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THE ANTIMICROBIAL ACTIVITY OF EXOGENOUS ANIONIC PHOSPHOLIPIDS AGAINST *Mycobacterium tuberculosis* AND *Escherichia coli*

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The effect of anionic phospholipids, namely, cardiolipin, phosphatidylglycerol and phosphatidic acid, on the growth of gram-negative bacteria E. coli BL21(DE3), as well as gram-positive bacteria M. tuberculosis H37Rv was investigated in this study. The influence of all anionic phospholipids tested on the bacteria growth was shown to be dose-dependent. Lipids at concentrations below 335 μM didn't affect, while at 335 μM and above they repressed bacteria growth and caused cellular death of both type of microorganisms. SOS response induction was observed by using strain E. coli CSH50 sfiA::lacZ during cultivation E. coli with cardiolipin, phosphatidylglycerol and phosphatidic acid. This indicates DNA damage through double-strand breaks. One reason of the DNA damage could be stabilization of transient complexes of DNA topoisomerase (types I and II) with DNA temporary broken by anionic phospholipids. However, neither phosphatidylglycerol nor phosphatidic acid affect the activity of types I and II DNA topoisomerases from E. coli in vitro. In contrast, cardiolipin inhibited DNA topoisomerase I and DNA gyrase (type II topoisomerase), but didn't stabilize transient complexes of the enzyme with DNA. It indicates that DNA damage due to anionic phospholipids exposure didn't result from inhibition of DNA topoisomerase activity through stabilization of the transient complex of the enzyme with DNA. The obtained results of cardiolipin, phosphatidylglycerol and phosphatidic acid bactericidal activity against gram-positive M. tuberculosis and gram-negative E. coli make it possible to use anionic phospholipids as individual antimicrobial agents or as a matrix of effective and non-toxic liposomal drugs for tuberculosis treatment.

Keywords: *anionic phospholipids, bactericidal activity, Mycobacterium tuberculosis, Escherichia coli, DNA topoisomerases.*

Introduction

Resistant strains of *Mycobacterium tuberculosis* (MBT, the causative agent of tuberculosis) with multiple and broad drug resistance (MDR and BDR, respectively) are widespread around the world. Therefore, searching new antituberculous preparations (ATP) is still of current interest. One of perspective directions is the creation of liposomal forms of antibiotics. We showed previously that "empty" liposomes (without an antibiotic) based on natural anion lipid cardiolipin (CL) isolated from bull heart have antibacterial activity with respect to sensitive strain of *M. tuberculosis* H37Rv [1] and resistant strain of *M. tuberculosis* MS-115 with MDR [2].

Anionic phospholipids (PLs): CL, phosphatidylglycerol (PG) and phosphatidic acid (PA) are structural components of the cellular membrane of the majority of pro- and eukaryotic cells. In particular, CL was found in the cytoplasmic membrane of gram-negative bacteria of *E. coli* [3] and gram-positive *M. tuberculosis* [4], as well as in the internal membrane of eukaryotic mitochondria [5]; PG – in the cytoplasmic membrane of *E. coli*. Besides, PG is the precursor in the biosynthesis of CL. Anionic PLs are unevenly located in the plasmatic membrane of bacteria in the course of cell division (mostly in the central part of the cell and at its poles) forming so-called "domains". It is especially important when amphiphilic proteins of the cellular cycle choose and recognize the center of cell division. Examples of such proteins are: DnaA (initiation of DNA replication in *oriC*) [6], MinD (a part of the MinCDE system preventing positioning of the divisome at the poles of *E. coli*) [7] and FtsA [a bacterial actin which is the linker protein for FtsZ protein (bacterial tubulin)] required for targeting Z-rings to the central membrane domain). CL and PG interact with components of the respiratory chain: succinate dehydrogenase, formate dehydrogenase N (FdnGHI) and nitrate reductase complex (NarGHI) [8]. In the latter case such interaction leads to activation of NarGHI due to the correct fixation of the hydrochinone substrate [8]. Besides, CL and FG interact with the proteins of the translocation complex SecYEG [9] and with the carrier proteins accounting for multiple drug resistance [10]. In the course of cellular respiration of CL acts as a proton trap due to its anion properties and binding with oxidizing phosphorylation proteins. Besides, it delivers protons to ATP synthase [11]. There are also data in the literature that the existence of anionic PLs, in particular PA, is necessary for normal functioning of the K^+-channel of KcsA in *Streptomyces lividans*, because this channel is closed in the absence of these PLs [12]. The participation of CL in the formation of spores by bacteria *Bacillus subtilis* [13] is not less interesting. It is also known that CL inhibits the *in vitro* activity of DNA topoisomerase I determining the topological state of DNA [14] and of Lon-protease [15] of *E. coli*.

PA is not as widespread component of membranes as PG and CL. However, despite of its small amounts in cells as compared to the total amount of PL it is a key intermediate in the biosynthesis of triacylglycerols. Besides, it is an important signaling molecule [16].

Thus, anionic PLs are normally present in all bacterial cells and perform important functions, for example, the signaling function (PA) or the construction and regulatory functions (PG and CL).

