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Effects of quinones and nitroazoles on free-radical fragmentation of glycerol-1-phosphate and 1,2-dimyristoyl-glycero-3-phosphatidyl-glycerol

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ABSTRACT

Effects of quinones and azoles on the formation of steady-state radiolysis products in aqueous solutions of glycerol-1-phosphate and aqueous dispersions of 1,2-dimyristoyl-glycero-3-phosphatidyl-glycerol has been investigated. The data obtained by LC–MS-ESI and spectrophotometric measurements shows that the compounds having quinoid structures, including the antitumor agent doxorubicin, and azoles having nitro groups effectively inhibit free-radical fragmentation of glycerol-1-phosphate and 1,2-dimyristoyl-glycero-3-phosphatidyl-glycerol, decreasing the radiation-chemical yields of either inorganic phosphate or phosphatidic acid respectively. The observed effects of blocking free-radical processes are believed to be related to the ability of the tested compounds to oxidize α-hydroxyl-containing carbon-centered radicals of starting substrates, which give rise to fragmentation reaction. The possibility of using the discovered properties of quinones, doxorubicin and nitroazoles to provide practical solutions in oncological radiotherapy and pathophysiology is discussed.

1. Introduction

Free-radical processes (FRP) play an important role in the functioning of an organism. At the same time, their activation leads to the onset or worsening of a number of diseases including neurodegenerative pathologies, ageing, inflammatory processes, cardiovascular problems, cancer etc. (Halliwell and Gutteridge, 2007). In biosystems, the FRP usually are induced by reactive oxygen species (ROS), which are generated either due to a failure in biochemical processes of oxygen utilization (internal factors) or as a result of external factors such as γ- or UV-irradiation of biosystems (Halliwell and Gutteridge, 2007; Von Sonntag, 2006). Highly reactive ROS such as ˙OH or RO˙ can react with biomolecules causing various transformations. In spite of this, the overwhelming amount of data has been devoted to the studies of oxidation reactions of biologically relevant compounds, primarily, cell membrane lipids (Frankel, 2005; Niki, 2009). It is believed that the hyperproduction of ROS and activation of lipid peroxidation (LPO) are the two main components of the oxidative stress in an organism. It is known at the same time that oxygen levels in various tissues, cells and even in cellular organellae vary within a wide range (Carreau et al., 2011). So, the amounts of O_2 in mitochondria are by an order of magnitude lower than in plasmatic membranes. This situation generated a need for introducing, along with «hypoxia» and «oxygenation», a new term «normoxia», which would characterize the oxygen levels necessary for normal functioning of bioobjects.

On interaction of ROS with biomolecules under conditions of low oxygen levels FRP may occur. In earlier studies, it has been shown (Edimecheva et al., 1997; Steenken et al., 1974; Von Sonntag, 1975) that glycerol-1-phosphate and hydroxyl-containing glycerophospholipids when interacting with OH-radicals underwent decomposition according to the following Scheme 1.

The key stage in this process (Scheme 1) is the fragmentation of the radicals (I) formed from the starting compounds. Elimination of phosphatidic acid (PA) was found to be extremely fast – the rate constant being 10^6–10^7 s^{-1}, which happens even in the presence of the highest physiological concentrations of O_2. The reaction (Scheme 1) results not only in destruction of the starting phospholipids but also the formation of PA. PA plays an important role in biosynthesis of membrane lipids, as well as in processes that ensure the control of cell cycle progression and cell growth (Wang et al., 2006).

