ISSN 1068-1620, Russian Journal of Bioorganic Chemistry, 2023, Vol. 49, No. 4, pp. 797–814. © Pleiades Publishing, Ltd., 2023.
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Structural Analogs of Dehydrozingerone Containing a Pyridoxine Fragment Exhibit Membrane-Modulating Properties and Synergistically Enhance the Antitumor Activity of Cytostatics

O. V. Bondar[a, 1], R. Karwt[a], T. Mohammad[a], R. S. Pavelyev[a], M. V. Pugachev[a], Be. B. Ygaiev[a], A. R. Kayumov[b], A. M. Aimaletdinov[a], and Y. G. Shtyrlin[a]

[a] *Scientific and Educational Center of Pharmaceutics, Kazan Federal University, Kazan, 420008 Russia*
[b] *Institute of Fundamental Medicine and Biology, Kazan Federal University, Kazan, 420008 Russia*

Received October 28, 2022; revised November 10, 2022; accepted November 12, 2022

Abstract—Previously we synthesized 10 novel structural analogs of dehydrozingerone based on the pyridoxine (vitamin B6) scaffold. Two lead compounds (compound **(I)** and compound **(II)**) expressed good cytotoxic activity against tumor cells and have shown higher selectivity than doxorubicin. In the present study, the mechanism of action of the leading analogues of dehydrozingerone, as well as the efficiency of their combinations with known cytostatics, was studied in more detail. We revealed a synergistic effect of leader dehydrozingerone analogs combinations with known cytostatics—doxorubicin, vinblastine and paclitaxel. It was established, that test compounds **(I)** and **(II)**, as well as curcumin and dehydrozingerone, possess membrane-damaging activity: cause cytoplasmic membrane depolarization and reduction in its microviscosity, which can explain the increase in toxicity of cytostatics. In addition, the test compounds were found to increase the ATPase activity of P-glycoproteins, likely acting as their substrates. It was also revealed that the test compounds increase the expression of BAX and E-cadherin, decrease the expression of Bcl-2 in cancer cells. Compound **(I)** does not cause blood cells hemolysis, does not possess DNA-damaging and mutagenic activity, and when administered intravenously to mice, the LD50 was 65 mg/kg. The investigated compounds are promising drug candidates to be further tested on animals with grafted tumors.

Keywords: pyridoxine, dehydrozingerone, curcumin, compositions with cytostatics, membrane-damaging activity

DOI: 10.1134/S106816202304009X

INTRODUCTION

Curcumin (diferuloylmethane) (Fig. 1) is a natural product extracted from *Curcuma longa* plant. Dehydrozingerone (DZG) known as feruloylmethane is a phytochemical obtained from the rhizomes of *Zingiber officinale* (ginger). DZG represents half of curcumin's chemical structure and a product of its metabolic degradation [1]. Aside from being employed as a spice, curcumin (CUR) has also been widely used in Ayurvedic and Chinese medicine for its antioxidant, antiseptic, analgesic, antimalarial and anti-inflammatory properties [2, 3]. But the most known property of CUR is an anti-cancer activity, CUR has been tested on multiple human cancer cells including breast, colon, melanoma, prostate and ovarian cancer cells [4–7]. According to epidemiological studies, the low incidence of colon cancer in India is attributed to the chemopreventive and antioxidant properties of diets rich in CUR [8].

The mechanism of the antitumor action of CUR is complex and includes an expression regulation of important transcription factors and signaling molecules. CUR suppresses the activation of NF-kB resulting in the downregulation of many NF-kB-regulated genes involved in inflammation, cellular proliferation and cell survival (COX-2, LOX, iNOS, TNF-α, Cyclin-D1, ICAM-1, c-myc, Bcl-2, IL-6, IL-8) [4, 9]. Since CUR neutralizes free radicals, it can also inhibit the initial stage of carcinogenesis—DNA damage by UV radiation or reactive oxygen species (ROS) [4]. CUR selectively induces apoptosis in tumor cells at the G2 phase via up-regulation of p53, p16, BAX, Bad, Bim expression and suppression of Cyclin D1, Cyclin E, Bcl-2, Bcl-xL, pAkt, mTOR expression [10, 11]. In addition, CUR induces autophagic cell death by inhibiting the Akt/mTOR/p70S6 kinase pathway and the ERK1/2 pathway, and supress angiogenesis and metastasis [12].

Phase I/II clinical trials showed that CUR is safe even at high doses (12 g/day) in humans but has poor bioavailability and fast metabolism [13, 14]. To

[1] Corresponding author: e-mail: oxanav.bondar@gmail.com.

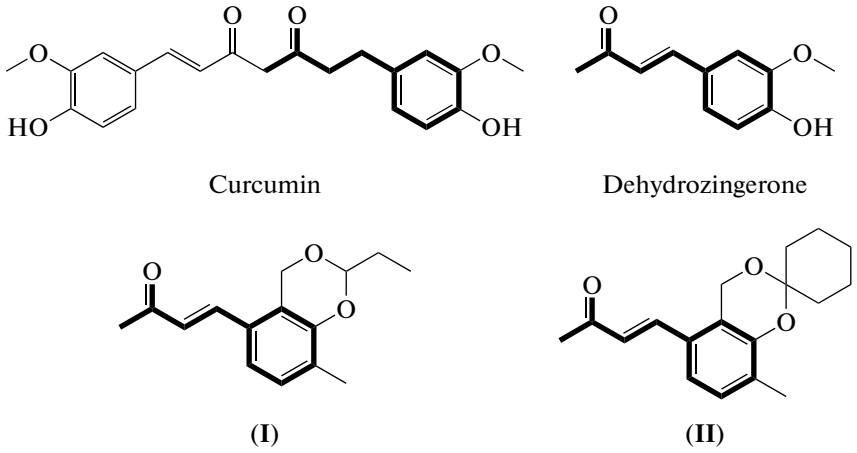

Fig. 1. Structures of test ((**I**) and (**II**)) and reference (CUR, DZG) compounds.

improve the bioavailability various structural analogues of CUR have been synthesized and studied. Mono-carbonyl analogs of CUR has been reported to have enhanced stability in vitro and an improved pharmacokinetic profile in vivo [15, 16], also symmetrical α,β-unsaturated and saturated ketones possessed on animal studies even better antitumor activity than TNP-470 drug, which is under the clinical trials [17]. Shibata et al. reported that new CUR analogs containing methoxymethyl group showed successful activity against colorectal cancer in vivo [18].

DZG, which is also a monoaromatic ring analogue of CUR, has proven to have anti-depressant, antimalarial, antifungal, antioxidant, anticancer activities and has a larger biological half-life than CUR itself [19, 20]. Fresh ginger root containing DZG is one of the most commonly used Chinese medicines in clinical practice [21]. Some in vitro studies have shown that DZG inhibits tumor promotion [22, 23]. It is known about the mechanism of action that DZG predominantly induced cell-cycle arrest at the G2/M phase accompanied by accumulation of intracellular ROS and up-regulation of p21 [20].

However, both DZG and CUR anti-cancer activity is considered to be limited in comparison with other therapeutic agents such as doxorubicin [24]. Nowadays, much effort has been focused on the co-administration of different drugs to achieve a synergistic therapeutic effect in cancer treatment. Recently, several studies have demonstrated that CUR has a synergistic effect on doxorubicin anticancer activity [25–27]. Curcuminoids (including curcumin, demethoxycurcumin and bisdemethoxycurcumin) sensitize the ABCG2 (ATP binding cassette subfamily G) expressing cells to conventional chemotherapeutic agents—mitoxantrone, topotecan, SN-38 and doxorubicin due to their influence on the function rather than the protein levels of ABCG2 [28]. Additionally, treatment of KB-V1 cells with CUR increases their sensitivity to vinblastine [29, 30].

Analyzing the reasons for the sensitization of tumor cells to cytostatics under the action of curcuminoids, we turned to the literature indicating the involvement of the membrane effects of the CUR in this sensitizing effect. Ingolfsson and co-authors showed that CUR significantly affects the single-channel lifetime of gramicidin in a 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) bilayer [31]. To understand this result, Hung and co-workers did a series of experiments with a DOPC bilayer and revealed that CUR not only thinned the lipid bilayer, but could also weaken its elasticity moduli. Hung and co-authors have shown that CUR can bind to the model membrane in two modes: a surface-associated mode at low concentrations and a transmembrane mode at high concentrations [32]. Follow up work by Barry showed that CUR is embedded deep into the membrane in a transbilayer orientation, anchored by hydrogen bonding to the phosphate group of lipids in a manner similar to cholesterol [33]. Many other studies have confirmed that CUR associates with or accumulates in cell membranes, forms highly ordered oligomeric structures in the membrane, induces dramatic changes in the fluidity of the lipid bilayer, may span, and likely thin, the bilayer [34, 38]. The observed effect of CUR on the physical properties of the membrane may explain its nonspecific and widespread influence on the activity of various membrane proteins.

The data available in the literature indicate that CUR regulates the action of membrane proteins not only by changing the physical properties of the membrane, but also through direct interactions with membrane proteins. In particular, CUR was found to be able to cross-link CFTR polypeptides and activate wild and mutant type ATP-binding CFTR chloride channels [39, 40]. For the hERG potassium channel, CUR exerts the pore-blocking effect [41]. In the mul-

tidrug-resistant human cervical carcinoma cell line KB-V1, CUR can significantly lower the P-gp expression and directly interacts with the drug-binding site of the transporter [29, 30]. Curcumin resistance has also been observed in some cancer cells, and efflux of CUR by ABC transporters is thought to be one of the factors causing resistance. In MCF-7/ADR cells overexpressing P-gp, CUR significantly enhances the cellular accumulation of Rhodamine-123 in a concentration-dependent manner, indicating that CUR significantly affects P-gp activity [42].