The aim of our work was studying the of effect of exogenous anionic phospholipids CL, PG and PA (in the form of liposomes) on the growth of *E. coli*, and also the effect of PG and PA on the growth of sensitive strain of *M. tuberculosis* H37Rv in order to determine the specificity of their action and to establish the possible mechanism of their action.

Experimental

Materials. The following materials were used in the work: disodium salt of cardiolipin from bull heart [the salt contains residues of linoleic (~ 87%) and oleic (~ 8%) acids (Avanti Polar Lipids, USA)]; PG and PA obtained by us with the use of fermentative semi-synthesis [17] by the action of phospholipase D from cabbage upon phosphatidylcholine (PC) from Lipoid S-100 soy (Lipoid GmbH, Germany); pUC19 plasmid (Invitrogene, USA); topoisomerase I of *E. coli* (New England Biolabs, USA); DNA gyrase provided by doctor Anthony Maxwell (John Innes Centre, Great Britain).

The following solvents were used in the work: chloroform, methanol, pure acetone (Chimmed, Russia).

The following microorganisms were used as objects of study: the standardized culture of laboratory strain *M. tuberculosis* H37Rv, strains of Escherichia coli BL21 (DE3) and CSH50 ***λ****sfiA::lacZ*.

Methods. Liposomes (large unilamellar vesicles, LUVs) from PLs (PA, PG and CL) were obtained by extrusion of multilamellar vesicles according to the method described in [1, 2].

Effect of PL anion on the growth of M. tuberculosis. *M. tuberculosis* H37Rv bacteria were cultivated during 2 weeks at 37°C and standardized with respect to medicinal sensitivity and colony-forming units (CFU). Then 150 μl of liposomes from PL (50, 100, 200, 335, 500, 669 μM) and 100 μl of *M. tuberculosis* H37Rv (5×10^5 CFU) were placed into a 96-hole plate in Dubos medium. *M. tuberculosis* H37Rv without preparations and Dubos medium were placed into the holes of the plate as control samples. The plate was placed into a CO_2 incubator, and the mycobacteria were cultivated at 37°C during 20 days. After each 1–2 days the optical density of the culture was measured at 600 nm (OD_{600}) with the use of a "Sigma" (USA) plate reader.

Effect of anionic PLs on the growth of E. coli. An overnight culture of *E. coli* BL21(DE3) cells was subcultured into fresh liquid medium LB (1:50) and cultivated at 37 °C until the logarithmic growth phase (OD_{600} = 0.1) was attained. Then 50 μl of *E. coli* and liposomes from PL (final concentration of a lipid of 100, 200, 335 and 500 μM) were placed into 96-hole plate. LB medium was added to attain a final volume of 100 μl. *E. coli* without preparations was used as a control sample. The plate was kept in an incubator at 37 °C during 5 h under 200 rpm stirring. The culture growth was tested hourly by measuring OD_{600}. After 5 h inoculation of a series of 10-fold dilutions on 1.5% LB agar was carried out. The Petri dishes were thermostated at 37°C during 24 h, and then CFU was calculated.

Studying the SOS DNA repair response of E. coli upon incubation with PL. A lawn of *E. coli* CSH50 λ *sfiA::lacZ* cells on the surface of agarized MacConkey medium (Difco, USA)

was obtained according to the technique described in [18]. The following preparations (5 μl) were applied on it: 1 and 1.5 mM of PL in the form of LUV, 12 μM of ciprofloxacin and 30 μM of microcin B (McB) as positive control; 20 μM of microcin C (McC) as negative control. The Petri dishes were kept in an incubator at 37°C during 24 h.

Obtaining and isolating supercoiled DNA for reactions with topoisomerase I and DNA gyrase. Supercoiled plasmid pUC19 was obtained and isolated by alkaline lysis according to the protocol by means of QIAGEN Plasmid Maxi Kit.

Obtaining relaxed DNA for reaction with DNA gyrase in the presence of ATP. Supercoiled pUC19 was treated with *E.coli* topoisomerase I overnight in a buffer solution, the composition of which is described below (see *DNA relaxation reaction catalyzed by topoisomerase I*). In order to stop the reaction the enzyme was inactivated by heating the reaction mixture at 65°C for 20 min.

The reactions with topoisomerases were carried out in a solid-state "Gnom" thermostat (DNA-Technology, Russia) designed for Eppendorf micro test tubes.

DNA relaxation reaction catalyzed by topoisomerase I. The reaction mixture with a total volume of 15 μl contained 2 μg of supercoiled pUC19 DNA, 5 μl of PL liposomes with concentrations 90, 300 and 600 μM and 2.5 units of active *E.coli* topoisomerase I in a buffer solution of the following composition: 50 mM of sodium acetate, 20 mM of tris-acetate (pH 7.9), 10 mM of magnesium acetate and 1 mM of dithiothreitol (DTT). The reaction was carried out at 37°C for 90 min and stopped by the addition of 15 μl of STEB buffer (40% sucrose, 100 mM of Tris-HCl, pH 7.5, 1 mM of EDTA, 2 mg/ml of bromophenol blue) and 15 μl of chloroform. Reaction products were analyzed by electrophoresis in 0.9% (w/v) agarose gel in a TAE buffer (40 mM of tris-acetate, 1 mM of EDTA) free of ethidium bromide during 2 h (70 V). When electrophoresis was complete, the gel was painted by ethidium bromide (10 μg/ml). The reaction products were analyzed by means of a transilluminator with the use of the gel documentation software (Helicon, Russia).