Therefore, finding substances that would significantly affect the destruction processes of glycerophospholipids resulting in the supernormal formation of PA in biosystems appears to be an important task. We have found earlier that the free-radical reactions (Scheme 1) were

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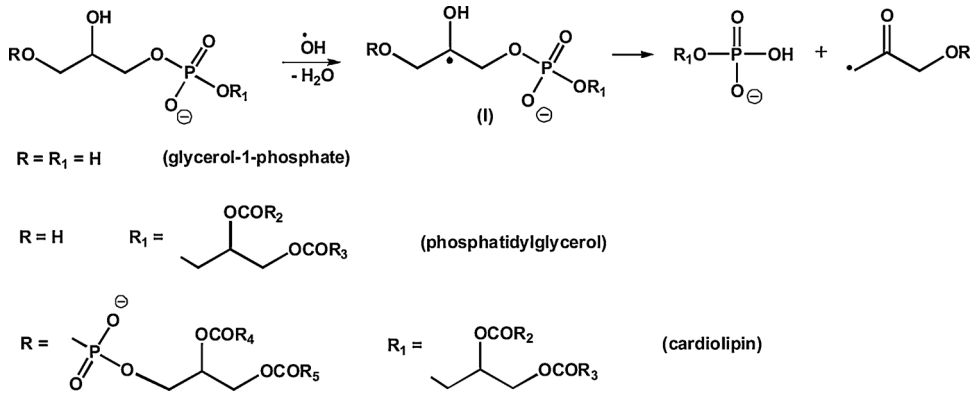


Scheme 1. Decomposition of glycerol-1-phosphate and hydroxyl-containing glycerophospholipids under the action of OH-radicals.

inhibited by oxidants, mostly by oxygen. We examined the effects of a number of quinones and nitroazoles on the yield of free-radical fragmentation of glycerol-1-phosphate and 1,2-dimyristoyl-glycero-3-phosphatidyl-glycerol in order to find novel regulators for these processes.

2. Experimental

2.1. Materials

1,4-Benzoquinone (**1**), 2,3,5-trimethyl-1,4-benzoquinone (**2**), tert-butyl-1,4-benzoquinone (**3**), coenzyme Q_0 (**4**), thymoquinone (**5**) and doxorubicin hydrochloride (**6**) (Fig. 1) were purchased from Sigma-Aldrich (Germany) and were used in this study without further purification.

Azoles represented in this study by imidazole (**7**), metronidazole (**8**), 1,2,4-triazole (**9**) and sanazole (**12**) were purchased from Sigma-Aldrich (Germany). Dinitrotriazoles – sodium 3,5-dinitro-1,2,4-triazolate (**10**) and 1-methyl-3,5-dinitro-1,2,4-triazole (**11**) (Fig. 2), were synthesized and purified according to the published procedures (Bagal et al., 1970; Haiges et al., 2015) in the Laboratory of chemistry of condensed systems (Research Institute for Physical and Chemical Problems of the Belarusian State University). Purity of the synthesized 1,2,4-triazoles was tested by mass spectrometry. Purity of all compounds under study was not less than 98%.

1,2-Dimyristoyl-sn-glycero-3-phosphate monosodium salt (DMP) (purity ≥99%), 1,2-dimyristoyl-sn-glycero-3-phospho-rac-(1-glycerol) ammonium salt (DMPG) (purity ≥97%), disodium salt of glycerol-1-phosphate (GP) (purity ≥95%), chloroform and ammonia solution 25% were obtained from Sigma-Aldrich (Germany). Methanol was from Fisher Chemical (UK). Deionized and then twice-distilled water was used in all experiments. All other chemicals were of the highest purity commercially available.

2.2. Preparation of samples for irradiation

Tested compound solutions were prepared by dissolving exactly weighed amounts of quinones or azoles in blank solutions of 10^{-1} M GP. The pH value of the solutions was adjusted to 7.0 ± 0.05 by adding suitable quantities of perchloric acid or sodium hydroxide. The solutions were transferred into ampoules, deaerated with high purity argon for 60 min and sealed. Concentration of the tested compounds was 10^{-3} M.

