA-induced injuries. The number of dead and alive cells was counted using Hoechst and propidium iodide staining of cells. Apoptosis markers were studied using labeled antibodies (MilliporeSigma, USA) to phosphatidylserine, BAD, activated caspases 8 and 9, protein p53, kinases ATR, CHK1, and CHK2 followed by marker detection using the Cytell cell imaging system and MagPix multiplex system. The study results showed that Xymedon and its derivative with L-ascorbic acid exerted an antiapoptotic effect significantly reducing the number of annexin-positive cells and apoptotic markers BAD (Ser112), activated caspases 8 (Asp384) and 9 (Asp315), protein p53 (Ser46), kinases ATR (total), CHK1 (Ser345), and CHK2 (Thr68). The results obtained showed that the cytoprotective effect of pyrimidine-derived drug Xymedon and its conjugate with *L*-ascorbic acid is realized by reducing apoptotic changes in cells. The revealed antiapoptotic effect of the compounds may be the key mechanism for the realization of the hepatoprotective effect of the compounds, which was shown in early studies. It was also shown that conjugation of Xymedon with *L*-ascorbic acid significantly enhanced the antiapoptotic effect.

Keywords Pyrimidine derivatives · Conjugate · Apoptosis · Hepatocytes · *d*-Galactosamine

1 Introduction

In modern society, nearly everyone faces the effects of industrialization, in particular, environmental pollution from toxic substances. One of the main organs that plays a key role in the excretion of exogenous and endogenous toxic substances from the body is the liver. High doses or long-term exposure to toxicants can lead to liver tissue transformations that are difficult to reverse (cirrhosis, necrosis, fibrosis, steatosis, etc.) [1]. Liver damage results in serious systemic disorders in various organs. The study focused on the pyrimidine-derived drug Xymedon (compound 1) and its conjugate with *L*-ascorbic acid (compound 2). The structural formula of compounds 1 and 2 is presented in Fig. 1.

Xymedon (1-(2-hydroxyethyl)-4,6-dimethylpyrimidine-2-one) is a Russian pharmaceutical compound, that stimulates tissue regeneration. This chemical compound was synthesized at the Institute of Organic and Physical Chemistry in 1966. Xymedon exerts a wide spectrum of effects including membrane stabilizing, regeneration, and immune stimulation [2].

Previously, successful conjugation of Xymedon with a few biogenic acids was shown; this included para-aminobenzoic

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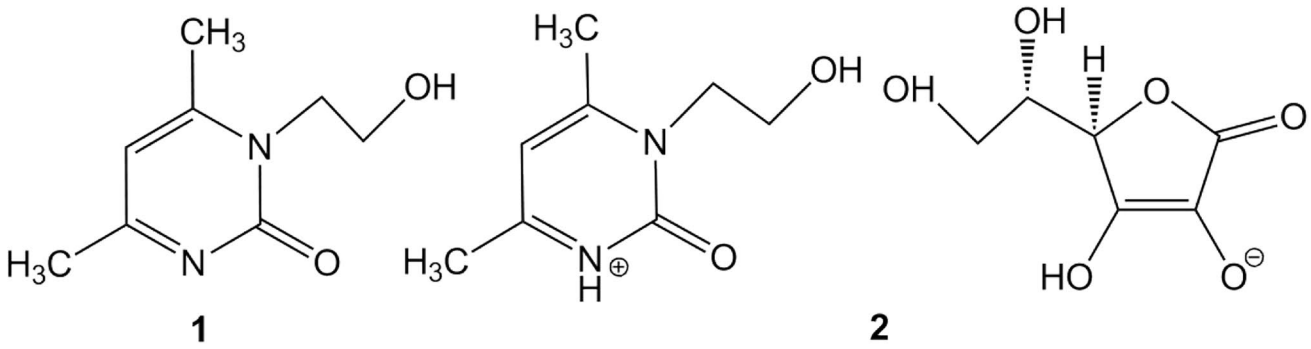

Fig. 1 Structural formula of 1—Xymedon (compound 1); 2—Xymedon conjugate with *L*-ascorbic acid (compound 2)

acid and succinic acid [3–9]. The effectiveness of Xymedon and its conjugate with *L*-ascorbic acid was shown in laboratory conditions in vivo to be promising hepatoprotectors [10]. Screening studies in vivo revealed that conjugation with *L*-ascorbic acid provided a more expressed and stable positive effect in comparison with the initial molecule of Xymedon or its conjugates with other biogenic acids. It was shown that Xymedon and its conjugate with *L*-ascorbic acid actively enhanced the regeneration of liver parenchyma, leading to the normalization of key biochemical markers in animal blood associated with liver damage induced by tetrachloromethane. These compounds were more effective than Thiotriazoline [10]. However, the mechanism of hepatoprotective effects of Xymedon and its conjugate with *L*-ascorbic acid has been understudied. The aim of the study was to establish the influence of Xymedone and its conjugate with *L*-ascorbic acid on apoptosis and biomarkers, which are involved in the process of apoptosis on the human hepatocyte cell line Chang Liver. Chang Liver cell damage was modeled by *d*-galactosamine (*d*-GLA), which causes a deficiency of UDP-glucose and UDP-galactose, leading to an increase in cellular oxidative stress, peroxidation of lipids, and ROI production, thereby contributing to cell damage [11]. The results of the study allowed the authors to evaluate the contribution of the mechanism of apoptosis in the manifestation of the hepatoprotective effect of pyrimidine-derived drug Xymedon and its conjugate with *L*-ascorbic acid and identify their influence on the key pathways of apoptosis.