DNA supercoiling reaction catalyzed by DNA gyrase in the presence of ATP. The reaction mixture with a volume of 15 μl consisted of 2 μg of relaxed DNA pUC19; 5 μl of PL liposomes with concentration 90, 300, and 600 μM, 1 and 1.5 mM, and 1.5 units of active DNA gyrase of *E.coli* in a buffer of the following composition: 35 mM of Tris HCl (pH 7.5), 24 mM of KCl, 4 mM of $MgCl_2$, 2 mM of DTT, 1.8 mM of spermidine, 1 mM of ATP, 6.5% (w/v) of glycerin and 0.1 mg/ml of albumin. The reaction was carried out at 37°C for 30 min. Analysis of reaction products was performed by the technique described for topoisomerase I. Ciprofloxacin was used as positive control.

Results and Discussion

Effect of anionic PLs on the growth of Mycobacterium tuberculosis H37Rv. As shown by us previously, natural phospholipid CL exhibits antibacterial activity towards tuberculosis mycobacteria [1, 2]. In this work we studied the effect of other anionic PLs – PG and PA – on the mycobacteria. These PLs are structural components of CL. We obtained these lipids by fermentative transphosphatidylation of PC of soya beans with the use of phospholipase D of cabbage. The choice of the soy PC as a source for obtaining PA and PG is caused by the fact that its fatty acid composition is similar to the composition of CL from bull heart: it contains mostly $C_{18:2}$ linoleic acid residues (70%) (Figure 1).

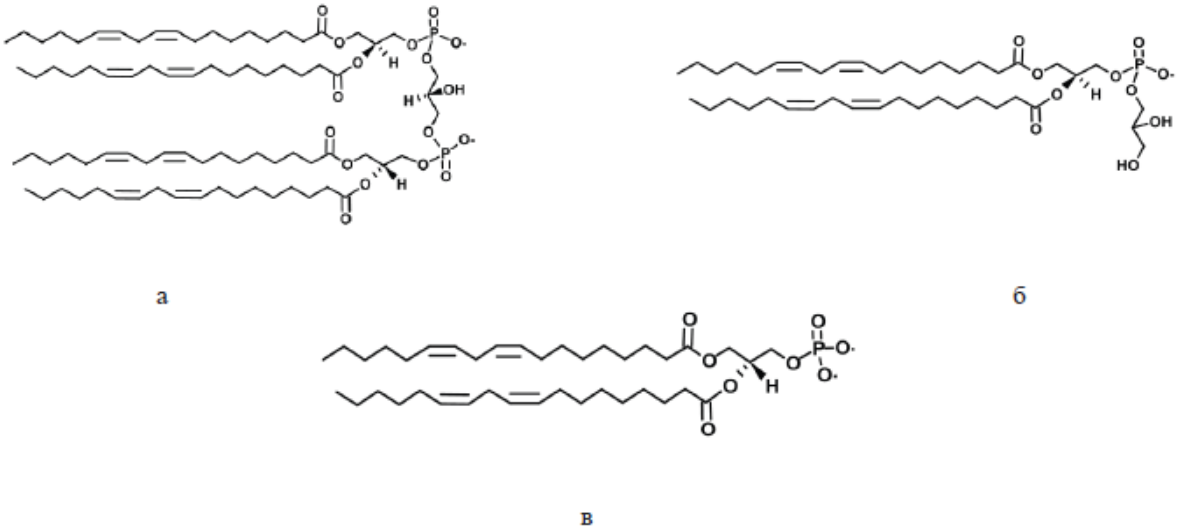

Figure 1. Structural formulas of phospholipids: cardiolipin (a), phosphatidylglycerol (b) and phosphatidic acid (c).
[а, б, в means a, b, c]

The effect of the above lipids on the growth of *M. tuberculosis* H37Rv strain sensitive to ATP was studied. The growth of the bacteria was judged by the optical density of the culture measured at 600 nm (OD_{600}). Figure 2 shows curves of *M. tuberculosis* H37Rv growth (initially 10^5 CFU) during 20 days in the presence of the PLs in concentrations 50, 335, 500 and 669 μM. The specified concentrations of the PLs were chosen taking into account that the minimal inhibiting concentration (MIC) of CL with respect to MBT determined by us *in vitro* is 335 μM [1].