In our previous work, we studied 10 novel structural modifications of DZG based on the pyridoxine (vitamin B6) scaffold [24]. Two of these compounds expressed a cytotoxic activity against 10 tumor cell lines that was comparable to doxorubicin. Moreover, two leading compounds (**I**) and (**II**) (Fig. 1) showed higher selectivity for cancer cells over normal cells than doxorubicin. Leading compound (**II**) and DZG have some similarities in the mechanisms of cytotoxic activity. Thus, they initiate cell cycle arrest in the G2/M phase, inhibit migration activity and, therefore, invasiveness of tumor cells. At the same time, compound (**II**) increases the ROS level in tumor cells, in contrast to DZG, which reduces it at nontoxic concentrations. Compound (**II**) also affects the functional activity of mitochondria by depolarization of their membranes. All of these effects finally contribute to the induction of drug-associated apoptosis in tumor cells. Our recent review describes systematic studies of pyridoxine derivatives, including their key pharmacological properties and approaches to the design of physiologically active compounds on their scaffold [43].

In the present research, we studied the antitumor activity of the revealed leader DZG analogs (**I**) and (**II**) in compositions with known cytostatics—doxorubicin, vinblastine and paclitaxel. A synergistic effect has been found from the combination of doxorubicin and vinblastine with DZG analogs. In order to identify the mechanisms of such synergism, we studied the effect of compounds (**I**) and (**II**) on the cytoplasmic membranes of tumor cells, as well as on the activity of membrane transporters. Also, several tests were performed to identify the mechanism of action of new DZG analogs, including an assessment of their effect on protein expression, tubulin polymerization, and blood hemolysis. In addition, we also evaluated the acute toxicity of the compounds in mice and the genotoxicity in microbial tests.

RESULTS AND DISCUSSION

Test Compounds

In the present research, we continued to study the antitumor properties of novel azaheterocyclic dehydrozingerone (DZG) analogs containing pyridoxine (vitamin B6) fragment in their structure. The structures of these compounds are shown in Fig. 1 versus curcumin (CUR) and DZG. Compounds (**I**) and (**II**) with the trans-configuration of the alkene fragment were obtained by the Wittig reaction in good isolated yields (70–88%) in the form of white crystalline substances with a purity of 98.5 and 97.3% respectively, according to HPLC study (Supplementary Information, Figs. S1 and S2). The synthesis of target compounds is presented in detail in our previous paper [24].

DZG analogs show in-vitro antitumor activity in composition with known commercial chemotherapeutic agents. Cytotoxic concentrations of test compounds against a broad panel of cancer cells in comparison with conditionally normal cells are given in [24]. The selectivity index for compounds (**I**) and (**II**) was 9 and 14, respectively, which is higher than that of DOX with 6.3. In the present work we studied the cytotoxicity of novel DZG analogs in compositions with known cytostatics against human cancer cells, in order to reveal a possible synergy from their combined use.

We investigated the effect of the leading DZG analogs – (**I**) and (**II**) and the reference compounds CUR and DZG on the doxorubicin (DOX), paclitaxel (PAC), and vinblastine (VIN) cytotoxicity against prostate adenocarcinoma (PC-3) cancer cells. Cells were cultured in the presence of a series concentrations of cytostatics with the addition of a series concentrations of study compounds added in a checkerboard pattern as described in [44, 45]. The baseline IC_{50} values of test compounds alone against PC-3 cells are shown in Table 1. Checkerboard pattern data of compositions are presented in Fig. 2 and Tables S1–S3 of the Supplementary Information. The studied compounds are inferior in cytotoxicity to commercial cytostatics, but cytotoxic concentration IC_{50} of CUR is 3.2–4.3 times higher and IC_{50} of DZG is 16.8–22.7 times higher than that of studied compounds.

Results showed that compounds (**I**) and (**II**) in non-toxic concentrations that inhibit cell proliferation by no more than 10–20% do not increase the cytotoxicity of DOX, and even protect cells from its toxic action. With an increase in the concentration of compounds (**I**) and (**II**) to IC_{40}, and then to IC_{50}, a sharp drop in the cytotoxic concentration of DOX from initial 0.35 μM (203 ng/mL) to 58 nM (33.6 ng/mL) and 80 nM (46.4 ng/mL) correspondingly, and then below 3 nM (1.7 ng/mL) was observed (Fig. 2a). In addition, DOX enhances the cytotoxicity of compounds 1 and 2, but only at concentrations above its IC_{50} (Supplementary Information, Table S1). The obtained FICI index for DOX and compound (**I**) at their IC_{50} concentrations is less than 0.05, and for compound (**II**) it is less than 0.15, which indicates a synergistic relationship. When combining DOX with reference compounds—CUR and DZG, similar patterns were revealed. CUR at a concentration of IC_{30} and DZG at a concentration of IC_{70} significantly enhance the cytotoxicity of DOX with a FICI < 0.13, but at non-toxic concentrations,

Table 1. Inhibitory concentrations (IC_{50}) of test compounds according to the MTT-assay on PC-3 cells, incubation time 72 h, values are the average of at least five separated MTT assay

IC_{50} (μM) on PC-3 cells						
Compound (**I**)	Compound (**II**)	CUR	DZG	DOX	VIN	PAC
12.7 ± 2.3	9.4 ± 2.8	40.2 ± 5.6	213.3 ± 43.0	0.35 ± 0.067	0.064.0 ± 0.006	0.072 ± 0.016

they produce a protective effect and rather impede the cytostatic action of DOX.

When combining compounds (**I**) and (**II**) with VIN, the following facts were revealed. Compounds (**I**) and (**II**) at their IC_{30}–IC_{40} concentrations increase the cytotoxicity of VIN by 2 times; with a further increase in concentrations to IC_{50}, the cytotoxicity of VIN increases by 15 times, and then more than 400 times when IC_{55}–IC_{60} is reached. IC_{50} of VIN decreased from initial 64 nM (58 ng/mL) to 31 nM (28 ng/mL), then to 12 nM (11 ng/mL) and then less to 0.15 nM (0.14 ng/mL) in the presence of compound (**I**) at concentrations of 9, 11 and 13 μM correspondingly. Much less effective are CUR and DZG, which increase the cytotoxicity of VIN starting from their IC_{70} and IC_{90}, respectively (Fig. 2b). In turn, VIN is able to enhance the cytotoxicity of the test compounds, however, at a non-toxic concentration of IC_{20} VIN acts weakly giving 15–20% increase in cytotoxicity, but when used at a concentration of IC_{60}, it enhances cytotoxicity by 3 or more times (Supplementary Information, Table S2). Thus, compositions of DZG analogs with VIN at their IC_{50} and higher concentrations for both components can be considered synergistic with a FICI < 0.5. When combined in lower concentrations, the effect is additive.

Compositions of the test compounds with PAC were found to be the least effective. On the one hand, all tested compounds significantly enhance the PAC cytotoxicity only at their IC_{70} concentration (Fig. 2c). On the other hand, PAC enhances the cytotoxicity of the compounds also when its IC_{70} concentration is reached (Supplementary Information, Table S3). That is, compositions with PAC are considered synergistic with a FICI < 0.5 when the components are combined at toxic IC_{70} concentrations.

It can be concluded that the test compounds (**I**) and (**II**), when combined at IC_{40}–IC_{50} concentrations, produce synergistic compositions with DNA intercalator DOX and with a blocker of mitotic spindle assembly VIN. However, to obtain synergistic compositions with PAC (inhibitor of tubulin disassembly), the concentration of compounds should be increased

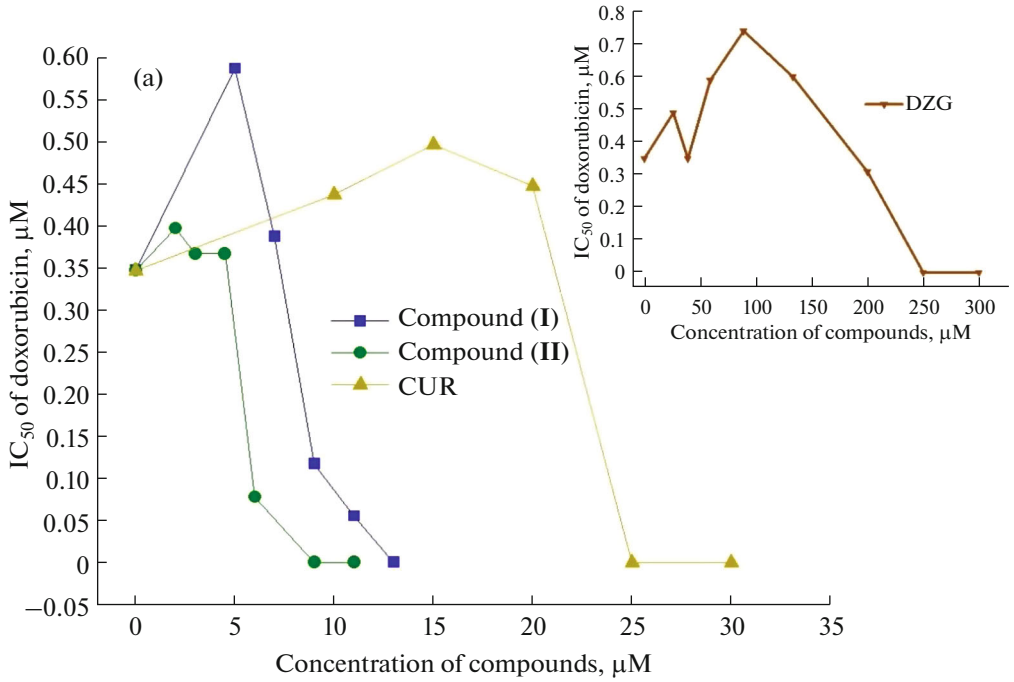

Fig. 2. Inhibitory concentrations (IC_{50}) of Doxorubicin (a), Vinblastine (b) and Paclitaxel (c) in compositions with compounds (**I**), (**II**), DZG and CUR according to the MTT-assay on PC-3 cells, incubation time 72 h.