2 Materials and Methods

2.1 Synthesis of the Studied Compounds

Xymedon (1-(2-hydroxyethyl)-4,6-dimethylpyrimidine-2-one) and Xymedon conjugate with *L*-ascorbic acid were synthesized by the previously described methods from 2-dihydro-4,6-dimethylpyrimidine-2-oh and 2-chloroethanol [4, 12].

2.2 Culturing of the Cell Line Chang Liver

The *Chang Liver* cell line culture (RRID:CVCL_0238) was obtained from the Russian Collection of Cell Cultures of the Institute of Virology named after D.I. Ivanovskiy (Moscow). For the study, the cells were cultivated in an incubator at 37 °C and 5% CO_2 in an Eagle's Minimum Essential Medium, 10% fetal bovine serum, 1% essential amino acids, and gentamicin [13].

2.3 Study of Protective Properties

The cells were separated from the cultural flask with a mixture of trypsin and versene at a ratio of 1:3. Living cells were counted in the obtained suspension using Goryaev's count chamber and trypan blue staining. Cell suspension with a concentration of 10^5 cells/mL was prepared and pipetted into a 96-well plate with 200 µL per well. To evaluate the cytoprotective properties in vitro, the studied compounds 1 and 2 at concentrations of 125, 250, and 500 µM were added together with the toxicant *d*-galactosamine (*d*-GLA) (Sigma-Aldrich, USA) at a concentration of 150 mM and incubated for one more day [14, 15].

To count living and dead cells, a complete medium with fluorescent stains was prepared. The medium included 198 µL of complete growth-promoting medium + 2 µL Hoechst 33,342 (Sigma-Aldrich, USA) (concentration of 1 mg mL) + 0.5 µL of propidium iodide (Sigma-Aldrich, USA) per well. Furthermore, cultural fluid was replaced with the prepared nutrient medium with stains and incubated for 45 min. After the incubation, dead and living cells were analyzed using the Cytell™ Cell Imaging System (GE Healthcare, USA) according to the standard protocol.

2.4 Method of Apoptosis Modeling for Chang Liver Cells at Different Concentrations of d-GLA

Right before the experiment, the cells were separated from the cultural flask with a mixture of trypsin and versene at a ratio of 1:3.. Living cells were counted in the obtained suspension using a Goryaev's count chamber and trypan blue staining. A

suspension with a concentration of 20^5 cells/mL was prepared. The obtained suspension was pipetted at a volume of 200 µL into the wells of a specialized plate (Agilent, USA) for the real-time cellular analyzer (RTCA) xCelligence (Agilent, USA). Twenty-four hours later, *d*-GLA was added at concentrations of 40, 45, 50, 55, 60, 65, and 70 mM in two replications. The registration of signals was automatically performed using the respective software every 10–15 min for 48 h (for 24 h, the cells were not exposed to *d*-GLA; for the following 24 h, the cells were exposed to *d*-GLA). Based on the results of electric impedance monitoring and automated calculation of the Cell Index, the dependency of the Cell Index from the time was calculated. In the case of an increase in biomass, the Cell Index increased. In the case of partial or the complete death of cells, it decreased. For the integral evaluation of the impact of various concentrations of *d*-GLA, after the introduction of the substance, we determined the inclination of the curve (change of the Cell Index) in time. The calculations were made automatically with RTCA software (Agilent, USA) which was supplied with the apparatus. The inclination was used to describe the rate of change of the curve within the set time interval. For each chosen well, the software calculated the curve inclination of the Cell Index of the wells in the chosen period, after fitting the points to the straight line. Thus, the curve inclinations were identified for each group of cells in the required time interval: between hours 24 to 48 of the experiment (time of *d*-GLA exposure). The positive dynamics of this parameter are associated with an increase in the Cell Index (increase in the cell biomass). The negative dynamics of this parameter show a decrease in the Cell Index (reduction of cell biomass). Also, it was found that the larger the deviation of this parameter was from zero on either the positive or negative side, the greater the rate of an increase or decrease of the Cell Index, respectively.

The count of annexin-positive cells revealed the transition of cells to apoptosis. The cells were stained according to the protocol of the Annexin V-Cy3 Apoptosis Detection Kit (Sigma-Aldrich, USA), with further detection and a count of the stained annexin-positive cells using the Cytell™ Cell Imaging System (GE Healthcare, USA) and its respective software.

2.5 Study of Molecular Markers of Apoptosis

The cells were pipetted into the wells of a 24-well plate at a concentration of 10^5 cells/mL (500 µL into each well) and incubated for 24 h. Furthermore, along with the studied compounds 1 and 2 at concentrations of 125, 250, and 500 µM, the toxicant *d*-GLA was introduced at a concentration of 55 mM. After which, the cells were incubated for another day. To specify the transition of the cells to the state of apoptosis, an annexin-positive cell count was made similar to the method described above.

To evaluate the effect of the studied pyrimidine-derived Xymedon and its conjugate with *L*-ascorbic acid on the molecular mechanisms of apoptosis, the changes in the markers of early apoptosis and markers of DNA damage were studied. The studied markers of early apoptosis included the proapoptotic protein BAD (Ser112), initiation-activated caspase-8 (CASP8, Asp384), and caspase-9 (CASP9, Asp315). The markers were studied in the lysates of cells according to the protocol of the Early Apoptosis Magnetic Bead Kit (MilliporeSigma, USA) with measurement of the parameters by MagPix (MerkMillipore, USA).

The studied markers of DNA damage included protein p53 (Ser46) and signal molecules: protein kinase ATR (total), phosphorylated (activated) kinases CHK1 (Ser345), and CHK2 (Thr68). The markers of DNA damage were studied in the lysates of cells according to the kit protocol DNA Damage/Genotoxicity Magnetic Bead Kit (MilliporeSigma, USA) with measurement of the parameters by MagPix (MerkMillipore, USA).