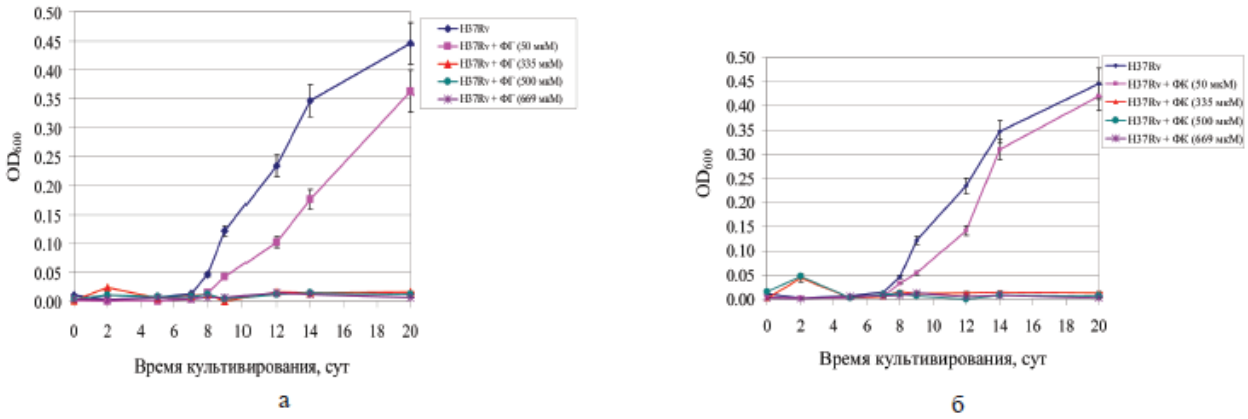

Figure 2. Curves of *M. tuberculosis* H37Rv growth (initially 10^5 CFU) in the presence of 50, 335, 500 and 669 μM of PF (a) and PA (b) at 37°C during 20 days. The results are presented as the average value of three independent experiments ± standard deviation (SD).
[а, б means a, b; Время культивирования, сут means cultivation time, days;
ФГ means PG; ФК means PA; мкМ means μM]

The culture of MBT cells without preparations (control) actively grew during 20 days. Cultivating MBT with 50 μM of PG resulted in insignificant inhibition of the mycobacteria growth, which was reflected in the average decrease of the culture OD_{600} by 0.1 units as compared to the control. PG in concentrations 335, 500 and 669 μM inhibited the cells growth completely, and OD_{600} of the culture during 20 days did not differ from the initial value. Incubating the tuberculosis mycobacteria with PA in concentration 50 μM did not affect the cells growth. This is indicated by the fact that the curve of the cells growth is similar to the curve of the control MBT culture growth, and PA in concentrations of 355, 500 and 669 μM completely inhibited the cells growth.

Thus, we established that anionic phospholipids PG and PA, along with CL, show inhibiting activity with respect to MBT in the same concentrations as CL. The data on the antimycobacterial effect of PA obtained by us are in agreement with the literature data. According to the latter exogenous the PA inhibits MBT reproduction in infected macrophages [19]. It is important that zwitterionic PC and glycosphingolipids, as found by us previously [20], did not affect the growth of MBT. This allows assuming that the negative charge of the lipid plays an important role for the bactericidal effect of PL on the mycobacteria.

Effect of anionic PLs on the growth of Escherichia coli BL21(DE3). We studied the effect of the same anionic lipids PG, PA and CL on the growth of fast-growing gram-negative bacteria of *E. coli*, which was estimated by the optical density of the culture at 600 nm. *E. coli* bacteria (initially 3×10^8 CFU) were cultivated with lipids in concentrations 100, 200, 335 and 500 μM at 37°C during 5 h (Figure 3).