Multilayer liposomes were prepared by dispersing thin lipid films in phosphate buffer (Bangham et al., 1965). To do this, the lipid was dissolved in chloroform/methanol mixture (2:1, v/v). The solvent was removed from DMPG solutions on a rotary evaporator and the resulting film samples were kept under vacuum for at least 1 h to ensure complete removal of the solvent. To the lipid film was added the appropriate quantity of phosphate buffer (50 mM, pH 7.4) containing sodium dodecyl sulfate (10^{-4} M), preliminarily deaerated with argon (99.9%) during 40 min. The mixture was shaken at 30 °C for 15 min on a Vortex mixer (IKA test tube vortex mixer, Severn Sales, Bristol, UK).The multilayer liposomes thus obtained were sonicated for 3 min using Bandelin Sonorex ultrasound unit (35 KHz, 60/150 W), whereupon transferred to ampoules, deaerated with high purity argon for 20 min and sealed. The similar procedure was applied to prepare liposome samples containing additives at concentrations of 5×10^{-4} M, being introduced with the phosphate buffer containing sodium dodecyl sulfate. The phospholipid concentration in the liposomes was 10^{-2} M.

2.3. Irradiation of the samples

Irradiation was performed on a unit MPX-γ-25 M with a ^{60}Co source. The dose rate absorbed during the experiment, measured with a Fricke dosimeter (Fricke and Hart, 1966), was 0.14 ± 0.01 Gy/s. The absorbed dose interval varied within the range of 0.17–1.01 kGy for solutions of GP and 0.42–2.35 kGy for multilayer liposomes.

2.4. Identification of radiolysis products and determination of their concentrations

2.4.1. LC–MS-ESI measurements

Lipids were extracted from the samples by shaking liposomes with a chloroform-methanol mixture (2:1, v/v) (Folch et al., 1957). After separation of layers by centrifugation, the lower layer was separated and

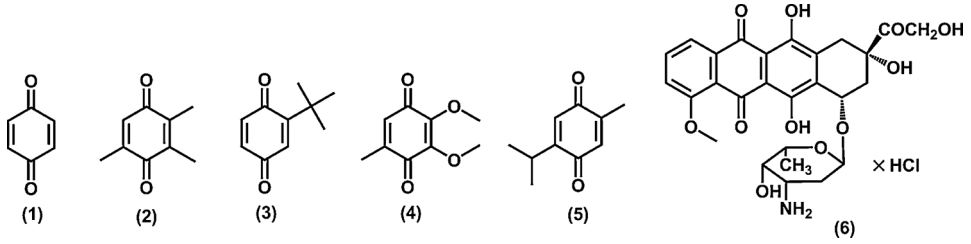


Fig. 1. Structures of the quinones examined in the study.

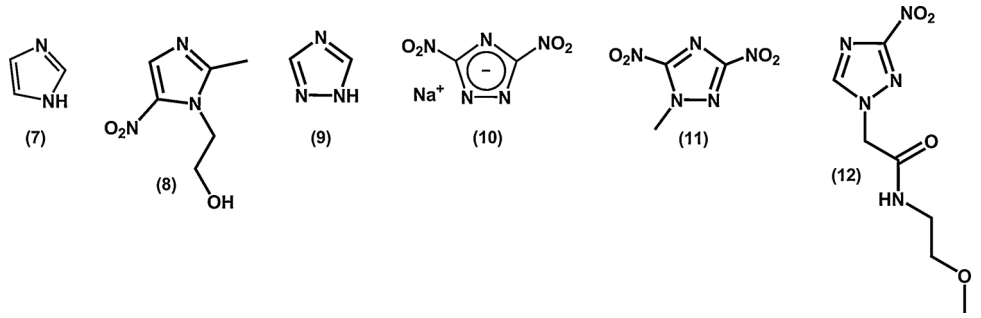


Fig. 2. Structures of the azoles examined in the study.

concentrated. The extraction procedure was repeated twice.

To determine the amount of DMPA resulting from the radiation-induced fragmentation of DMPG, a Shimadzu LCMS-2020 instrument was used (Mizuno et al., 2012), featuring a liquid chromatograph equipped with a Shim-pack XR-Sil column (100 mm length, 2.0 mm ID, 2.2 μm particle size) from Shimadzu (Kyoto, Japan) coupled to a mass spectrometer with a quadrupole detector. The equipment performance was computer-controlled using a LabSolutions software Version 5.42 SP4. The injected sample/standard volume was 1 μl. Mobile phase consisted of chloroform/methanol/ammonia/water (55:39:1:5, v/v/v/v). Isocratic elution mode was used with a flow rate of 0.2 ml/min; the column was thermostated at 33 °C.