For the study of apoptotic markers, preliminary lysis of cells was performed. The cells were exposed to the studied compounds and the toxicant according to the instructions for the MicroRotofor Lysis Kit (BioRad, USA). The Protease Inhibitor Cocktail (Sigma-Aldrich, USA) was also added. Furthermore, all samples were aligned according to the concentration of total protein up to 100 µg/mL. This was done by measuring the levels of protein in the samples using Bradford's technique, the Quick Start Bradford Protein Assay Kit (BioRad, USA), and microplate spectrophotometer Epoch (BioTech, USA). To make the calibration graph, the authors used bovine serum albumin (Amresco, USA).

2.6 Statistical Analysis

All the obtained experimental data were included in the statistical analysis. The mean values and standard error of the mean were calculated. The figures show mean values for each standard parameter and standard error. For each sampling, the normality of the distribution was assessed with the Kolmogorov–Smirnov test. In the case of normal distribution, the samplings were compared using the Student's *t*-test. In the case of non-normal distribution, the samplings were compared using the Mann–Whitney test. The statistical processing was performed with the software SPSS 13.0. The visualization of the data was made by the Origin Lab 2018 software and MS PowerPoint 2010.

3 Results

3.1 Cytoprotective Effect of the Tested Compounds

The results of the study on the protective properties of compounds 1 and 2 revealed a significant increase in the survivability of *Chang Liver* cells exposed to the toxicant

d-GLA at a concentration of 150 mM (Fig. 2). All the studied compounds exerted a positive effect at all the studied concentrations.

3.2 Selection of the Concentration of a Toxicant for Apoptosis Induction in Chang Liver Cells

Before the experiment on the influence of compounds 1 and 2 on apoptosis, the authors performed some tests to determine the concentration of *d*-GLA that provided the lowest count of dead cells and the highest count of apoptotic cells. The tests were done with RTCA xCelligence. Figure 3A shows an inclination graph of the changes in the Cell Index for the time interval between 24 and 48 h (the time of exposure of *d*-GLA) determined by the respective software.

The graph (Fig. 3A) shows that the number of cells in the control group (0 mM *d*-GLA) continued growing because the rate of changes in the Cell Index was positive. In the groups of cells that were exposed to *d*-GLA at concentrations of 70, 65, 60, and 55 mM in the nutrient medium, there was partial or complete death of cells within 24 h. At concentrations of 50, 45, and 40 mM, *d*-GLA led to an insignificant increase in the Cell Index over time, which indicated inhibition of cell culture growth in comparison to the control group.

Based on the results obtained from the measurement of electric impedance using RTCA xCelligence, it was suggested that the concentration of *d*-GLA that induced apoptosis and minimal death of *Chang Liver* cells ranged between 50 and 55 mM. The evaluation of the influence of *d*-GLA on the initiation of apoptosis showed that a *d*-GLA concentration of 50 mM induced apoptosis in 18.7 ± 2.3% of cells. When the concentration of *d*-GLA was 55 mM, the number of cells in a state of apoptosis was 1.7 times higher (32.3 ± 3.3%). The analysis of the dependence of the number of dead and annexin-positive cells from the concentration of *d*-GLA showed that an increase in the concentration of *d*-GLA by each 5 mM led to a decrease in the number of apoptotic cells by 1.3 times and an increase in the number of dead cells by 1.27 times (Fig. 3B).

The study results showed that at a concentration of 55 mM *d*-GLA there was the largest number of apoptotic cells and the least number of dead cells. Thus, this concentration was chosen for the evaluation of the influence of the studied compounds (pyrimidine derivatives) on the mechanism of apoptosis.

3.3 Influence of the Tested Substances on the Apoptosis of Chang Liver Cells

The study results showed that in comparison to the control *d*-GLA group (the share of annexin-positive cells was 20.21 ± 5.6%), compound 2 at concentrations of 500, 250, and 25 μM significantly contributed to a decrease in the share of detected annexin-positive cells by 2.3 (8.45 ± 0.43%), 3.7 (5.4 ± 0.25%), and 3.96 times (1.46 ± 0.32%); and compound 1 at concentrations of 500 and 250 μM—by 4.3 (4.63 ± 0.42) and by 3.6 times (5.8 ± 0.54%), respectively. At a concentration of 125 μM, compound 1 was ineffective (Fig. 3C). Thus, it was shown that for the studied derivatives of pyrimidine, the most effective concentrations were 500 μM for compound 1 and 125 μM for compound 2.

3.4 Influence of the Tested Substances on the Markers of Early Apoptosis

The studied markers of early apoptosis included the protein BAD (Ser112), activated caspase-9 (Asp315), and caspase-8 (Asp384). A significant decrease in the intensity of fluorescence of protein BAD (Ser 112) in the cell samples exposed to compound 2 and *d*-GLA at 55 mM was observed in comparison to the control group which was exposed only to *d*-GLA at 55 mM (MFI = 695 ± 89). Thus, the