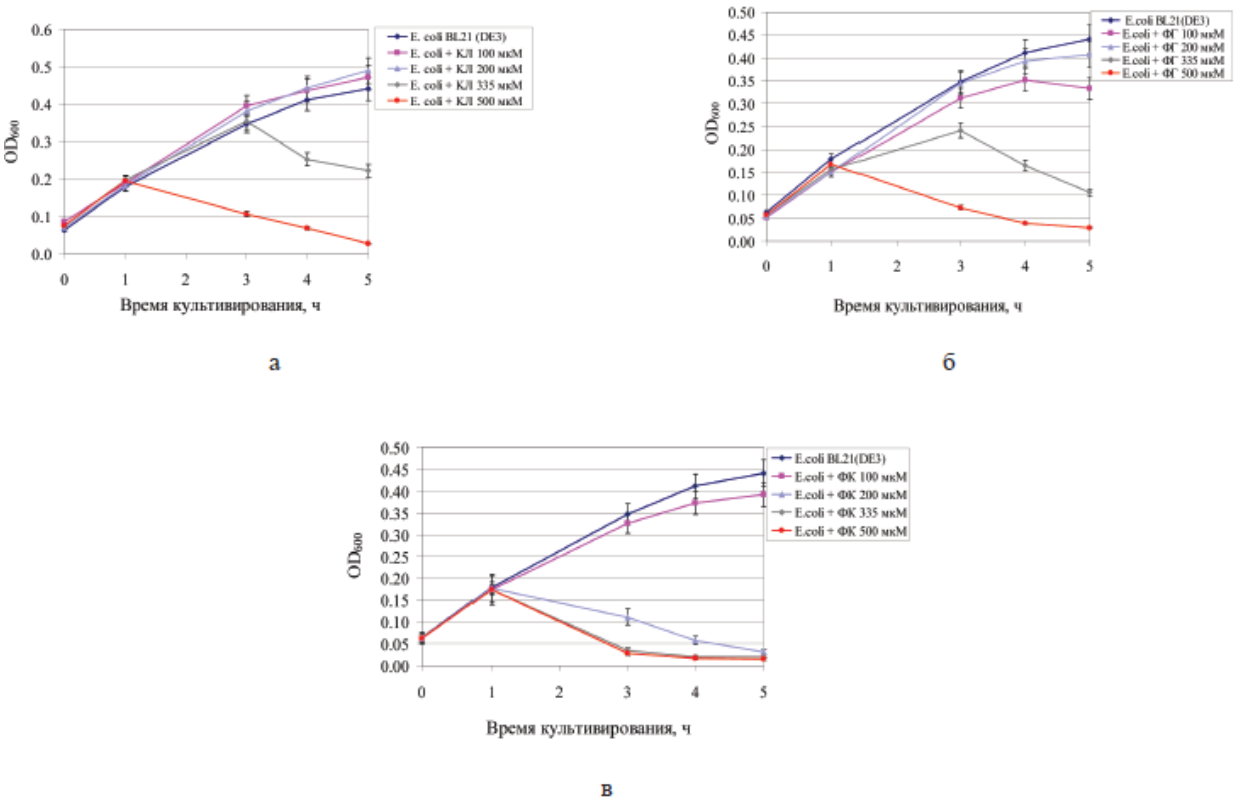

Figure 3. Curves of *Escherichia coli* BL21(DE3) growth (initially 3×10^8 CFU) in the presence of 100, 200, 335 and 500 μM of CL (a), PG (b) and PA (c) at 37°C during 5 h. The results are presented as the average value of three independent experiments ± standard deviation (SD).

[а, б, в means a, b, c; Время культивирования, ч means cultivation time, h;
КЛ means CL; ФГ means PG; мкМ means μM]

The control culture of *E. coli* (without preparations) grew rather actively, and cultivating the cells with CL proved the dose-dependent effect of the lipid: CL in concentrations 100 and 200 μM did not affect the growth of the cells, but CL in concentrations 335 and 500 μM inhibited the growth (Figure 3A). It is interesting to note the shape of the curves of *E. coli* cells growth in the presence of the inhibiting concentration of CL. When incubating with 335 μM of CL, the growths of the cells during the first 3 h occurred at the same rate as in case of the control culture. However, by the 4th hour the cells died. CL in concentration 500 μM did not affect the growth of the cells within the first hour. However, by the 3rd hour cell death occurred, and by the 5th hour of their cultivation lysis occurred, because OD_{600} of the culture decreased to 0.027±0.019, whereas OD_{600} at the beginning of the experiment (0 h) was equal to 0.075±0.004. Probably the cells died by the end of the experiment and underwent lysis. This statement is confirmed by the decrease of CFU after 5 h of cultivating the cells with the lipids by a factor of 10^3–10^5 as compared to the initial quantity of the cells (Figure 4).

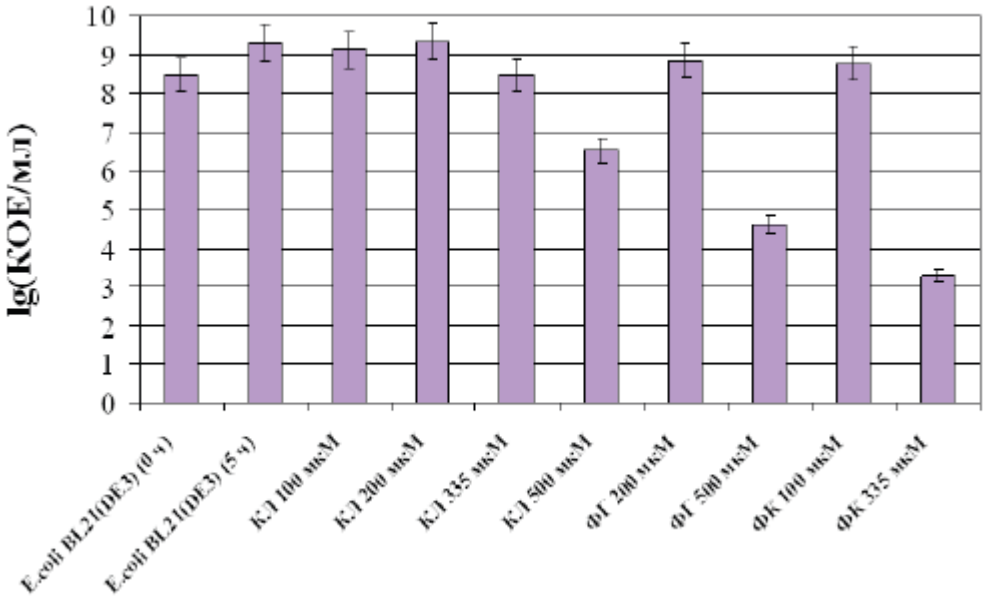

Figure 4. Change of the quantity of CFU/ml of *E. coli* calculated after passage of the cells to 1.5% agar LB after cultivating the bacteria for 5 h with PL at 37°C and stirring at 200 rpm. The results are presented as the average value of three independent experiments ± standard deviation (SD). [КОЕ/мл means CFU/ml; КЛ means CL; ФГ means PG; ФК means PA; мкМ means μM]

The fact that the cells normally grew within the first several hours with lethal doses of the lipid can be possibly explained, firstly, by the high initial concentration of the cells (10^8 CFU), whereas in case of 10^5 CFU the cells growth is not observed in the presence of toxic concentrations of the lipid throughout all the experiment (data are not provided). Secondly, it is possible that the cells try to adapt to the stressful conditions (to exogenous CL) at the initial stage of the experiment.