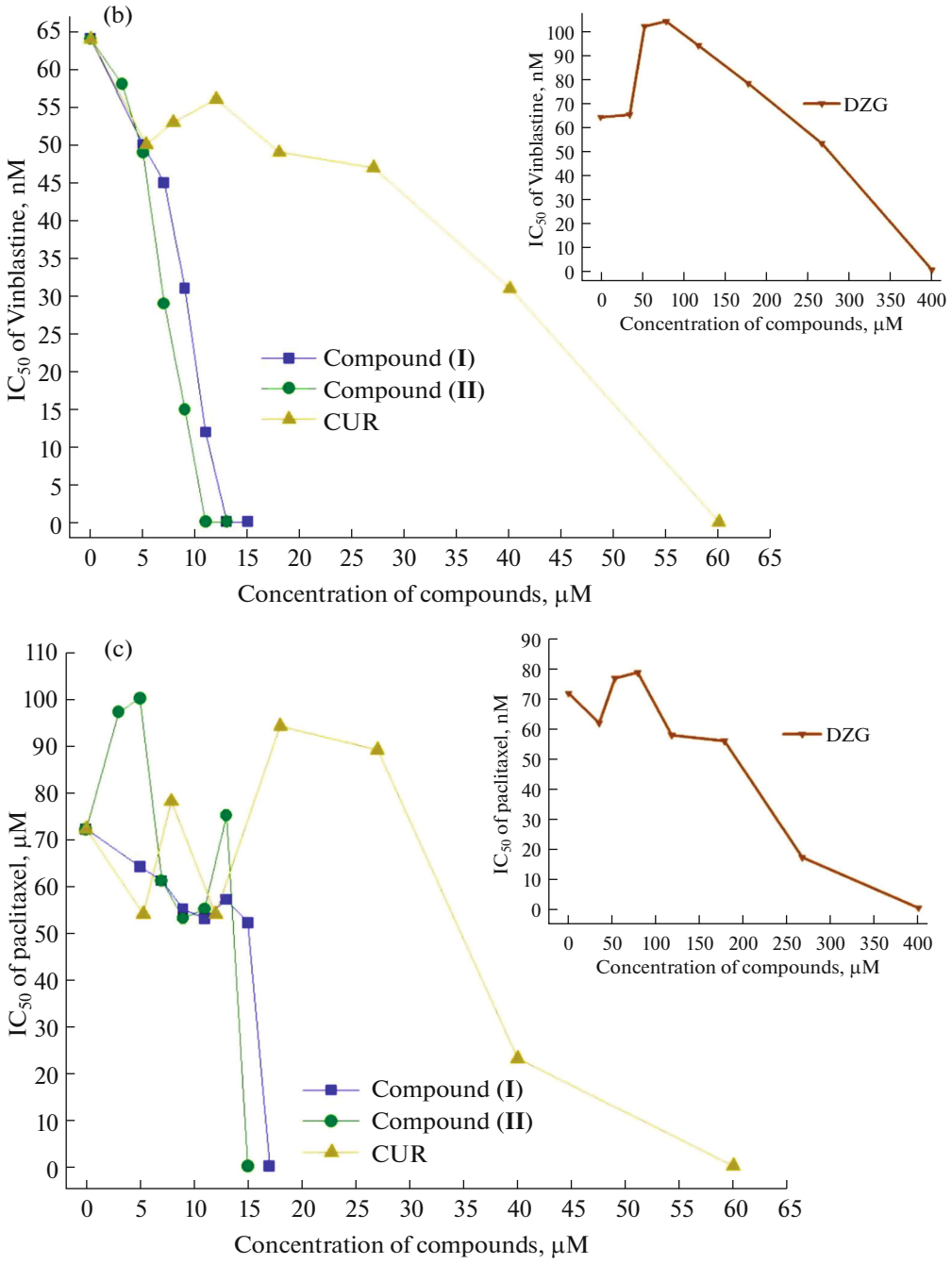

Fig. 2. (Contd.)

up to IC_{70}. It is of interest to identify the reasons for this synergism at the cellular level.

DZG analogs influence the intracellular accumulation of doxorubicin. In order to identify the reasons for the revealed synergism, we studied the influence of DZG analogs on the intracellular uptake of DOX. DOX possesses its own fluorescence; therefore, its intracellular accumulation was estimated using a flow cytometer. Cancer PC-3 cells were treated with DOX at 5 μM and its compositions with test compounds at 5 and 20 μM for 1 h, then the cells were washed, trypsinized and analyzed. Figure 3 shows the degree of DOX fluorescence in treated cells. We found a small but statistically significant increase in the intracellular accumulation of DOX under the action of compounds **(I)** and **(II)** at a concentration of 20 μM. The reference compounds CUR and DZG produce an even more pronounced effect on the DOX transport in tumor cells. It can be assumed that the study compounds increase the accumulation of DOX by enhancing its

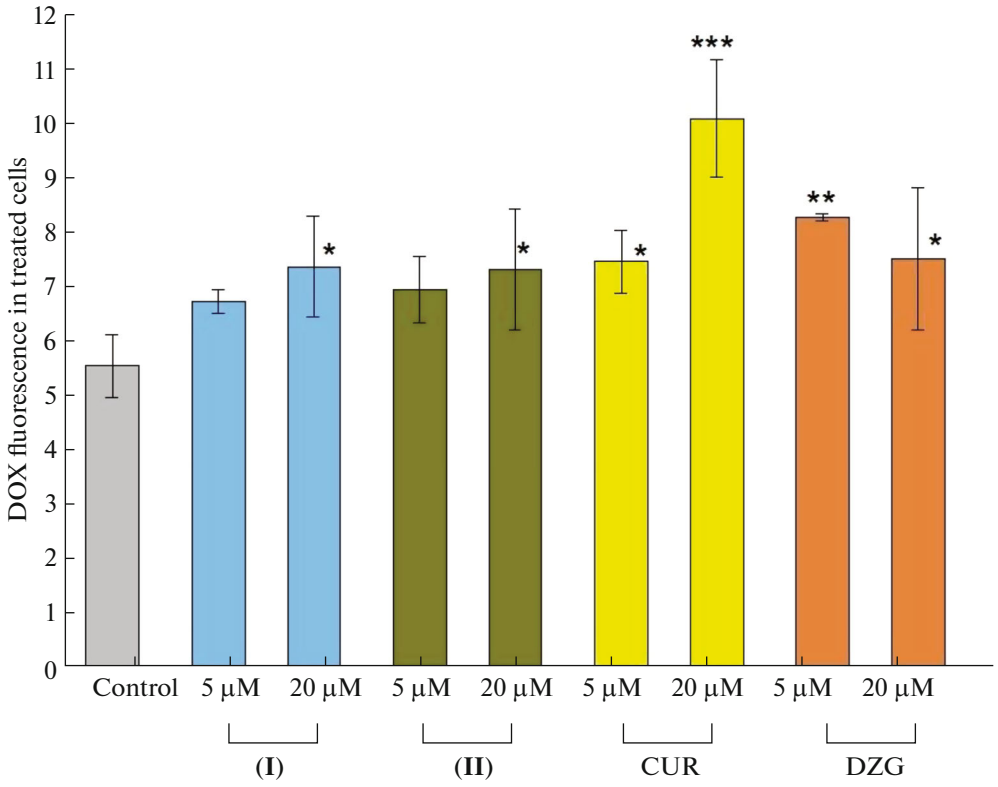

Fig. 3. Doxorubicin fluorescence in PC-3 cells treated with compounds (**I**), (**II**), CUR and DZG according to the flow cytometry results. Control treated with doxorubicin only.

intracellular transport across the lipid membrane or by decreasing its elimination from cells through the action on P-glycoprotein membrane transporters.

Membrane Modulating Activity of New DZG Aanalogs

Action on the trans-membrane potential of the plasma membrane. We proposed that new DZG analogs (**I**) and (**II**) act on the cytoplasmic membrane. In this regard, we tested the total membrane potential of PC-3 cells using the fluorescent indicator DiOC6(3), which accumulates in cell membranes (cytoplasmic membrane and membrane of organelles) in direct proportion to their potential [46–48].

Suspension of PC-3 cells was treated with various concentrations of the test compounds and the DiOC6(3) for 20 min. As reference compounds, we used nonionic surfactants—Triton X-100 and Tween-20. Fluorescence of DiOC6(3) in treated cells is shown in Fig. 4.

The data obtained showed that the studied DZG analogs significantly reduce the DiOC6(3) fluorescence in the treated cells. We believe that this effect is primarily due to the influence of test compounds on the cytoplasmic membranes. Since the test is short-term, the effect on the membranes of mitochondria and other organelles is less pronounced. Thus, a decrease in DiOC6(3) fluorescence of treated cells indicates a drop in the cytoplasmic membrane potential or cell depolarization. The reference compounds DZG, Triton X-100, Tween-20, however, affect the membrane potential more significantly, but CUR acts similarly to compounds (**I**) and (**II**). In our previous work, under conditions of longer exposure to the cell, the studied compound (**II**) had a depolarizing effect on the mitochondrial membranes [24].

The revealed effect of the studied compounds on the membrane potential probably explains the increase in the intracellular delivery of DOX to cancer cells.

Action on the plasma membrane microviscosity. Membrane microviscosity is a measure of the mobility of lipids in the bilayer, which plays an important role in membrane permeability and the functioning of membrane proteins. In this regard, it is of interest to study the effect of the test compounds on the plasma membrane microviscosity with the help of lipophilic indicator diphenylhexatriene (DPGT). An increase in membrane fluidity is accompanied by a decrease in DPGT fluorescence polarization [49, 50].

PC-3 cells in a suspension were treated with DPGT and various concentrations of compounds (**I**) and (**II**) (as well as reference compounds CUR, Triton X-100, Cholesterol). The experimental results are shown in Fig. 5.