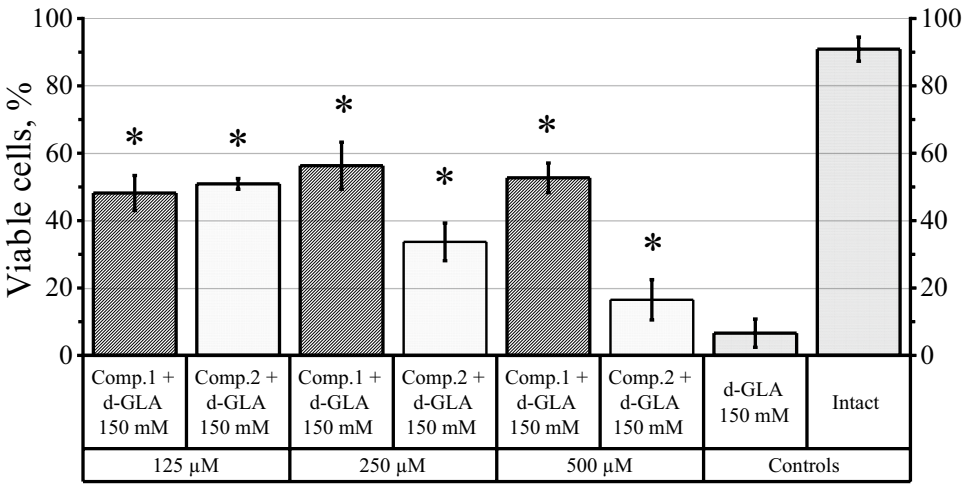

Fig. 2 Cytoprotective effect of compound 1 and compound 2. The asterisk indicates differences from the control group *d*-GLA. Mann–Whitney test, $p < 0.05$

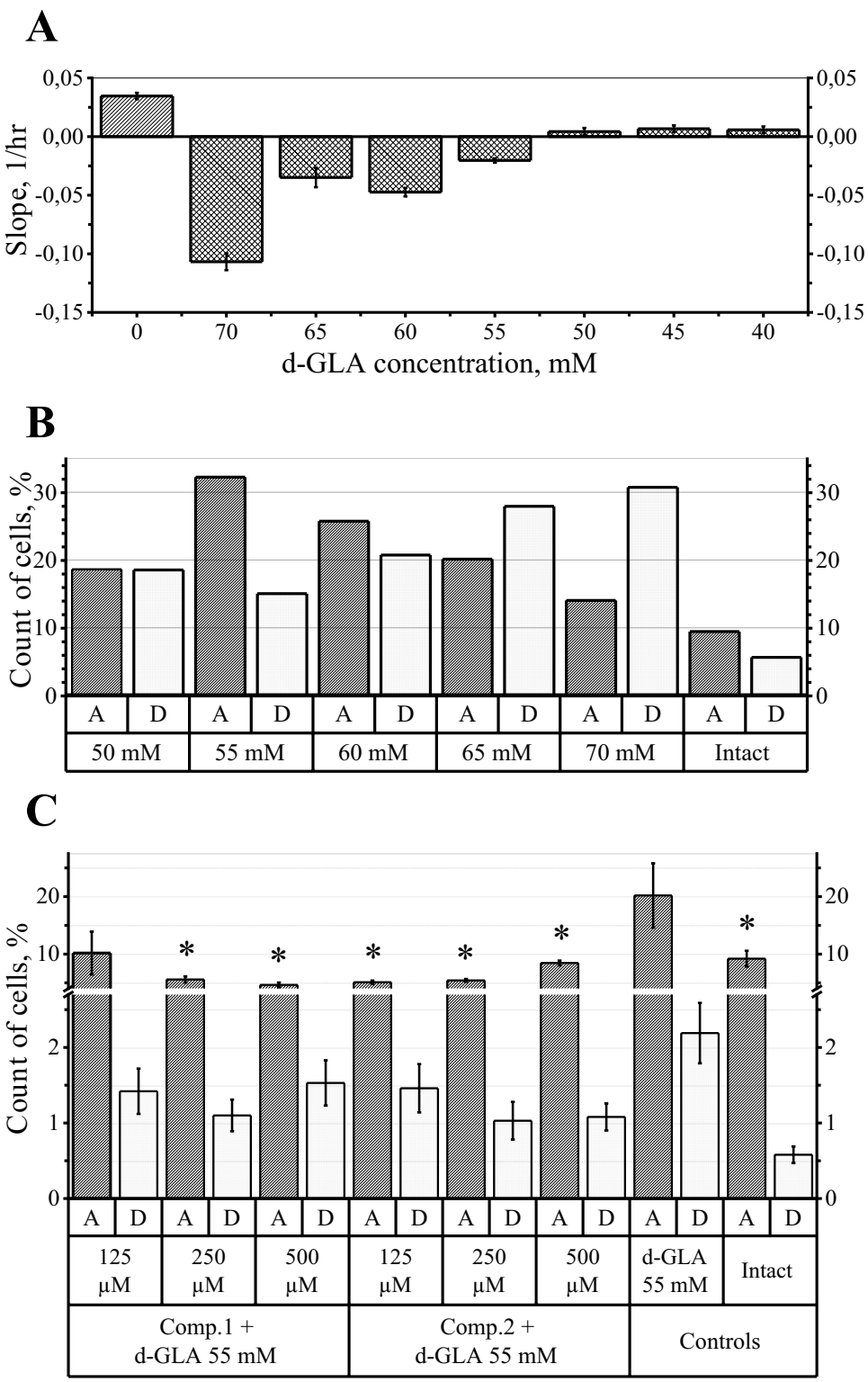

Fig. 3 Influence of *d*-GLA and compounds on the apoptosis of the cell line *Chang Liver.* **A** Influence of *d*-GLA on the rate of the *Chang Liver* Cell Index changing measured by RTCA xCelligence. **B** Apoptotic cell count when affected by different concentrations of *d*-GLA, where A—annexin-positive cells; D—dead cells. **C** Antiapoptotic effect of compound 1 and compound 2, where A—annexin-positive cells; D—dead cells. The asterisk indicates differences from the control group *d*-GLA are significant as shown by the Mann–Whitney test, $p < 0.05$

application of compound 2 at a concentration of 125 µM led to a decrease in the intensity of protein BAD fluorescence (Ser112) by 13.9 times (MFI = 47 ± 3.7) as was detected by MagPix. The introduction of compound 2 at concentrations of 250 and 500 µM reduced the intensity of protein BAD (Ser112) fluorescence by 5.9 times (MFI = 117 ± 3) and by 1.7 times (MFI = 417 ± 30), respectively (Fig. 4A). When *Chang Liver* cells were exposed to compound 1, a decrease in the expression of BAD protein (Ser112) was observed only at a concentration of 125 µM, wherein the intensity

of fluorescence of BAD protein (Ser112) decreased by 3.3 times (MFI = 205 ± 29) (Fig. 4A).