We found that the effect of the other negatively charged lipids, PG and PA, on the growth of *E. coli* is also dose-dependent (Figure 3B, C). Thus, PG in concentrations 100 and 200 μM had no essential impact on the cells growth, and in concentrations 335 and 500 μM it inhibited the cells growth (Figure 3B). The shape of the curves of *E. coli* growth in the presence of PG reminded the shape that was observed when cultivating the cells with CL: by the 5th hour cells death occurred, which was manifested in the decrease in OD_{600} and CFU (Figure 4). In addition, 500 μM of PG caused lysis even of cells that had been taken initially for the experiment: OD_{600} was equal to 057±0.005 at 0 hour, and by the 5th hour it decreased to 0.029±0.004.

In contrast to PG, PA suppressed the reproduction of *E. coli* even at concentration 200 μM, that is, it turned out to be a more effective antibacterial agent (Figure 3B). The cells growth within the first hour in the presence of PA did not differ from the growth of the control culture of the cells, and complete lysis of the cells occurred by the 5th hour.

So, we showed that anionic PLs studied by us – CL, PG and PA – show antibacterial activity towards both gram-positive *M. tuberculosis* and gram-negative *E. coli*.

The following stage of our work was to establish the mechanism of the bactericidal effect of the anionic phospholipids on *E. coli*. For this purpose we studied the effect of PL on the bacterium DNA: the SOS DNA repair response of the cells – a process starting in bacteria in response to DNA damages that occurred in the cell cycle [21].

Induction of the SOS DNA repair response in E. coli cells under the influence of PLs. The effect of anionic PLs on the integrity of DNA was determined with the use of a test system based on registration of the level of the expression of the reporter gene of β-galactosidase (*LacZ*). DNA damage in the cells results in the induction of the SOS DNA repair response followed by transcription of the genes normally repressed by LexA transcription regulator. We used the strain of *E. coli* CSH50 *sfiA::lacZ* containing a reporter gene *lacZ* under the control of the LexA-dependent *sfiA* promotor, the genome of which has a deletion of the chromosomal *lac* operon [18]. Ciprofloxacin (CFX) and microcin B (McB) were used as positive controls. They inhibit the growth of cells due to blocking DNA gyrase and induce SOS response. Microcin C (McC) was used as a negative control. It inhibits the growth of cells due to inactivation of aminoacyl tRNA synthetases, but does not induce SOS response. Induction of the SOS response was determined by the emergence of bright red rims around the zones of cell growth inhibition [18].

CL, PA and PG suppressed the growth of the cells in different ways (Figure 5) and induced SOS DNA repair response, which was indicated by the existence of cell growth inhibition zones of different diameters and a bright red aura around these zones. As judged by the larger diameter of the cell growth inhibition zone, PG was the most effective antibacterial agent. In addition, the control preparation CFX was the most effective antibiotic inducing the SOS response.

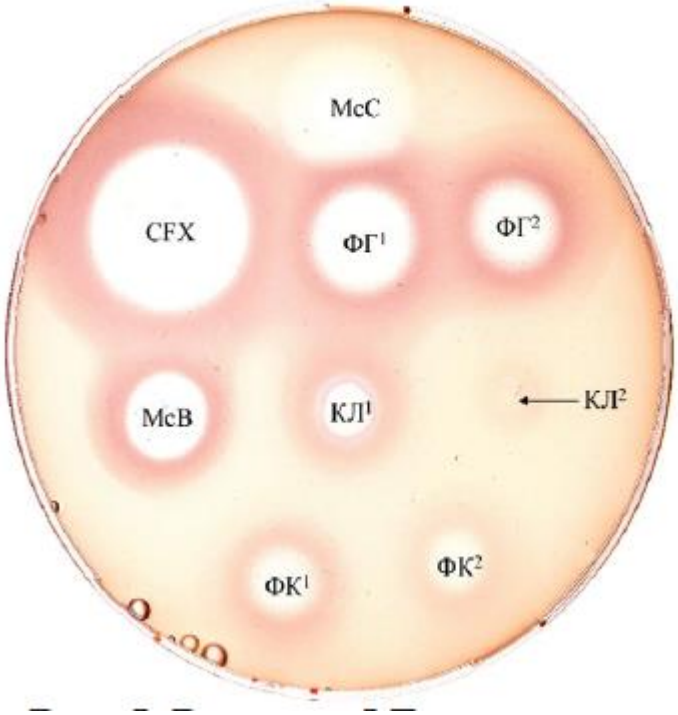

Figure 5. Effect of PL on the induction of SOS DNA repair response in *E. coli* cells.
Photograph of a Petri dish with a lawn of bacteria *E. coli* CSH50 sfiA::lacZ reporter strain placed on it. PG^1 – 1.5 mM, PG^2 – 1 mM, CL^1 – 1.5 mM, CL^2 – 1 mM, PA^1 – 1.5 mM, PA^2 – 1 mM. Positive controls: CFX – ciprofloxacin (12 μM), McB – microcin B (30 μM); negative control: McC – microcin C (20 μM). [КЛ means CL; ФГ means PG; ФК means PA]