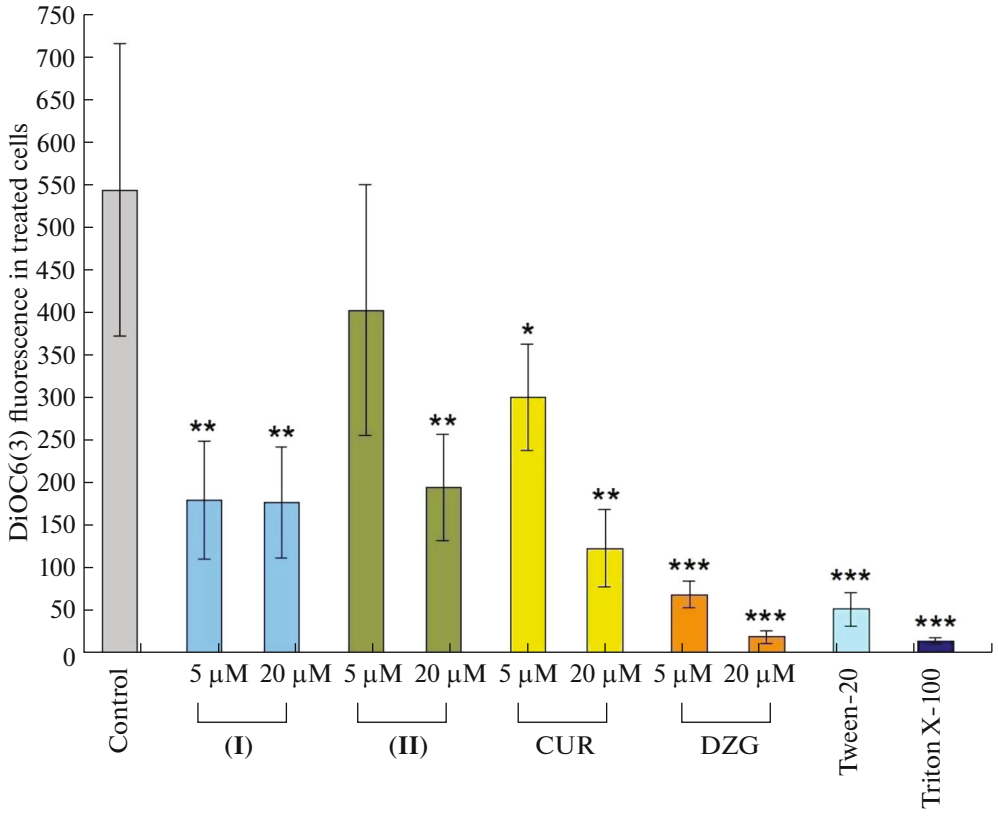

Fig. 4. DiOC6(3) fluorescence in PC-3 cells treated with the studied DZG analogs and control compounds (Triton X-100 and Tween-20), emission 525 ± 30 nm. Control treated with DiOC6(3) only. Tween-20 and Triton X-100 were used at a concentration of 0.1 mg/mL.

When cells were treated with compounds (**I**) and (**II**) at concentrations of 20 and 100 μM, we observed statistically significant decreases in the DPGT fluorescence polarization, which indicates a decrease in the cancer cell membrane microviscosity. Compounds (**I**) and (**II**) had no significant effect on microviscosity at a concentration of 5 μM. The revealed effect on microviscosity is dose-dependent. CUR at a concentration of 20 μM compared with compounds (**I**) and (**II**) had a less pronounced effect on the microviscosity. Since DZG possesses fluorescence in the studied wavelength range, it was not included in this assay. Triton X-100 significantly reduced the polarization of DPGT fluorescence, while cholesterol, on the contrary, increased, which is consistent with the known effects of these compounds on the membrane.

According to the results of the last two tests, it can be concluded that the probable cause of the increase in the intracellular accumulation of DOX in cancer cells is a change in the physicochemical characteristics of cell membrane under the influence of the test compounds.

Red blood cells hemolysis induction. The revealed membrane-damaging activity of DZG analogs caused us concern about the possible hemolysis of erythrocytes upon their intravenous administration. In this regard, the hemolysis of human erythrocytes was evaluated in vitro during incubation with test compounds at concentrations of 30, 300, and 3000 μM. Triton X-100 0.2% was used as a positive control inducing complete hemolysis of erythrocytes. The optical density of hemoglobin in the supernatant was measured and converted to the hemolysis percentage; the results are shown in Fig. 6.

It has been shown that compound (**II**) at high concentration of 3 mM (this concentration will be achieved with intravenous administration of compound (**II**) at a dose of 64 mg/kg) causes pronounced blood hemolysis (77%); at a concentration of 300 μM (corresponds to dose 6.4 mg/kg) possesses 16% hemolysis of erythrocytes; at 30 μM does not exhibit significant membrane-damaging activity. It's useful to note that compound (**I**) does not cause blood hemolysis even at a concentration of 3 mM, which can be achieved with intravenous administration of compound (**I**) at a dose of 55 mg/kg approaching it LD_{50} (64.8 mg/kg). While DZG has a weak membrane damaging activity at a concentration of 3 mM (17% hemolysis). Thus, with the possible intravenous use of compound (**II**), severe side effects are possible; however, compound (**I**) does not cause blood hemolysis and is safe from this point of view.

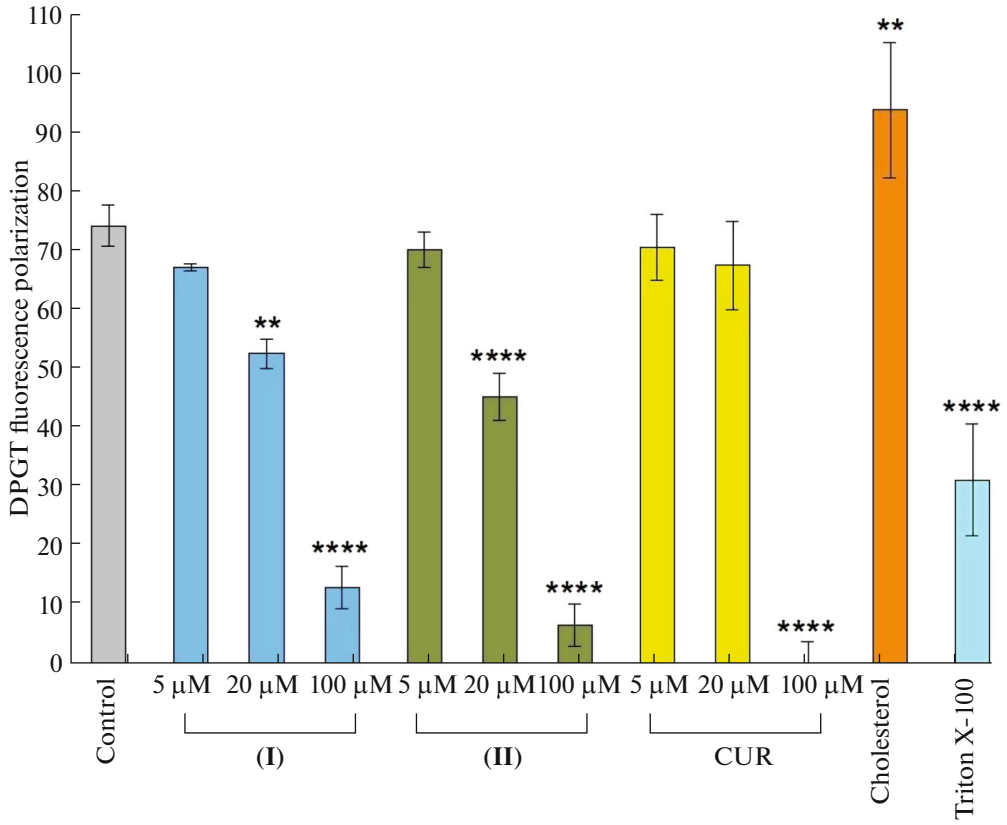

Fig. 5. Fluorescence polarization of diphenylhexatriene (DPGT) in the suspension of PC-3 cells treated with test and reference compounds after 60 min of exposure. The cell suspension density is 2×10^6 cells/mL, the temperature is 25°C. Cholesterol concentration was 0.1 mg/mL, Triton X-100 0.05%.

DZG analogs influence on P-glycoprotein ATPase activity. Data available in the literature indicate that CUR regulates the action of membrane proteins not only by changing the physical properties of the membrane but also through direct interactions with membrane transporters [28–30, 42]. P-Glycoprotein (P-gp) is able to "pump" out of the cell many chemotherapeutics, including DOX, and can cause insensitivity to treatment; P-gp overexpression is associated with the development of multiple drug resistance. Taking into account all of these facts, we decided to study P-gp ATPase activity in the presence of CUR, DZG and test compounds (**I**) and (**II**).

The ATPase activity of the membrane P-glycoprotein was studied using a commercial preparation of isolated membranes of insect cells Spodoptera frugiperda (line Sf9) overexpressing human recombinant P-glycoprotein (MDR1) according to the manufacturer's protocol. ATP hydrolysis during the catalytic activity of P-glycoprotein is accompanied by the formation of inorganic phosphate (Pi). The ATPase activity of P-glycoproteins was expressed in the amount of inorganic phosphate Pi (pmol)/mg protein/min.

P-Glycoprotein overexpressing membranes exhibit background ATPase activity, which increases in the presence of transported substrates (verapamil, vinblastine, etc.). Non-competitive inhibitors or slow transporting substrates decrease the ATPase activity observed in the presence of an activator. Therefore, the effect of the test compounds on the ATPase activity was investigated in the presence of the transported substrate - verapamil.