The analyte concentrations were determined in the total ion current measurement mode. Analytical conditions: ionization method–electrospray with negative ion generation; the interface and detector voltages were −3.5 kV and 0.20 kV, temperatures of the heating and desolvation blocks were 400 °C and 300 °C, respectively; the sprayer gas flow was 1.5 l/min. The scan mass range was *m/z* 300–900. The detected species were deprotonated molecules ((M − H))–.

2.4.2. Spectrophotometric measurements

Inorganic phosphate (IP) in the presence of the organic phosphate was determined spectrophotometrically on a Specord S600 UV–vis instrument using a modified procedure described in (Gin and Morales, 1977). To obtain calibration curves, aqueous solutions of KH_2PO_4 were used. To 0.5 ml of the solution to be examined were added 1 ml 1.8% solution of $(NH_4)_2MoO_4 \times 4H_2O$ in 1 M H_2SO_4 and 0.2 ml of 10% $FeSO_4 \times 7H_2O$ in 0.075 M H_2SO_4, and 4 ml of twice-distilled water. The solutions were stirred for 5 min on an auto-shaker. Optical density of the solutions was measured at λ = 720 nm using quartz cells with optical path length of 10 mm. The plot of optical density vs. concentration of IP was linear within the range of 10^{-5} to 10^{-3} M.

Concentrations of the tested compounds in the solutions of GP before and after γ-irradiation were determined spectrophotometrically using quartz cells with optical path length of 1 mm, if not stated otherwise.

2.5. Calculations of radiation-chemical yields

Radiation-chemical yields (G, mol/J) were calculated from linear portions of plots of the product concentrations vs. dose absorbed. The results obtained in three independent experiments were used to calculate the yields. The error in determinations of radiation-chemical yields was calculated by the least squares method using the confidence coefficient of 0.95.

2.6. Statistical analysis

The data obtained were statistically analyzed using Statistica v. 10 software (StatSoft, Poland). The results were expressed as the mean ± one standard deviation (SD) from three independent parallel experiments. The data was analyzed using the *t*-test for independent samples. The differences were considered to be significant at $p < 0.05$.

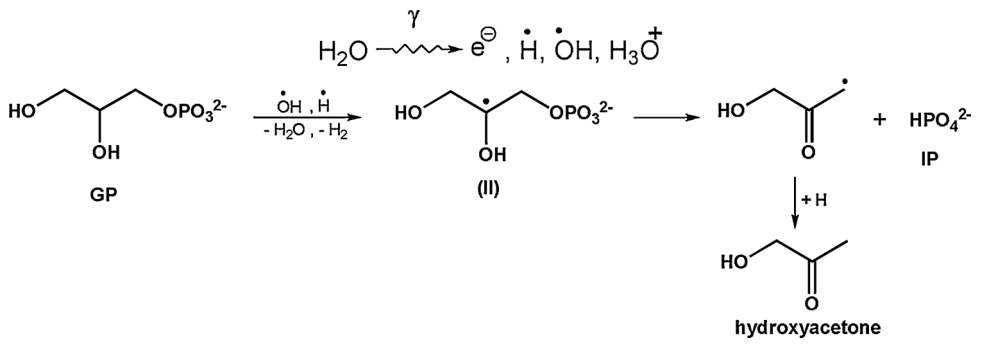
3. Results and discussion

3.1. Steady-state radiolysis of aqueous solutions of GP in the presence of the tested additives

GP is an important metabolite of the cell, being involved in both lipid synthesis and in other metabolic processes (Lehninger et al., 2012). The major intermediates formed on radiolysis of aqueous solutions of GP are α-hydroxyl-containing carbon-centered radicals (α-HCR) (II). Fragmentation of these radicals results in the dephosphorylation of GP giving IP and hydroxyacetone (Scheme 2).