Effect of anionic PLs on the activity of topoisomerase I and DNA gyrase from E. coli in vitro. The induction of SOS DNA repair response in the cells is caused by accumulation of double-stranded breaks of DNA, which can occur, for example, due to inhibition of DNA topoisomerases – enzymes of the replicative system determining supercoiling of DNA and bringing temporary gaps into it [22]. Another cause can be interaction of DNA with active forms of oxygen, in particular, very toxic hydroxyl radical [23]. Thus, fluoroquinolones inhibit DNA gyrase (II type topoisomerase is ATP-dependent and catalyzes splitting of two chains of DNA) stabilizing transition complexes with DNA split by this enzyme. Due to this DNA gyrase is blocked and cannot cross-link the temporarily split DNA, which eventually leads to accumulation of double-stranded breaks. The effect of camptothecin on topoisomerase I is analogous: it does not depend on ATP and causes a break in one DNA chain. In this case accumulation of single-stranded breaks is a prerequisite for the emergence of double-stranded breaks of DNA, which induce the SOS DNA repair response. Besides, it is known from literature that the CL inhibits DNA topoisomerase I of *E. coli in vitro* [16]. We showed previously that the inhibiting effect of CL extends also to the activity of DNA gyrase [24]. Thus, the bactericidal

action of PL anion towards *E. coli* can result from inhibition of DNA topoisomerases by lipids due to stabilization of transition complexes of the enzyme with DNA temporarily split by it as in case of fluoroquinolones. In order to confirm or refute this assumption we studied the influence of PA and PG on the reaction of DNA relaxation catalyzed by DNK topoisomerase I (Figure 6), as well as the supercoiling of DNA catalyzed by DNA gyrase in the presence of ATP (Figure 7). The conclusion about inhibition of the enzyme activity by lipids was made after carrying out electrophoresis of the reaction products in 0.9% agarous gel on the basis of the existence of a strip corresponding to the DNA initial form, or strips corresponding to the topoisomers of DNA close to the DNA initial form on the electrophoregram. CFX antibiotic was used as a positive control in the reactions with DNA gyrase. PG and PA did not affect the activity of both enzymes even in the maximum concentrations of 335 and 500 μM (Figures 6 and 7).

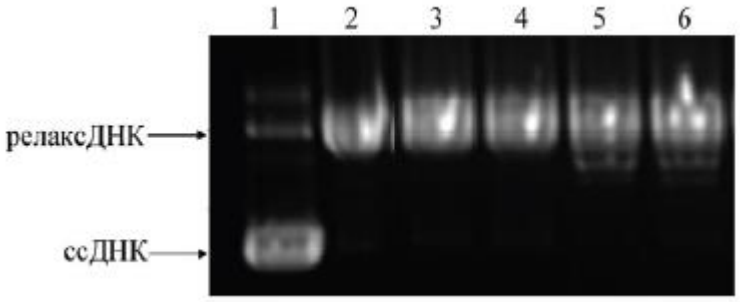

Figure 6. Effect of anionic PLs on the activity of DNA topoisomerase I (topo I) from *E. coli in vitro.* Path No. 1 is the supercoiled plasmid DNA pUC19 (scDNA), No. 2 is scDNA + topo I, No. 3 is scDNA + topo I in the presence of PA in concentration 335 μM, No. 4 – PA 500 μM, No. 5 – PG 335 μM, No. 6 – PG 500 Mm. (*Hereinafter*: relaxDNA (релаксДНК) is the relaxed form of DNA, scDNA (ссДНК) – the supercoiled form of DNA).

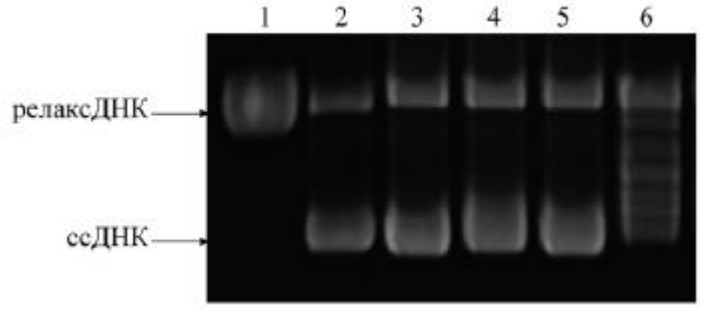

Figure 7. Effect of anionic PLs on the activity of DNA gyrase from *E. coli* in the presence of ATP *in vitro*. Path No. 1 is the relaxed plasmid DNA of pUC19 (relaxDNA), No. 2 – relaxDNA + gyrase, No. 3 – relaxDNA + gyrase in the presence of PA in concentration 500 μM, No. 4 – PG 335 μM, No. 5 – PG 500 μM, No. 6 – CFX 4 μM (positive control).

At the same time, as we showed previously, CL inhibits the activities of both DNA topoisomerases [24]. In order to check whether this fact is the cause of DNA damage we studied the ability of CL to stabilize the transitional complex enzyme – split DNA, which is followed by accumulation of DNA with single- and double-stranded breaks.