The results showing the influence of compounds (**I**) and (**II**) and their natural analogs CUR and DZG on the membrane P-gp ATPase activity are presented in Figs. 7a and 7b, respectively. It was revealed that all study compounds did not decrease, but, on the contrary, increase the P-gp ATPase activity; the effect is dose-dependent and it escalates when increasing the concentration of the compounds from 10 to 50 μM. Thus, compound (**II**) at a concentration of 50 μM increases the P-glycoprotein ATPase activity up to 221%. Reference compounds CUR and DZG similarly affect ATPase activity. It can be assumed that compounds (**I**) and (**II**), like DZG and CUR, may be substrates of P-gp and can be transported across the membrane with the expenditure of the ATP hydrolysis energy.

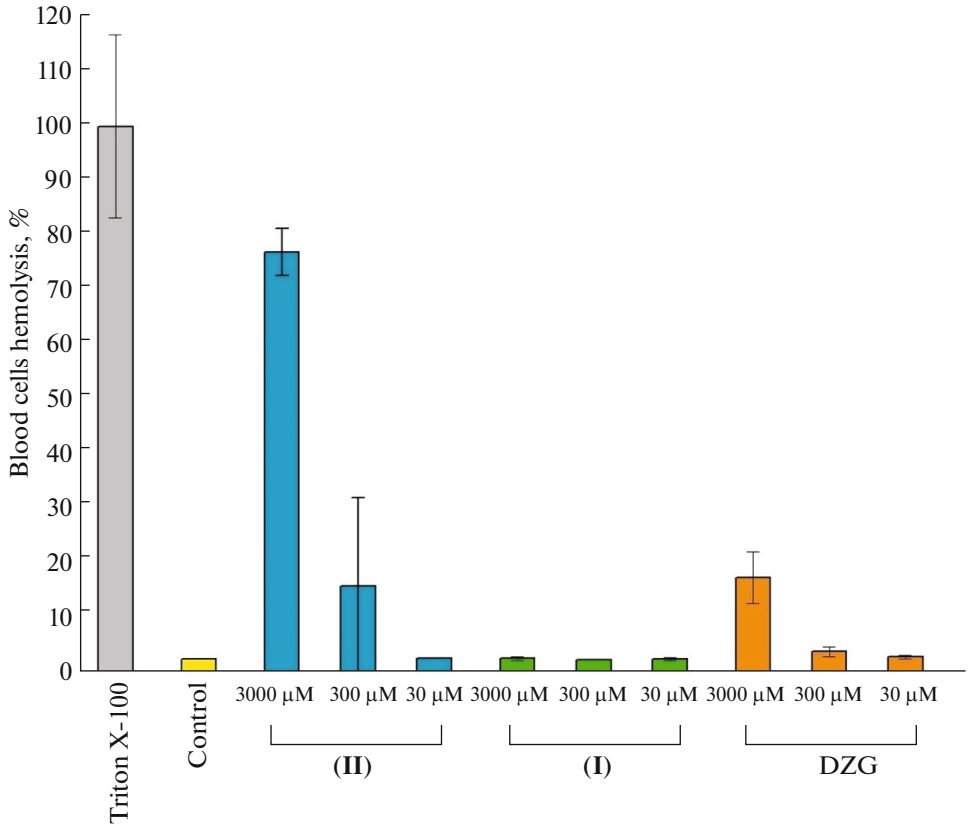

Fig. 6. Hemolysis of human erythrocytes treated with the test compounds and Triton X-100 at 37°C and shaking for 4 h. Hemolysis of erythrocytes treated with 0.2% Triton X-100 was taken as 100%.

Thus, it was found that the studied DZG analogs are not P-glycoprotein inhibitors, but rather act as their substrates and can increase the P-gp ATPase activity. However, it can also be assumed that the studied compounds can compete with DOX for the active sites of the transporter and reduce DOX reverse transport from cells due to this mechanism.

DZG analogs influence on tubulin polymerization. Tubulin polymerization is a vital process in the events related to cell division and proliferation. Affecting this process is one the mechanisms by which an antitumor agent can induce cell death. Some studies have reported that curcumin can affect tubulin polymerization pattern [51]. This raised the question of the ability of compounds (**I**) and (**II**) to affect this process.

In order to identify the molecular mechanisms of action of test compounds (**I**) and (**II**), their effect on tubulin polymerization was evaluated using a cytoskeleton test kit. The kit is based on the observation that microtubules scatter light to a degree proportional to the microtubule polymer concentration. We studied polymerization of highly purified porcine neuronal tubulin at 37°C in the presence of test compounds and inducers of polymerization—GTP and glycerol. We used paclitaxel, a polymerization enhancer, and vinblastine, a tubulin polymerization inhibitor, as positive and negative controls, respectively. Received curves are represented in Fig. 8.

The control polymerization curve represents three phases of microtubule polymerization, namely nucleation, growth and steady-state equilibrium, the V_{max} calculated from a slope of the curve is 6.7 ± 1.0 mOD/min. In the presence of paclitaxel, the nucleation phase proceeds faster, the slope of the straight line is more obtuse, V_{max} was 8.0, which is 1.2 times higher than the control reaction. In the presence of vinblastine we observed a significant (3-fold) inhibition of tubulin polymerization, V_{max} reached 2.4 mOD/min. However, the studied compounds (**I**) and (**II**), as well as the reference CUR and DZG, did not significantly affect the polymerization of tubulin; the maximum reaction rate varied within the error limits. For compounds (**I**) and (**II**) V_{max} was found to be 6.3 and 7.2 correspondingly, for DZG and CUR was 6.7 and 7.4 mOD/min. Thus, it was found that the investigated DZG analogs did not significantly affect the polymerization of tubulin and probably act on cancer cells via other mechanisms.

DZG analogs change the expression of apoptosis markers. The influences of compounds (**I**) and (**II**) on the expression of apoptosis markers (BAX, Bcl-2, Bcl-xL, procaspases 3 and 7), adhesion molecule E-cad-

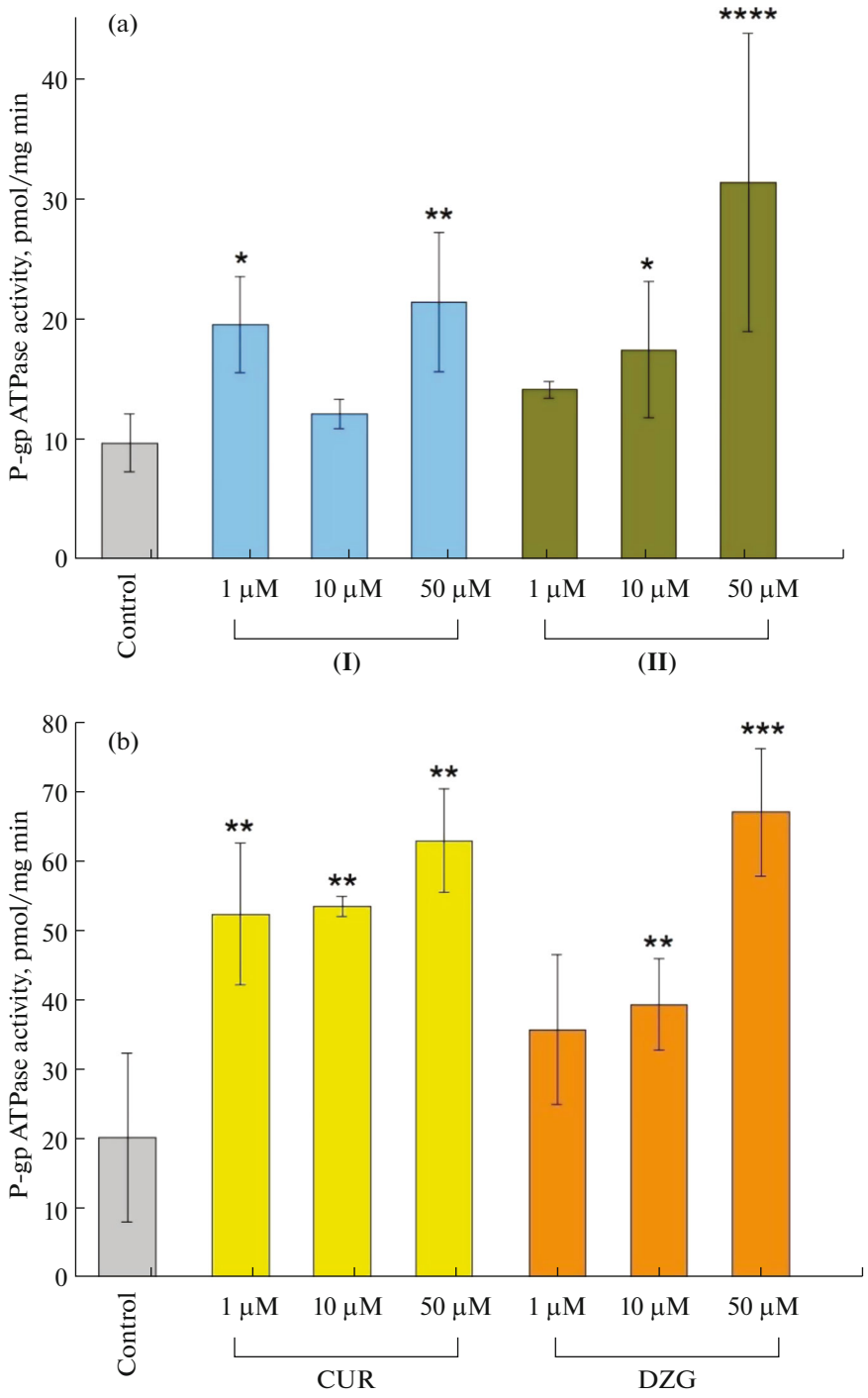

Fig. 7. P-Glycoprotein (MDR-1) ATPase activity (pmol/mg protein/min) in the presence of test compounds (**I**) and (**II**) (a) and reference compounds CUR and DZG (b). The control activity represents a fully activated reaction with the use of verapamil as a transported substrate.

herin, proinflammatory cyclooxygenase 2, as well as oncogene c-Myc were assessed using Western blot. The PC-3 cells were treated with test compounds for 3 days, then their whole lysate was obtained. The loading of protein into the gel was controlled by subsequent staining of the membrane with colloidal Coomassie G-250. Images of blots are shown in Fig. 9. The intensity of the Western blot bands was analyzed quantitatively, taking into account the correction for the protein load of the wells, the data are shown in Table 2.