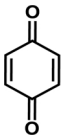
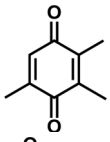
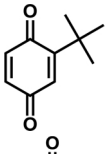
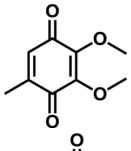
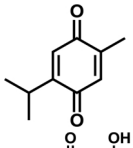
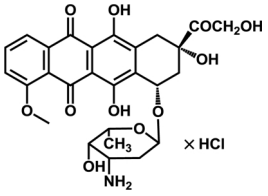
IP is the main stable final product of GP radiolysis in deaerated aqueous solutions at pH 7 (Schuchmann et al., 1995), unlike hydroxyacetone, which is initially formed as a radical intermediate.

At the additive/GP ratios of 1/100 (10^{-3} M–10^{-1} M), the radiolysis products of water (˙H and ˙OH) would predominantly react with GP to form carbon-centered radicals. Therefore, the data on the effects of quinones and nitroazoles on the yields of phosphate anions will provide information about the possible interactions of the additives with radicals (II) formed from GP.



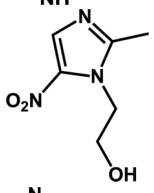
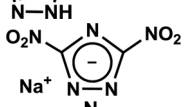
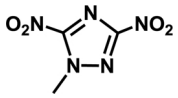
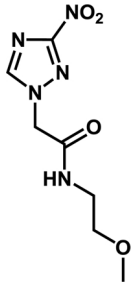
Scheme 2. Dephosphorylation of GP during radiolysis of its aqueous solutions.

Table 1
Effects of quinones on radiation-chemical yields of inorganic phosphate formed in deaerated aqueous 10^{-1} M glycerol-1-phosphate solutions at pH 7.

Additive		Radiation-chemical yields (G) × 10^7, mol/J	
		Inorganic phosphate	Consumption of the additive
No additive		4.51 ± 0.15 a	–
	(1)	2.39 ± 0.16 bcd	– 4.16 ± 0.24 b
	(2)	2.55 ± 0.16 b	– 1.33 ± 0.04 e
	(3)	2.27 ± 0.07 cd	– 4.66 ± 0.20 a
	(4)	2.37 ± 0.04 bc	– 1.69 ± 0.06 d
	(5)	2.14 ± 0.15 d	– 2.32 ± 0.16 c
	(6)	2.59 ± 0.24 b	– 0.39 ± 0.04 f

Results are expressed as means ± SD (n = 3).
(a–f) – means in the column followed by different letters are significantly different ($p < 0.05$) according to the evaluating software used.

Table 2
Effects of azoles on radiation-chemical yields of inorganic phosphate formed in deaerated aqueous 10^{-1} M glycerol-1-phosphate solutions at pH 7.

Additive		Radiation-chemical yields (G) × 10^7, mol/J	
		Inorganic phosphate	Consumption of the additive
No additive		4.51 ± 0.15 a	–
	(7)	3.76 ± 0.10 c	0 c
	(8)	2.00 ± 0.09 d	– 1.59 ± 0.04 a
	(9)	4.08 ± 0.12 b	0 c
	(10)	2.18 ± 0.18 d	– 1.21 ± 0.12 b
	(11)	1.93 ± 0.09 d	–
	(12)	2.14 ± 0.05 d	– 1.13 ± 0.04 b

Results are expressed as means ± SD (n = 3).
(a–d) – means in the column followed by different letters are significantly different ($p < 0.05$) according to the evaluating software used.

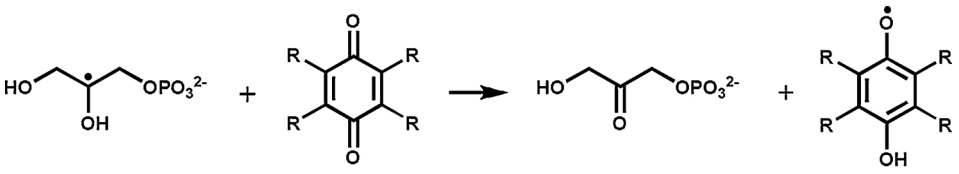
The radiation-chemical yields of IP formed in deaerated 0.1 M aqueous solutions of GP in the presence and in the absence of benzoquinones and doxorubicin are summarized in the Table 1.