The measurement of fluorescence intensity of the activated caspase-9 (Asp315) with MagPix showed that in all the studied concentrations, compound 2 reduced the expression of this protein in the apoptosis-induced samples with *d*-GLA 55 mM in comparison to the control group with *d*-GLA (MFI = 1552 ± 31). Thus, the intensity of fluorescence of caspase-9 (Asp315) in samples with compound 2 at the concentration of 125 µM decreased by 5.7 times (MFI = 271 ± 2.5), at 250 µM—by 11.2 times (MFI = 138 ± 8.5), and at 500 µM—by 2.6 times (MFI = 584 ± 46.5), respectively (Fig. 4B). In the groups of cells treated with Xymedon, the influence on the marker caspase-9 (Asp315) was observed only at a concentration of 125 µM which caused a decrease in the intensity of fluorescence of caspase-9 (Asp315) in comparison to the control group by 3.6 times (MFI = 424 ± 56) (Fig. 4B).

The level of caspase-8 (Asp384) fluorescence intensity in the test groups exposed to *d*-GLA at a concentration of 55 mM and compound 2 at concentrations of 125, 250, and 500 µM or Xymedon at concentrations of 125 and 250 µM was lower than in the control group exposed to *d*-GLA at a concentration of 55 mM. However, the revealed differences were not statistically significant (Fig. 4C).

3.5 Influence of the Tested Substances on the Makers of DNA Damage

The studied markers of DNA damage included protein p53 (Ser46), kinase ATR (total), CHK1 (Ser345), and CHK2 (Thr68).

The obtained data showed a decrease in the fluorescence intensity of protein p53 (Ser46) in the samples of cells treated with Xymedon conjugate with *L*-ascorbic acid at all the studied concentrations associated with exposure to *d*-GLA at 55 mM. In comparison to the control group with *d*-GLA (MFI = 754 ± 33.5), fluorescence intensity decreased by 1.4 times when treated with compound 2 at a concentration of 125 µM (MFI = 557 ± 30.5) and by 1.3 times at concentrations of 250 and 500 µM (MFI = 586 ± 24 and 565 ± 34, respectively). In the group of cells treated with Xymedon and *d*-GLA at a concentration of 55 mM, a significant decrease (by 1.8 times) in fluorescence intensity of p53 (Ser46) was observed only at the concentration of 125 µM, (MFI = 427 ± 184) (Fig. 5A).

In the group of cells treated with compound 2, at all the studied concentrations and exposed to *d*-GLA, a significant decrease in fluorescence intensity (MFI) of kinase ATR (total) was observed in comparison to the control group wherein only *d*-GLA 55 mM was used (MFI = 583 ± 29) (Fig. 5B). Fluorescence intensity decreased in groups treated with compound 2 at a concentration of 125 µM by 2.2 times