Compounds (**I**) and (**II**) were found to downregulate the expression of the anti-apoptotic regulator Bcl-2,

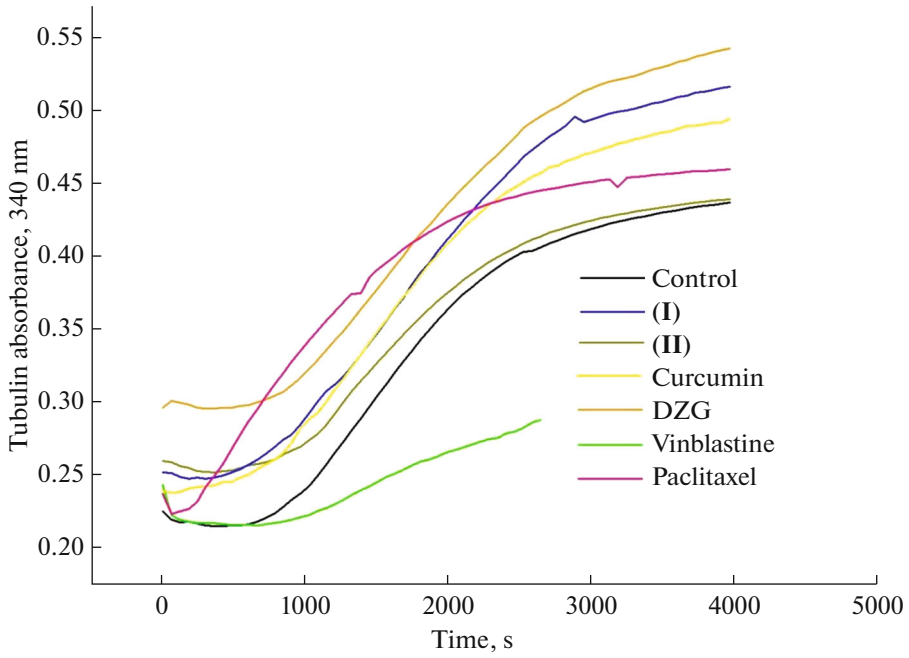

Fig. 8. Tubulin polymerization curves in the presence of study compounds ((**I**), (**II**), CUR, DZG), positive (paclitaxel) and negative (vinblastine) controls (all at a concentration of 10 μM).

but no suppression of the anti-apoptotic Bcl-xL was observed. Together, the studied derivatives increase the expression of the pro-apoptotic regulator BAX. We also studied the effect of compounds (**I**) and (**II**) on the expression and activation of apoptotic caspase 3. The amount of inactive procaspase 3 in cells, treated with compounds at a concentration of 15 μM, decreased, but this event does not indicate the caspase activation through proteolysis, because no increase in active caspase p17 and p19 amounts was found. Thus, it was revealed that the studied DZG analogs do not induce apoptosis by execution of caspase 3, but influence the expression of pro-apoptotic regulators BAX and anti-apoptotic regulator Bcl-2.

The investigated compounds (**I**) and (**II**) increase the expression of the membrane glycoprotein E-cadherin, which is a tumor suppressor protein, since it is involved in the regulation of intercellular adhesion and cell motility. The effect on E-cadherin expression explains the inhibitory effect of compounds (**I**) and (**II**) on cell migration, which was found in [24]. Also, the studied compounds do not reduce the expression of the transcription factor c-Myc, which is an oncogene involved in the activation of metabolism and proliferation of tumor cells. The studied compounds activate the expression of the pro-inflammatory enzyme cyclooxygenase 2 (COX-2), which can be explained by their stressful effect on the cancer cell.

Assessment of acute toxicity in mice. It was of interest to evaluate the safety of the lead compound in animal model. Since many anticancer drugs are administered intravenously, the acute toxicity of compound (**I**) was assessed after intravenous administration to female white ICR (CD-1) mice weighing 20–28 g.

Table 2. Western blot band intensities divided by the overall Coomassie staining ratio. Explanatory table for Fig. 9

Protein marker	Control	Compound (**I**)		Compound (**II**)	
		5 μM	15 μM	5 μM	15 μM
E-cadherin	4732	8378	8805	9575	16286
Bax	1095	4357	8415	3826	6453
Bcl-2	3559	1336	718	1979	295
Bcl-xL	10531	14264	11901	10295	15779
c-Myc	875	1352	1328	1153	622
COX-2	530	887	1654	889	221
Procaspase 3	431	203	170	199	111

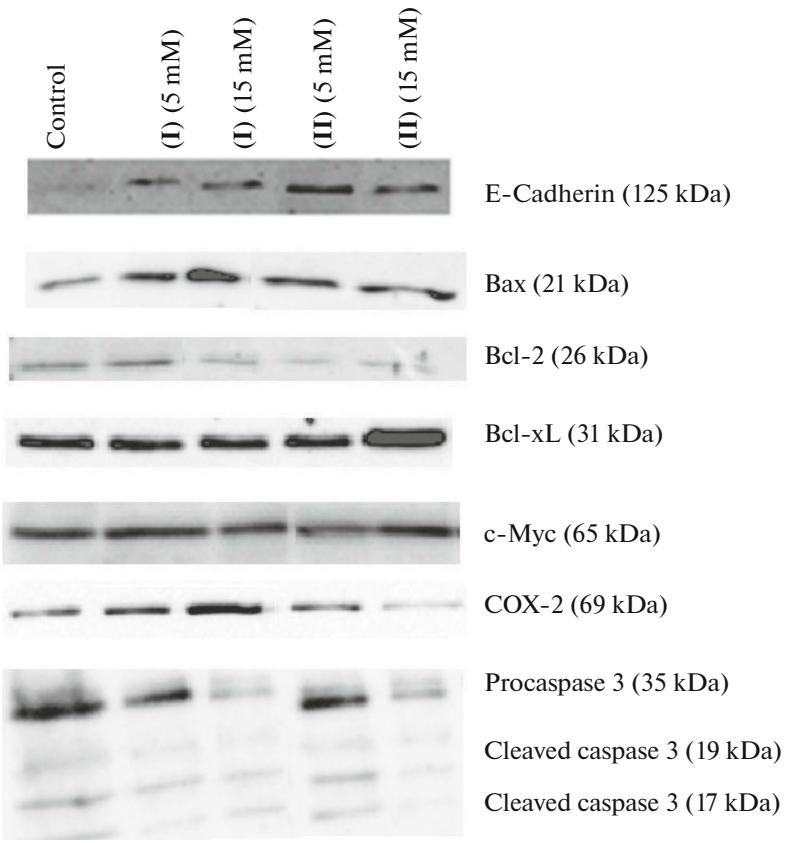

Fig. 9. Expression of proteins (E-cadherin, BAX, Bcl-2, Bcl-xL, c-Myc, COX-2, procaspases 3) in PC-3 cells treated with compounds (**I**) and (**II**) for 72 h according to the Western blot.

The results of the experiment are presented in Supplementary Information, Table S4. No deaths were recorded in the 50 mg/kg group. However, depression, blinking, ataxia, erratic movement in the cage, tremors of the muscles of the ears and back, and shortness of breath were observed. After 5 min, an improvement in the condition of the experimental animals was observed. After 1–1.5 h, the mice began to actively move around the cage. After 2 h, food and water began to be taken. In the group receiving 60 mg/kg one mouse died, in the group receiving 65 mg/kg 3 mice died, and at a dose of 70 mg/kg all mice died. Bronchospasm, salivation, convulsions were added to the listed effects at these doses. However, the symptoms of intoxication in surviving animals began to disappear after 1–2 h. The lethal dose 50 for compound (**I**) was 65 mg/kg with a confidence interval of 61.5:68.7. The LD_{50} of doxorubicin in CD-1 mice is 12 mg/kg, thus test compound (**I**) is 5.4 times less toxic than doxorubicin.

Assessment of genotoxicity. To evaluate genotoxicity a SOS-chromotest was performed using *Salmonella typhimurium* TA1535/pSK1002 as described by Oda [52]. The β-galactosidase activity means (OD420) were normalized by the growth factor (OD600) and the induction ratio (IR) of SOS-response was calculated as the ratio of activity in presence of compounds and the solvent control (Supporting Materials, Table S5). The concentrations of compounds (**I**) and (**II**) were 3×, 15×, 30×, and 150× of their CC_{50}. No significant dose-dependent increase more than 2-fold of IRs in presence of compounds (**I**) and (**II**) occurred in comparison with Mitomycin C demonstrating the absence of DNA-damaging activity of studied DZG analogs under concentrations tested.

To further assess the genotoxicity of compounds (**I**) and (**II**) the Ames test was performed using the *S. typhimurium* TA98 and *S. typhimurium* TA100 [53]. Samples were taken in concentrations of 1× and 5× of their CC_{50}. No increase in the number of revertant colonies was detected, as well as no dose-dependence could be observed (Supplementary Information, Table S6), suggesting no mutagenic potential of compounds (**I**) and (**II**).