The data presented in Table 1 shows that the radiation-chemical yields of IP significantly decreased (on average 2 times) in the presence of quinones, including the antitumor agent doxorubicin (**6**). This provides evidence for their ability to block the fragmentation reaction of the radicals (II), in spite of the high reaction rate constant ($> 10^6\ s^{-1}$) for the dephosphorylation (Scheme 2) (Schuchmann et al., 1995).

It also follows from the data of Table 1 that benzoquinones (**2**, **4**, **5**) and doxorubicin (**6**), while blocking fragmentation of GP, were consumed in relatively low yields. This fact points to the possibility of their regeneration from products back to the starting compounds. High consumption yields of 1,4-benzoquinone (**1**) and t-butyl-1,4-benzoquinone (**3**) as measured by the loss of quinoid conjugation system by UV–vis spectrophotometry are probably due to additional free-radical reactions that might be involved, because of the presence of unsubstituted semi-aromatic C═C bonds in the quinoid structures.

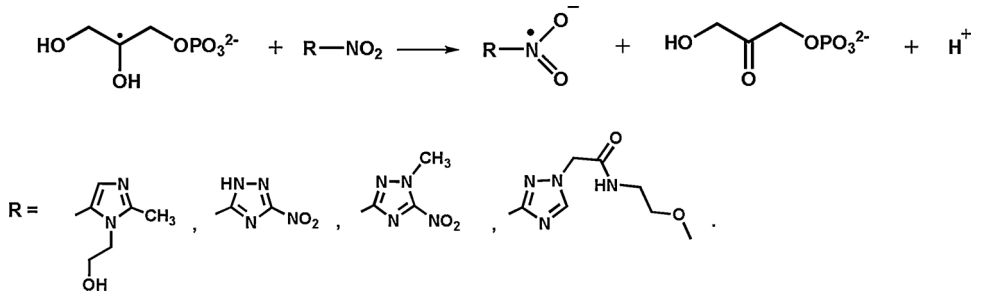
Quinones are known to inhibit processes of the homolytic fragmentation of hydroxyl-containing organic compounds due to the oxidation of α-HCR (~$10^9\ M^{-1} \times s^{-1}$) (Al-Suhybani and Hughes, 1986; Simic and Hayon, 1972). Therefore, the average two-fold decrease in the radiation-chemical yields of IP in the presence of the tested quinones (Table 1) might be caused by realization of the following Scheme 3.

The radiation-chemical yields of IP formed in oxygen-free 0.1 M aqueous solutions of GP in the presence of imidazole (**7**), metronidazole



R = -H, -CH_3, -$CH(CH_3)_2$, -$C(CH_3)_3$, -OCH_3.

Scheme 3. Interaction of the tested quinones with α-HCR of GP.



Scheme 4. Interaction of the tested nitroazoles with α-HCR of GP.

(**8**), as well as 1,2,4-triazole (**9**) and its derivatives (**10–12**) are summarized in the Table 2.

It follows from the data shown in the Table 2 that the studied nitroazoles (**8**, **10–12**) suppressed homolytic dephosphorylation of GP, decreasing the radiation-chemical yield of IP two fold. Low consumption yields of the nitroazoles suggest the possibility of their regeneration.

Meanwhile, there were only slight changes in the radiation-chemical yields of IP in the presence of imidazole (**7**) and 1,2,4-triazole (**9**) and no consumption of the additives was observed. These findings show low reactivity of these additives towards radicals (II) formed from GP.

The phenomena observed in the presence of additives (**8**, **10–12**) were associated with the presence of nitro group(s) in their respective structures. Therefore nitro group(s) is responsible of nitroazoles oxidative properties (Samovich et al., 2018) and enabled them to participate in the following oxidation reactions with α-HCR (Scheme 4).

Hence, the data presented above give reasonable grounds to conclude that the tested benzoquinones, including doxorubicin, as well as nitroazoles, effectively inhibit radiation-induced processes of the dephosphorylation of GP.