Study of accumulation of open ring and linear DNA under the influence of CL. We studied accumulation of open ring DNA (with a single-stranded break) in the reaction with topoisomerase I and linear DNA (with a double-stranded break) in the reaction with DNA gyrase in the presence of anionic PLs. CFX was used as positive control in the reactions with gyrase. It causes accumulation of linear DNA due to stabilization of the transition complex with DNA gyrase.

The reaction products were analyzed after electrophoresis in a 0.9% the agarous gel containing 0.5 μg/ml of ethidium bromide. The latter intercalates into DNA. As a result the relaxed form of DNA containing ethidium bromide gets positive supercoiling, and its position on the electrophoregram intermixes with the position of the negatively supercoiled DNA. After linking with ethidium bromide the open ring and linear DNA forms cannot get positive supercoiling. Therefore, their position in the gel is clearly distinguishable. The strip corresponding to the linear DNA is below that corresponding to the open ring DNA, but both strips are much above the strips corresponding to the supercoiled and relaxed DNA forms (Figure 8).

Figure 8. Study of accumulation of DNA with single- and double-stranded breaks upon the action of anionic PLs on DNA topoisomerase I (A) and DNA gyrase (B). **A.** Path No. 1 is scDNA (pUC19), No. 2 – scDNA + topo I, paths No. 3, 4, 5 – scDNA + topo I in the presence of 500 μM and 1 μM of CL, 1 μM of PC (negative control), respectively. **B.** Path No. 1 is relaxDNA (pUC19), No. 2 – relaxDNA + gyrase, paths No. 3 and 4 – relaxDNA + gyrase in the presence of 500 μM of CL and 10 μM of CFX (positive control), respectively. Electrophoresis was carried out during 2 h in a 0.9% agarous gel containing 0.5 μg/ml of ethidium bromide. [orDNA (окДНК) is open ring DNA (with a single-stranded break), linDNA (линДНК) is linear DNA (with a double-stranded break)]. [А, Б means A, B]

In all the reactions catalyzed both by topoisomerase I and by DNA gyrase the action of CL resulted in the formation of open ring and linear DNA, respectively, in quantities comparable

to the basic level observed in the reaction without preparations (Figure 8). As for CFX, it completely transformed the initial DNA in reactions with the participation of DNA gyrase into the linear form, that is, it stabilized the gyrase complex with the split DNA.

Thus, the presence of CL in the reaction mixture both with topoisomerase I and with gyrase did not lead to obvious accumulation of open ring and linear DNA, respectively. This proved that CL did not stabilize the complex enzyme – split DNA, and the mechanism of the bactericidal action of CL differs from the mechanism of the action of fluoroquinolones (on DNP gyrase) or of camptothecin (on topoisomerase I).

Thus, we showed that PG and PA do not affect the activity of DNA topoisomerases, whereas CL does not cause accumulation of single-stranded and double-stranded breaks of DNA, although it inhibits both enzymes [24]. Apparently, such difference in the inhibiting activity of PLs can be explained by their different structures. Thus, CL, unlike PA and PG, contains two phosphate groups and four residues of linoleic acid. Taking into account the literature data [16] and our experimental results it is most probable that CL interacts with the enzyme itself. Presumably, cardiolipin, due to its structure having distributed negative charge [25], competes with DNA for places of bonding with DNA topoisomerases, the active center of which is rich with positively charged amino-acid residues providing fixation of the substrate (DNA or CL) due to electrostatic interaction [26].

Conclusion

In this work the effect of anionic phospholipids PG and PA on the growth of gram-positive pathogenic bacteria *M. tuberculosis* (the causative agent of tuberculosis) and the effect of CL, PA and PG on the growth of gram-negative bacteria *E. coli* were studied. It was established that the above negatively charged PLs exercise antibacterial effect towards both microorganisms. In case of *E. coli* such activity of CL, PG and PA is caused by the damage of cell DNA (emergence of breaks). The exact reason of this has not been established yet. One of causes of the DNA damage could be inhibition of enzymes of the replicative system of DNA topoisomerases by the lipids due to stabilization of transition complexes of the enzyme with the split DNA as observed in case of fluoroquinolones. However, studies performed by us showed that PA and PG did not affect the activity of topoisomerases, and CL inhibiting both enzymes did not stabilize the transition complexes. Therefore, the mechanism of the bactericidal effect of the anionic PLs differs from the mechanism of the effect of fluoroquinolones. Besides, it is obvious that DNA is not a primary target of PA, PG and CL, because, possessing negatively charged phosphate groups, the PLs and DNA hardly interact with each other. Another presumable reason

of the DNA damage under the action of anionic PLs is the action of active forms of oxygen. This is what our further studies will be devoted to.

The manifestation of bactericidal activity of the liposomal forms of these anionic lipids added exogenously to the cultures of different bacteria (tuberculosis mycobacteria and colibacillus) opens opportunities of creating new, effective and less toxic antibacterial preparations based on them, especially for treating tuberculosis. It is important to note that it is possible to inject liposomal drugs by means of inhalation, thus accelerating the process of the preparation transfer into the site of the pathology course – the alveolar macrophage infected with *M. tuberculosis*.

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