CONCLUSIONS

In the present work, we continued to study the biological efficacy of two new promising DZG derivatives with pronounced antitumor activity in comparison with their natural analogs—DZG and CUR. We tested these compounds in compositions with known cyto-

statics: doxorubicin, vinblastine and paclitaxel. DZG analogs (**I**) and (**II**) at concentrations corresponding to their IC_{40}–IC_{50} synergistically increased the cytotoxicity of doxorubicin and vinblastine for PC-3 prostate adenocarcinoma cancer cells. However, to obtain synergistic compositions with paclitaxel, the concentration of compounds should be increased up to IC_{70}. Reference compounds DZG and CUR possess the same activity, but the cytotoxic concentration IC50 of CUR is 3.2–4.3 times higher and IC_{50} of DZG is 16.8–22.7 times higher than that of studied compounds. To identify the reasons for the revealed synergistic effect of compositions, we studied the influence of DZG analogs on the intracellular transport of doxorubicin, the physicochemical characteristics of cell membranes, and also investigated their effect on the activity of membrane P-glycoproteins.

The data obtained indicate that compounds (**I**) and (**II**) increase the intracellular accumulation of doxorubicin in tumor cells. Additionally, test compounds (**I**) and (**II**), as well as CUR and DZG cause tumor cell membrane depolarization and significantly reduce membrane microviscosity. The membrane-damaging effect of CUR is well known, but this effect was revealed for the first time for DZG and its new analogs. Thus, the synergism of the use of DZG analogs in composition with cytostatics (doxorubicin and vinblastine) is explained by their effect on the physicochemical characteristics of the cytoplasmic membrane of cancer cells. Due to membrane-damaging activity, compound (**II**) can cause red blood cells hemolysis in concentrations higher than 300 μM, however, compound (**I**) is safe up to 3000 μM. Compounds (**I**) and (**II**) showed no inhibitory effect on the membrane P-glycoprotein ATPase activity; on the contrary, they probably serve as substrates for P-gps and thus can compete with doxorubicin for transporter binding sites. It was also revealed that the studied compounds, in contrast to vinblastine and paclitaxel, do not affect the polymerization of tubulin in vitro. Certain effects of compounds (**I**) and (**II**) on protein expression were also revealed, it was found that they increase the expression of pro-apoptotic BAX and decrease the expression of anti-apoptotic Bcl-2, as well as increase the expression of E-cadherin, which is a protein of adhesion and intercellular contacts.

We assessed the acute toxicity of compound (**I**) after intravenous administration to female white ICR (CD-1) mice, the LD_{50} was found to be 65 mg/kg; test compound (**I**) is 5.4 times less toxic than doxorubicin. SOS-chromotest revealed the absence of DNA-damaging activity of studied DZG analogs. According to the Ames test, compounds (**I**) and (**II**) has no mutagenic potential on *S. typhimurium*. Therefore, the study drug candidates are relatively safe.

Thus, the investigated compounds affect the cell in a complex manner, change the functional state of the cytoplasmic and mitochondrial membranes, induce apoptosis, and affect the adhesion and motility of the cancer cell. It can be concluded that new DZG analogs are promising drug candidates to be further tested on animals with grafted tumors, including their compositions with doxorubicin and vinblastine.

EXPERIMENTAL

Synthesis of Test Compounds

General information. Compounds (**I**) and (**II**) were obtained by the Wittig reaction. Chromatographic purification of compounds was carried out using column chromatography on Acros silica gel (60–200 mesh). Reaction progress and purity of compounds were monitored by TLC on Sorbfil PTLC-AF-A-UF plates. Melting points of the products were determined using a Stanford Research Systems MPA-100 OptiMelt apparatus. ^1H, ^{13}C NMR spectra were recorded on a Bruker Avance 400 spectrometer (400.17 and 100.62 MHz). HPLC/MS-experiment was carried out using a TripleTOF 5600, AB Sciexsuperhigh resolution mass spectrometer (Germany) from the solution in methanol by turboionic spray (TIS) ionization method with the collision energy with nitrogen molecules of 10 eV. Analytical reversed-phase HPLC was used for determination of uncalibrated purity of the compounds and conducted using a Atlantis T3 C18 column (5 μm, 150 × 4.6 mm); eluent A water; eluent B methanol; gradient elution (0 min A : B = 60 : 40 to 20 min A : B = 0 : 100); flow rate was 1.0 mL/min. HPLC analysis was performed at 40°C during 20 min at 280 nm.

(*E*)-4-(2-Ethyl-8-methyl-4*H*-[1,3]dioxino[4,5-*c*]-pyridin-5-yl)but-3-en-2-one (I). 2-Ethyl-8-methyl-4*H*-[1,3]dioxino[4,5-*c*]pyridine-5-carbaldehyde (0.53 g, 2.56 mmol) and Et3N (1.36 mL, 9.80 mmol) were successively added to a solution of (2-oxopropyl)triphenylphosphonium chloride (1.00 g, 2.56 mmol) in 30 mL of CH_2Cl_2, and the reaction mixture was stirred at 70°C for 24 h. The solvent was removed in vacuo, the residue was dissolved in ethyl acetate (50 mL), and the insoluble residue of triphenylphosphine oxide was filtered off. The solvent was evaporated in vacuo, and the product was purified by column chromatography on silica gel (eluent diethyl ether). Yield 70% (0.44 g), white crystals, mp 86°C. Spectra NMR ^1H and ^{13}C were identical as in [24]. HPLC analysis: retention time 11.9 min; purity 98.5%.

(*E*)-4-(8'-Methyl-4'*H*-spiro[cyclohexane-1,2'-[1,3]dioxino[4,5-*c*]pyridin-5-yl)but-3-en-2-one (II). 8'-Methyl-4'*H*-spiro[cyclohexane-1,2'-[1,3]dioxino-[4,5-*c*]pyridine-5-carbaldehyde (0.63 g, 2.56 mmol) and Et3N (1.36 mL, 9.80 mmol) were successively added to a solution of (2-oxopropyl)triphenylphosphonium chloride (1.00 g, 2.56 mmol) in 30 mL of CH_2Cl_2, and the reaction mixture was stirred at 70°C for 24 h. The solvent was removed in vacuo, the residue was dissolved in ethyl acetate (50 mL), and the insoluble res-

idue of triphenylphosphine oxide was filtered off. The solvent was evaporated in vacuo, and the product was purified by column chromatography on silica gel (eluent diethyl ether). Yield 88% (0.65 g), white crystals, mp 80°C. Spectra NMR ^{1}H and ^{13}C were identical as in [24]. HPLC analysis: retention time 15.1 min; purity 97.3%.

Preparation of solutions. Stock solutions of DZG analogues with a concentration of 20 mM were prepared in DMSO/H_2O 1 : 1; an equimolar amount of HCl was added to the stock solutions of the compounds to obtain their hydrochlorides, which are better soluble in water. Solutions of dehydrozingerone hydrochloride, curcumin and a commercial chemotherapeutic agent were prepared in DMSO.

Cytotoxicity study of new DZG analogs compositions with known commercial chemotherapeutic agents. Human Prostate Carcinoma (PC-3 GSM136316) cells were kindly provided by Fox Chase Cancer Center (Philadelphia, USA). PC-3 cells were cultured and maintained in standard conditions as described in [24]. PC-3 cells were cultured in a 96-well plate in the presence of a series concentrations of cytostatics with the addition of a series concentrations of study compounds added in a checkerboard pattern as described in [44, 45]. Further, cell viability was assessed using the MTT-test as described in [24], cytotoxic concentrations IC_{50} of DOX, PAC, and VIN alone and with the addition of test compounds were obtained as well as IC_{50} of test compounds alone and with the addition of cytostatics were obtained.

The fractional inhibitory concentration index (FICI) was calculated using the following formula:

$$\mathrm{FICI} = \frac{\mathrm{IC_{50}\ Drug\ } A \mathrm{\ in\ combination\ with\ } B}{\mathrm{IC_{50}\ Drug\ } A} + \frac{\mathrm{IC_{50}\ Drug\ } B \mathrm{\ in\ combination\ with\ } A}{\mathrm{IC_{50}\ Drug\ } B}.$$

If the FICI index is <0.5, then the relationship between two compounds in tested concentrations is considered synergistic, if 0.5 < FICI < 4 the relationships are additive, if FICI > 4 then antagonistic.

Assessment of doxorubicin accumulation in tumor cells. PC-3 cells at a concentration of 10000 cells/mL were cultured in 12-well plate in 2 mL of medium for 24 h. After that compounds (**I**), (**II**) and CUR were added to the cells at a final concentration of 5 μM and 20 μM. Then Doxorubicin was added at a concentration of 5 μM to control wells and each well with the test compound and incubated for 1 h. After that cells were washed 3 times with DBPS and harvested using Trypsin-EDTA, centrifuged and re-suspended in DPBS. The fluorescence was analyzed using Guava® easyCyte™ 8HL flow cytometer.

Evaluation of the DZG Analogs Effect on the Cytoplasmic Membrane

Influence on the cytoplasmic membrane potential. The transmembrane potential of the plasma membrane was assessed using the DiOC6(3) (3,3'-dihexyloxacarbocyanine iodide) indicator and the methodology described in [46–48]. PC-3 cells were cultured under standard conditions, harvested in Trypsin-EDTA solution, washed three times in DPBS buffer and then suspended in DPBS at 1×10^6 cells/mL. DZG analogs as well as DZG and CUR were added to the cell suspension to a final concentration of 5 and 20 μM and incubated for 10 min at 37°C. Nonionic surfactants Triton X-100 and Tween-20 were used as positive controls at concentrations of 0.1 mg/mL. Then the indicator DiOC6(3) was added to the treated cells to a final concentration of 200 nM and additionally incubated for 20 min at 37°C until equilibrium was reached. The stained cells fluorescence was detected on a Guava® easyCyte ™ 8HL flow cytometer.

Influence on the cytoplasmic membrane viscosity. The microviscosity of the plasma membrane of PC-3 cells was measured using the membrane indicator Diphenylhexatriene (DPGT) fluorescence anisotropy [49, 50].