3.2. Steady-state radiolysis of aqueous dispersions of 1,2-dimyristoyl-glycero-3-phosphatidyl-glycerol (DMPG) in the presence of the tested additives

It has been found (Shadyro et al., 2004a,b) that, similar to GP, hydroxyl-containing lipids undergo homolytic decomposition during radiolysis of their aqueous dispersions, resulting in the formation of phosphatidic acid (PA). In the case of DMPG, the following processes take place (Scheme 5).

We studied the correlation of the amounts of accumulated 1,2-dimyristoyl-sn-glycero-3-phosphatidic acid (DMPA) with the absorbed dose during γ-irradiation of DMPG in oxygen-free 10^{-2} M aqueous dispersions in the presence and in the absence of the tested benzoquinones, doxorubicin and nitroazoles (Table 3).

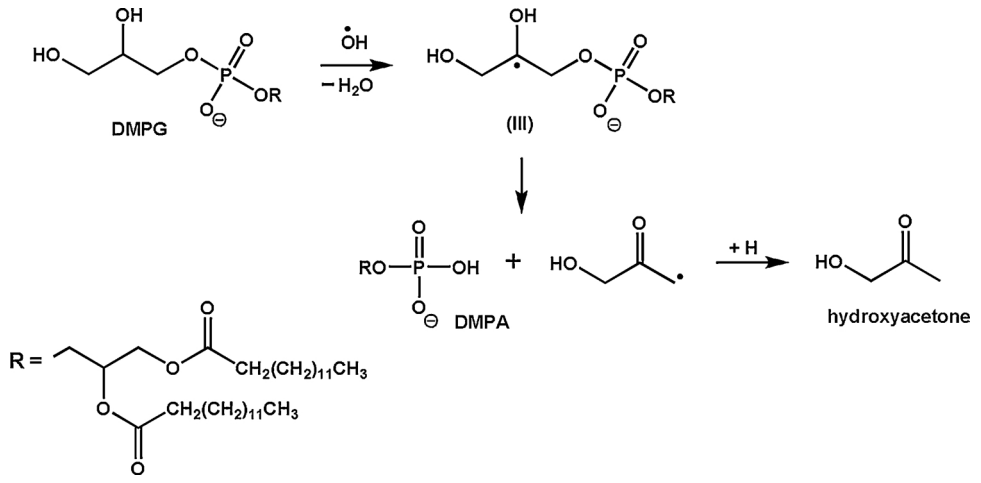
The radiation-chemical yield of DMPA in the additive-free DMPG system was $(0.54 \pm 0.05)\times10^{-7}$ mol/J, which is substantially lower than the yield of corresponding product – IP ($(4.51 \pm 0.15)\times10^{-7}$ mol/J) formed on radiolysis of aqueous solutions of GP (Table 1). This phenomenon requires an additional investigation. It is apparently associated with the influence of structural organization of liposomal systems on both the probability of interaction of ˙OH and ˙H with the polar component of the lipids and the fragmentation reaction rate of the radicals formed from the starting compounds.

It is apparent from the data shown in Table 3 that the amounts of accumulated product resulting from free-radical fragmentation of DMPG were substantially lower in the presence of the tested compounds. The radiation-chemical yields of DMPA observed in cases where quinones (**2**, **4**, **5**) were used as additives decreased by a factor of 2.7 vs. the control values, while in cases of nitroazoles (**8**, **12**) the factor was 1.9. The largest effect was produced by the anticancer drug doxorubicin (**6**), which lowered the DMPA yield by a factor of 3.4.

Since the additive/lipid ratio was 1/20, the tested compounds reacted predominantly with the corresponding α-HCR (III) formed in the polar part of the lipid, and this process competed with fragmentation reactions of these radicals. The decrease in yields of DMPA was due to the ability of quinones and nitroazoles to oxidize the primary radicals of DMPG according to Schemes 3 and 4.