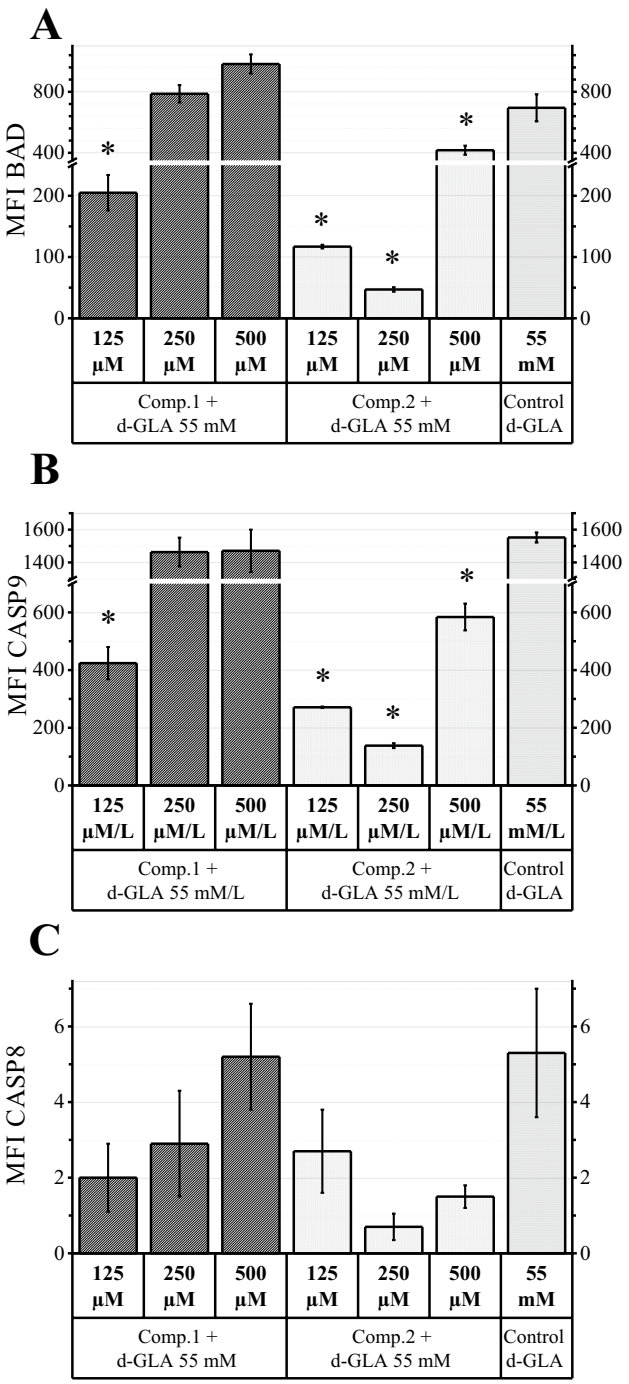

Fig. 4 Influence of compounds on the markers of early apoptosis associated with damage caused by *d*-GLA. **A** Mean fluorescence intensity (MFI) of BAD protein (Ser112). **B** Mean fluorescent intensity (MFI) of activated caspase-9 (Asp315). **C** Mean fluorescent intensity (MFI) of activated caspase-8 (Asp384). All: the asterisk indicates differences from the control group *d*-GLA are significant according to the Student's *t*-test, $p < 0.05$

(MFI = 265 ± 17), 250 µM—by 2.4 times (MFI = 248 ± 4), and 500 µM—by 2.5 times (MFI = 235 ± 24), respectively. The application of compound 1 and toxic *d*-GLA showed a

similar effect, in particular, a decrease in MFI ATR (total) by 2.7 times (MFI = 216 ± 19.5) was observed only at the concentration of 125 µM.

MFI of phosphorylated kinase CHK1 (Ser345) associated with the exposure to *d*-GLA and compound 2 at a concentration of 125 µM decreased by 35 times (MFI = 0.5 ± 0.5), and at a concentration of 500 µM, MFI was not detected in comparison to the control group *d*-GLA (MFI = 17.5 ± 1.5) (Fig. 5C). When toxicant *d*-GLA was used with compound 1 at concentrations of 125 and 250 µM, MFI CHK1 (Ser345) decreased by 8.75 (MFI = 2.0 ± 2.0) and by 3.5 times (MFI = 5.0 ± 0.0), respectively, in comparison to the control group with *d*-GLA (MFI = 17.5 ± 1.5) (Fig. 5C).

A significant decrease was observed in MFI of phosphorylated kinase CHK2 (Thr68) when compounds 1 and 2 were used with toxicant *d*-GLA (Fig. 5D). When cells were treated with compound 2 at a concentration of 125 µM, MFI decreased by 1.8 times (MFI = 80.0 ± 4.7), 250 and 500 µM—by 2.7 times (MFI = 58.2 ± 2 and MFI = 58.5 ± 5.5, respectively) in comparison to the control group with *d*-GLA 55 mM (MFI = 158 ± 7.5). The effect of compound 1 on the expression of phosphorylated CHK2 (Thr68) was less expressed than the effect of compound 2. Thus, the application of compound 1 at a concentration of 125 µM led to a decrease in MFI by 2.5 times (MFI = 61.0 ± 9.5), 250 and 500 µM—by 1.5 times (MFI = 105.0 ± 2.5 and MFI = 106.0 ± 10.5, respectively) in comparison to the control group with *d*-GLA 55 mM (MFI = 158.0 ± 7.5) (Fig. 5D).

4 Discussion

The results obtained in the present study revealed a cytoprotective activity of Xymedon and its conjugate with *L*-ascorbic acid in *Chang Liver* cells exposed to the toxic effect of *d*-GLA, which confirmed the previously obtained data on hepatoprotective properties of the studied compounds in in vivo studies. At the same time, the number of apoptotic cells which appeared was significantly lower in comparison to the control group.

Detection of apoptotic cells stained with annexin V allowed the authors to reveal cells with phosphatidylserine on the external surface of the cell membrane (in normal conditions, phosphatidylserine remains on the internal surface of the cell membrane). "Holding" of phosphatidylserine on the internal surface of the membrane is an active process. Its result depends on the balance of activity between two ATP-dependent membrane enzymes flippase and flippase [16, 17]. The former actively transports phospholipids from the external membrane to the internal, and the latter is involved in an opposite process (transportation of phospholipids from the internal to the external membrane). The initiation of apoptotic processes leads to a shift of balance of enzymatic activity to the transport of phosphatidylserine to the external part of plasmalemma [17].

This study showed that the conjugation of Xymedon with *L*-ascorbic acid led to a more expressed manifestation of