PC-3 cells were harvested using Trypsin-EDTA solution, centrifuged, suspended at 2×10^6 cell/mL in DPBS buffer, transferred in 96-well black plate. DPGT was then added at 1 μM final concentration. After 30 min of incubation at room temperature, the baseline fluorescence polarization was measured using Infinite F200 PRO TECAN plate reader. Then compounds (**I**), (**II**), CUR and DZG were added at concentrations of 5, 20 and 100 μM and the fluorescence polarization was measured every 10 min for 1 h. Non-treated PC-3 cells were used as negative control, 100 μg/mL cholesterol and 0.05% Triton X-100 were used as positive controls.

Evaluation of the blood cells hemolysis. Erythrocytes were isolated from freshly isolated blood of conventionally healthy donors. The blood was centrifuged at 1000 *g* for 15 min at 4°C. The supernatant plasma was removed, and the red blood cell pellet was washed three times by centrifugation in cooled isotonic buffer (154 mM NaCl, 50 mM HEPES acid, 1 mM EDTA, pH 7.4). The isolated erythrocyte fraction was diluted 100-fold in isotonic buffer. Test compounds ((**I**), (**II**), DZG, CUR) at a concentration of 10, 30, 300, 3000 μM and 0.2% Triton X-100 (positive control, complete hemolysis) were added to the erythrocyte suspension. Samples were incubated for 4 h at 37°C on a shaker, then erythrocytes were removed by centrifugation at 300 *g*. The amount of hemoglobin in the supernatant was determined spectrophotometrically at 540 nm. The measurements were carried out on a Infinite M200 PRO TECAN plate reader.

Evaluation of the P-glycoprotein ATPase activity. The ATPase activity of membrane P-glycoproteins

(P-gp) was studied according to the manufacturer's protocol (Sigma-Aldrich) using commercial preparation of isolated insect cell membranes of Spodoptera frugiperda (cell line Sf9, Cat. no. SBAT01-1EA), overexpressing human recombinant P-glycoprotein (MDR1).

Briefly, membrane preparation (whole protein 0.2 mg/mL) in a reaction buffer was mixed with the study compounds in concentrations of 1, 10 and 50 μM in the presence of an activator substrate (verapamil 100 μM) for 5 min at 37°C. Then Ca^{2+}-stabilized ATP (5 mM) was added to the mixture and incubated for an additional 60 min at 37°C. The reaction was stopped by the addition of 100 μL of 5% SDS. ATPase activity was assessed by the detection of free inorganic phosphate with the use of colorimetric ascorbic acid/ammonium molybdate reaction [54]. The samples were mixed using a vortex, then 130 μL of Pi-reagent and 100 μL of ascorbic acid 2% were added. The signal was read immediately after the addition of reagents and for another 1 h with an interval of 5 min using a Infinite M200 PRO TECAN plate reader at 880 nm. ATPase activity was expressed in pmol Pi/mg protein/min.

Evaluation of the tubulin polymerization. To evaluate tubulin polymerization in vitro, we used the Cytoskeleton Tubulin Polymerization Assay Kit (Cat. no. BK006P). The standard polymerization reaction contains 100 μL of 3 mg/mL porcine tubulin in 80 mM PIPES pH 6.9, 0.5 mM EGTA, 2 mM $MgCl_2$, 1 mM GTP, 7% glycerol. Test compounds and controls (vinblastine and paclitaxel) were used at a concentration of 10 μM. Polymerization was initiated by heating to 37°C and followed by absorbance readings at 340 nm on Infinite M200 PRO TECAN plate reader each minute for 80 min.

Western blot analysis. The PC-3 cells were treated with compounds (**I**) and (**II**) at concentrations of 5 and 15 μM for 72 h. The treated and non-treated control cells were harvested, washed in PBS and lysed in ice-cold RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCI, 0.1% SDS, 1% sodium deoxycholate, 1% Nonidet P-40, and protease inhibitors) at 4°C for 30 min. Cell lysates were normalized (Lowry assay) and a total of 40 μg protein was resolved by denaturing 12 or 6% (for low and high molecular weight proteins correspondingly) SDS-PAGE gel electrophoresis and transferred to Immuno-Blot PVDF membrane using Trans-Blot SD Semi-Dry or Wet (in case of high molecular weight protein) Transfer Cell (BioRad, USA). Membranes were blocked with PBS-T containing 5% (mass/vol) nonfat dried milk for 2 h at 4°C, incubated with primary anti-E-cadherin (Thermo Scientific), anti-BAX (Abcam), anti-Bcl-2 (Abcam), anti-Bcl-xL (Abcam), anti-caspase-3 (Santa Cruz), anti-c-Myc (Santa Cruz), anti-COX-2 (Thermo Scientific) antibodies overnight at 4°C, and then with Anti-Mouse, Anti-Rabbit, Anti-Goat IgG-HRP antibody (Santa Cruz) for 2 h at room temperature. Blots were developed with Clarity Western ECL substrate (Bio-Rad, USA) and documented using ChemiDoc XRS Plus (Bio-Rad, USA). Equal protein loading was confirmed with colloidal Coomassie G-250 staining. Western blot band density and Coomassie bands total density were determined using the ChemiDoc system software and their ratio was calculated.

Assesment of acute toxicity in mice. Female white mice ICR (CD-1) were purchased from "Nursery for laboratory animals" (Russian Academy of Sciences, Pushchino) and were quarantined for 2 weeks before dosing. The animals were randomized into groups, the weight variation was 20–28 g. The mice were housed in stainless steel cages and fed complete extruded compound feed for keeping rats, mice, hamsters (Laboratorkorm). Food and water were available *ad libitum*. The mice were housed in a room with a temperature of 22–26°C, relative humidity of 30 to 70%, and a 12 h light/dark cycle. In the acute toxicity study, mice were injected intravenously through the tail vein with a total dose of 10, 50, 60, 65, 70 mg/kg of compound (**I**) in saline. The volume of administration was calculated individually for each animal, based on the bodyweight recorded immediately before the administration of the substance. The animals were observed individually after administration for 30 min, then at least once an hour for 4 h, then every day once a day for 14 days, after which they were euthanized and subjected to gross necropsy. If an animal dies during the study, the time of death is established and documented as accurately as possible. The animal is weighed and opened as early as possible.

Ames test and SOS-chromotest. *Salmonella typhimurium* strains TA98 and TA100 [52, 53] were grown overnight in 5 mL of LB medium, diluted 4-times by pre-warmed LB then incubation was continued for 2 h. Cells were harvested, washed once by 1× salt base solution (g/L: Sodium citrate·$3H_2O$—0.5; $K_2HPO_3 \cdot 3H_2O$—14; KH_2PO_3—6; $(NH_4)_2SO_4$—1; $MgSO_4 \cdot 7H_2O$—0.5) and resuspended in 6 mL of 1× salt base. Bacterial suspension 100 μL was mixed with top agar (0.5% agar, 0.5% NaCl, 50 mM L-histidine, 50 mM biotin, pH 7.4, 42°C) in a final volume of 3 mL and the substance to be tested. Each mixture was then seeded onto the minimal agar plates (1.5% agar in the 1× salt base supplemented with 0.5% glucose and ampicillin 10 μg/mL). Next, the plates were incubated at 37°C for 72 h and colonies were counted. The sodium azide (10 μg/plate) was used as a positive control.

The SOS-chromotest was performed by using the *Salmonella typhimurium* TA1535/pSK1002 as described in [52]. Briefly, aliquots of 0.5 mL of an overnight culture of the tester strains were diluted in 5 mL of LB medium and then incubated with vigorous agitation in presence of the ficin substances. The Mitomycin C (Sigma) at concentration of 1 μg/mL was used as a positive control. After 4 h of incubation,

the cell density (A600) and the β-galactosidase activity was measured by the Miller's protocol [56] with modifications. Cells were harvested from 0.5–1.5 mL of culture liquid, redissolved in 800 μL of Z-buffer (60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, and 1 mM $MgSO_4 \cdot 7H_2O$ (pH 7.0)) containing additionally 0.005% cetyl trimethylammonium bromide (CTAB) and 50 mM β-mercaptoethanol. After preincubation at 30°C for 5 min, the reaction was started by adding of 200 μL of 4 mg/mL *o*-nitrophenyl-β-*D*-galactopyranoside in Z-buffer. When the yellow color appeared, the reaction was stopped by 500 μL of 1 M Na_2CO_3. For the blank solution, the Na_2CO_3 was added prior the incubation. The β-galactosidase activity was measured at 420 nm. To calculate the Miller units, we used the following formula: (A420/(A600 of 1 : 10 dilution of cells × time of incubation)) × 1000.

Statistical analysis. The results were calculated and statistically analyzed using GraphPad prism 9. One-way analysis of variance (ANOVA) or the Student's t-test were used to analyze the results, and differences were deemed statistically significant at a p-value < 0.05. The analyzed variables were presented as mean ± standard deviation. The significance of differences relative to control is shown above the bars, p-value: 0.1234 (ns), 0.0332 (*), 0.0021 (**), 0.0002 (***), <0.0001 (****). Each experiment was repeated at least three independent times. Figures represent average data or data from the most representative experiment.

FUNDING

Cytotoxicity study was performed by the subsidy allocated to Kazan Federal University for the state assignment in the sphere of scientific activities, project no. 0671-2020-0053. All other experiments of the study were funded by RFBR, project no. 20-33-70175.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflicts of interest.

Statement on the welfare of animals. The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of Kazan (Volga region) Federal University (protocol code 24 and date of approval 22.09.2020) for studies involving animals.

All manipulations with animals were carried out in accordance with GOST 33215-2014 of Russian Federation "Guidelines for the maintenance and care of laboratory animals. Rules for equipping premises and organizing procedures for working with laboratory animals". Additionally, this research was carried out in accordance with the ARRIVE guidelines [55]. The acute toxicity study of compound (**I**) was agreed with the local ethics committee of the Kazan (Volga region) Federal University, protocol no. 24 of 22.09.2020.

SUPPLEMENTARY INFORMATION

The online version contains supplementary material available at https://doi.org/10.1134/S106816202304009X.

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