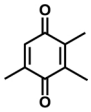
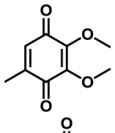
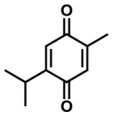
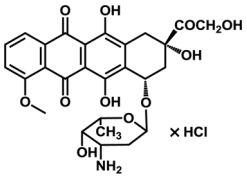
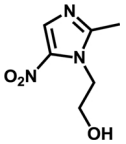
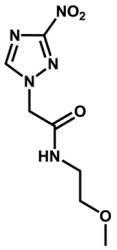
Thus, investigation of the effects of the tested compounds on radiolysis product yields of GP and DMPG has led us to the conclusion that quinones and doxorubicin, as well as nitroazoles, are effective inhibitors of free-radical fragmentation of the starting substrates by suppressing the formation of IP and DMPA, respectively.

The data presented in this paper, as well as obtained earlier (Edimecheva et al., 1997; Shadyro et al., 2004a), provide convincing evidence of the possibility of the formation of PA due to the ˙OH-



Scheme 5. Homolytic decomposition during radiolysis of DMPG aqueous dispersions resulting in the formation of DMPA.

Table 3
Effects of quinones and nitroazoles on radiation-chemical yields of phosphatidic acid formed in deaerated 10^{-2}M aqueous dispersions of 1,2-dimyristoyl-glycero-3-phosphatidyl-glycerol.

Additive		Radiation-chemical yields of phosphatidic acid (G) × 10^7, mol/J
No additive		0,54 ± 0,05 a
	(2)	0,21 ± 0,02 c
	(4)	0,20 ± 0,01 c
	(5)	0,20 ± 0,01 c
	(6)	0,16 ± 0,02 d
	(8)	0,28 ± 0,04 b
	(12)	0,29 ± 0,03 b

Results are expressed as means ± SD (n = 3).
(a–d) – means in the column followed by different letters are significantly different ($p < 0.05$) according to the evaluating software used.

induced decomposition of hydroxyl-containing glycerophospholipids, including cardiolipin (Shadyro et al., 2004a,b; Yurkova et al., 2008). The latter is one of the components of mitochondrial membranes, in which the processes of four-electron reduction of oxygen to water take place, generating ROS. The mitochondrially-derived ROS can interact with lipids, which would result in accumulation of PA, causing changes in the cardiolipin/PA ratio. This also will affect the processes of fusion and division of mitochondria (Kameoka et al., 2018), which are important for maintaining physiological processes at a normal level.

Prior to our work, only biochemical processes leading to the formation of PA were taken into consideration. These are: the reaction of phospholipid cleavage, catalyzed by phospholipase D; the reaction of diacylglycerol phosphorylation, catalyzed by DG kinase; as well as the reaction of acylation of lyso-PA, catalyzed by LPA acyltransferase (Bruntz et al., 2014; Cho and Han, 2017; Peng and Frohman, 2012).

Hyperproduction of ROS under the influence of internal and external factors could cause an increase in PA levels in an organism, and, as a consequence, overloading its utilization pathways, for example, a biochemical process catalyzed by PA phosphatase. The latter one is a fat-regulating enzyme and could hold the key to obesity, diabetes and other diseases (Carman, 2011). Hence, it is important to know how one can effectively lower the PA levels by inhibiting the processes of its formation. Based on the data obtained in this study, it appears reasonable to perform the search for inhibitors of non-enzymatic reactions leading to the formation of PA among organic oxidizers, such as nitroazoles and compounds having quinoid structures.

The obtained data are also of interest for oncotherapy, in particular, when using cytostatic agents and radiation, which produce ROS in cancer cells thereby destroying them. In such cases, it is necessary to regulate the ROS-generating processes leading to the formation of PA. This is important due to the finding (Foster, 2009) that PA favors the cancer cell survival and enhances their division. These facts suggest that while using radiotherapy for the treatment of cancer patients it is advisable to use radiosensitizers, which would suppress the radiation-induced formation of PA. We have found that there are inhibitors of PA formation among nitroazoles (metronidazole, sanazole) that possess radiosensitizing properties, as confirmed by radiotherapeutical practice (Wardman, 2007). Among cytostatic agents, tetracycline antibiotics, such as doxorubicin, suppress the formation of PA, and this may be an additional factor determining their antitumor properties along with causing damage to the nucleic acids of cancer cells (Halliwell and Gutteridge, 2007).

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