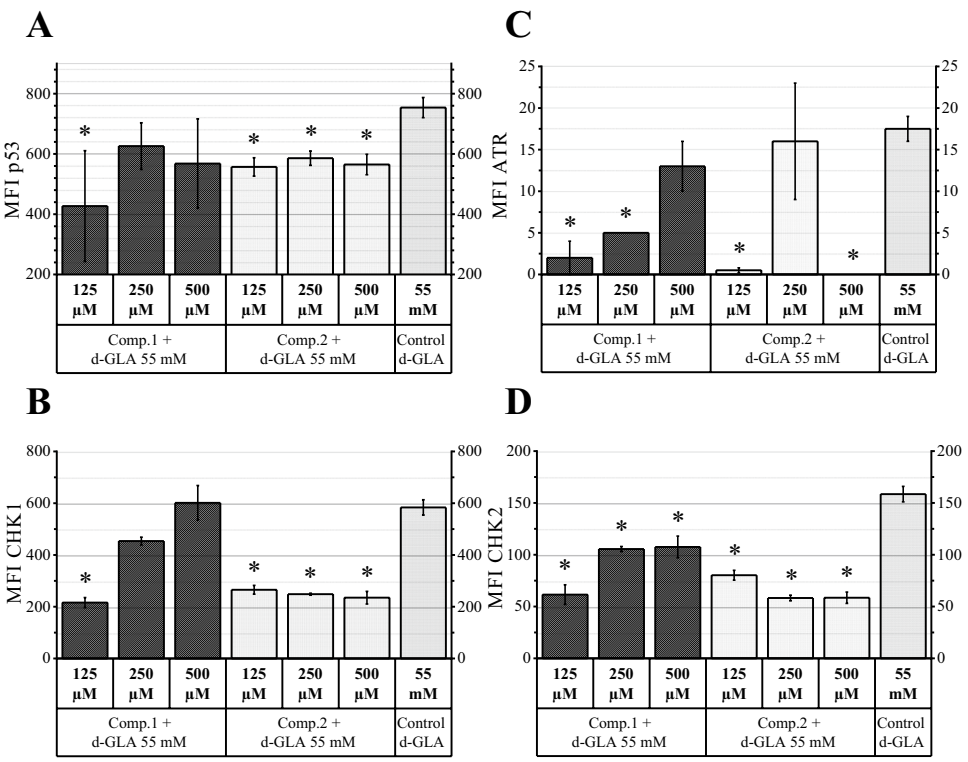

Fig. 5 Influence of the compounds on markers of DNA damage associated with damage caused by *d*-GLA. **A** Mean fluorescence intensity (MFI) of p53 protein (Ser46). **B** Mean fluorescence intensity (MFI) of ATR kinase (total). **C** Mean fluorescence intensity (MFI) of CHK1 kinase (Ser345). **D** Mean fluorescence intensity (MFI) of CHK1 kinase (Thr68). All: the asterisk indicates differences from the control group *d*-GLA are significant according to the Student's test, $p < 0.05$

antiapoptotic properties when compared to Xymedon. It should be mentioned that a lower dose of the conjugate was effective in comparison to Xymedon. However, an increase in the concentration of Xymedon led to an enhancement of the antiapoptotic effect, and an increase in the conjugate led to an increase in the number of annexin-positive cells. Taking into account that high concentrations of the conjugate led to a decrease in the number of living cells associated with the exposure of *d*-GLA, it can be suggested that, in high doses, it will exert an inhibiting effect.

The analysis of molecular markers of early apoptosis showed complete identity in the changes of the level of caspase-9 and protein BAD markers. Since caspase-9 is involved in the internal pathway of apoptosis, it is activated after the release of cytochrome C from mitochondria and the assembly of the apoptosome. As a result of homotypic interactions of inactive kinase-9 with CARD on an apoptosome, caspase-9 becomes catalytically active [18]. Furthermore, effector caspase-3 can be activated by an active caspase-9 [19, 20]. After that, apoptotic processes in a cell continue. In turn, BAD (BCL family protein that contains only a BH3 subunit) inhibits antiapoptotic proteins BCL-2 and BCL-xL and activates proapoptotic proteins of the BCL family (BAX and BAK) [21, 22]. Thus, the intensity of BAD fluorescence in the control group and the group of cells that were exposed to Xymedon was highest at concentrations of 250 and 500 μM and *d*-GLA. The beginning of apoptotic processes in these groups could be as follows: a release of cytochrome C from mitochondria, assembly of apoptosomes, activation of caspase-9, and an increase in fluorescence intensity. The application of Xymedon conjugate with *L*-ascorbic acid led to a significant decrease in fluorescence of BAD protein and activated caspase-9, which can indicate lower damage of mitochondrial membranes and the release of cytochrome C which activates the apoptotic processes.

It should be mentioned that the studied compounds did not show a significant influence on the fluorescence intensity of caspase-8 which is characterized as an enzyme that activates the external pathway of apoptosis via death receptors [23]. Insignificant fluctuations of fluorescence intensity of caspase-8 can be explained by its activation via the internal pathway of apoptosis, which was confirmed by several studies [24, 25] that showed the activation of caspase-8 via proteolysis with the help of caspase-3 and caspase-6 that are also activated via the internal pathway.

An increase in the expression of p53 protein can indicate DNA damage and the initiation of apoptosis. This protein can be detected both in the cell nucleus and mitochondria after damage to DNA and the activation of caspases [26]. Also, p53 can bind with BCL-2 and BCL-xL contributing to the release and activation of BAX and BAK [27] and enhancing the process of apoptosis. Under the influence of Xymedon conjugate with *L*-ascorbic acid in all of the studied concentrations, a decrease in fluorescence intensity is observed in comparison to the control group, which can indicate lesser damage to the genetic apparatus and mitochondrial membranes. Thus, when Xymedon conjugate with *L*-ascorbic acid is used, there are indirect signs that the initiation of effector caspases is less expressed because an increase in the p53 occurs after the activation of caspases. The application of Xymedon led to a less expressed effect contributing to a decrease in fluorescent intensity of p53 only at a concentration of 125 μM.

The observed decrease in the expression of ATR by the effect of Xymedon conjugate with *L*-ascorbic acid in all the studied concentrations (125, 250, and 500 μM) and Xymedon 125 μM along with the toxicant confirmed the suggestion that these compounds exert a protective effect on cells and the genetic apparatus. ATR is a protein kinase (signal molecule) that is actively expressed in a cell when single-strand breaks occur [28]. Thus, lower expression of ATR can indicate less expressed DNA damage. And so, the protective effect of the studied compounds (especially Xymedon conjugate with *L*-ascorbic acid) that reduces the occurrence of single-strand breaks was also confirmed by a dependency between ATR and phosphorylated CHK1 (activated via ATR), because this kinase is a substrate for ATR [28]. A further effect of activated (phosphorylated) CHK1 is observed by a stop of the cellular cycle in G1/S, intra-S phase, and G2/M phases via the inhibition of the activity of cyclin-dependent kinase, which elongates the time available for the reparation of DNA before replication or mitosis [29].

It should be noted that the application of Xymedon conjugate with *L*-ascorbic acid and Xymedon in all the studied concentrations (125, 250, and 500 μM) together with the toxicant reduces the occurrence of double-strand breaks. This indicates a decrease in fluorescence intensity of the phosphorylated (activated) kinase CHK2 in comparison to the control group. This kinase acts similar to CHK1 but is activated by a different signal molecule of DNA damage—ATM [28]. Unlike ATR, ATM active expression is initiated by DNA double-strand breaks [29].

According to the published data [30], the mechanism of action of the Xymedon molecule is associated with the regulation of adenylate cyclase (AC) activity because there are data on Xymedon having the capacity to reduce the activity of this signal molecule in immune-competent cells [30]. The description of further consequences of activation or inhibition of AC should include the fact that there are different isoforms of AC with G-protein-mediated regulation. The regulation of AC activity via G-protein subunits is topospecific, i.e., it depends on the isoform of AC and the type of cells [31]. The activity of AC is directly associated with the production of cAMP [32], which, in turn, activates regulatory processes in a cell depending on its type [33]. For example, there are data that high concentrations of cAMP stimulate

the proliferation of epithelial cells, hepatocytes, adipocytes, and pancreatic β-cells [33]. At the same time, the high production of cAMP by fibroblasts, flat muscle, and neoplastic B-cells suppresses proliferation [34]. To sum up, it can be suggested that Xymedon in hepatic cells activates AC, which leads to an increase in the number of cAMP that can improve carbohydrate and lipid metabolism in the hepatocytes [35], protect hepatocytes from apoptosis caused by the release of cytochrome C from mitochondria, inhibit the activation of caspases [36], and contribute to a decrease in the level of antiinflammatory cytokine TNFα [37].

Xymedon and its conjugate with *L*-ascorbic acid can also contribute to the changes in the levels of other secondary messages, which reduces the effects of apoptosis. It was shown that *d*-GLA caused UDP-glucose and UDP-galactose deficiency and changed the homeostasis of intracellular calcium, affecting cellular membranes, cellular organelles, energetic metabolism, and synthesis of proteins and nucleic acids [11]. This resulted in an increase in cellular oxidative stress, lipid peroxidation, and reactive oxygen intermediates (ROI) production [11]. H_2O_2 is one of the ROI forms and can act as a secondary messenger in a cell's death [38]. Another messenger that contributes to the induction of apoptosis in cells is ceramide [38, 39]. It was established that *d*-GLA could increase the level of some of its fractions in the liver cells [40]. Thus, apart from a possible regulation of adenylyl cyclase and cyclic AMP, the studied compounds can exert a protective effect through a decrease in lipid peroxidation and ROI production or enhancement of ROI metabolism [38]. A decrease in the level of ROI under the influence of the studied compounds contributed to a reduction in DNA damage, which was evident in the levels of ATR, CHK1, and CHK2 kinases.

It should also be mentioned that the activation of the PI3K pathway, in particular, Akt-kinase, as a result of the exposure to the studied compounds, could lead to the inactivation of BAD and the reduction of autophagy processes [41]. The inactivation of BAD, in turn, contributed to a decrease in mitochondrial membrane damage and caspase activation.

An enhancement of the protection and antiapoptotic properties of Xymedon conjugate with *L*-ascorbic acid in comparison to Xymedon can be explained by different factors. First, it can be associated with the high bioavailability of ascorbic acid for cells [42], which contributes to an increase in the bioavailability of the Xymedon molecule. Vitamin C conjugation is a potential tool for improving the bioavailability and absorption of medicine [43, 44]. Secondly, there is evidence that L-ascorbic acid can act in synergy with other substances. For example, ascorbic acid in combination with phenylephrine and metaproterenolol can enhance the synthesis and proliferation of primary hepatocyte culture DNA [45]. Phenylephrine and metaproterenolol alone do not accelerate the synthesis and proliferation of primary hepatocyte culture DNA [45]. Thirdly, the enhanced efficacy of the conjugate, in comparison with Xymedon, can be due to the antioxidant properties of ascorbic acid, the concentration of which decreases under the influence of *d*-GLA [11].

5 Conclusion

The results obtained showed that the cytoprotective effect of pyrimidine-derived drug Xymedon and its conjugate with *L*-ascorbic acid is realized by reducing apoptotic changes in cells. The studied substances contribute to the reduction of apoptosis markers such as BAD and caspase-9 and DNA damage markers such as p53, ATR, CHK1, and CHK2. The revealed antiapoptotic effect of the compounds may be the key mechanism for the realization of the hepatoprotective effect of the compounds, which was shown in early studies. It was also shown that the conjugation of Xymedon with *L*-ascorbic acid significantly enhanced the antiapoptotic effect. It was found that a minimal dose of the conjugate was more effective than the same of Xymedon.

Author Contribution Methodology: Parfenov Andrey, Vyshtakalyuk Alexandra, Semenov Vyacheslav; design and synthesis of test compounds: Semenov Vyacheslav, Galyametdinova Irina; formal analysis and investigation: Parfenov Andrey; writing—original draft preparation: Parfenov Andrey; writing—review and editing: Vyshtakalyuk Alexandra, Semenov Vyacheslav, Zobov Vladimir; funding acquisition: Vyshtakalyuk Alexandra.

Funding The reported study was funded by the Russian Foundation for Basic Research (RFBR) according to the research project no. 20–315-90039.

Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

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