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Liposomes loaded with lipophilic prodrugs of methotrexate and melphalan as convenient drug delivery vehicles

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Liposomal formulations prepared by extrusion from natural phospholipids and 1,2-dioleoylglycerol conjugates of methotrexate and melphalan (egg phosphatidylcholine–phosphatidylinositol–prodrug, 8:1:1, by mol.) were characterized by size, composition and stability. Both prodrugs were shown to incorporate completely into unilamellar liposomes with the mean size below 100 nm and form stable dispersions containing the drug concentrations relevant for systemic injections in animals. For long-term storage, the dispersions can be subjected to deep freezing (- 196°C) and stored at - 70°C; before usage, they should be defrosted and treated shortly in an ultrasonic bath. According to the example of methotrexate conjugate, stability of prodrug ester bond in liposomal formulation towards hydrolysis by human plasma esterases during 24-h incubation were established. Also, liposomes bearing methotrexate conjugate were shown to overcome resistance of human leukemia cells related to impaired transport of initial drug across the membrane.

Key words: Liposomes – Methotrexate – Melphalan – Lipophilic prodrugs – Long-term storage – Ester bond hydrolysis – Stability in plasma – Tumor cell resistance.

Inclusion of anticancer drugs in nanoparticulate carriers decreases the general toxicity and improves the efficacy of treatment due to the reduction in circulating free drug concentration, as well as the phenomenon of passive transport of vehicles in tumors induced through the enhanced permeability and retention (EPR) effect caused by fenestrated neovascular walls [1, 2]. Liposomes, being amongst the most biocompatible particles, were given new impulse after development of the Stealth liposome technology [3, 4], where membrane surface pegylation prevents premature withdrawal of liposomes from the circulation by cells of the reticuloendothelial system. In clinics, the improved therapy was shown for some anticancer antibiotics included in the water volume of Stealth liposomes, first of all the point concerns doxorubicin (marketed products Doxyl, Myocet, Caelyx) [5]. In the production of these liposomal formulations, remote loading technique is employed, i.e. cationic lipophilic drug molecules can be accumulated in pegylated liposomes (due to exposition against gradient of ammonium sulfate). Such technique works for a confined number of drugs, e.g. for anthracycline antibiotics. The alternative methods of preparation of liposomes bearing the drug in the interior are not practically feasible.

The most widely used anticancer agents are hydrophilic substances, which could be incorporated in the lipid bilayer of liposomes after lipophilic derivatization. Inclusion of a drug as a biodegradable lipid conjugate (lipophilic prodrug) in liposomal membrane simplifies and makes the technology of liposome preparation universal. Moreover, the delivery of cytotoxic agents in malignant cells as lipophilic prodrugs in liposomes has the following advantages: i) it results in a decreased leakage of drugs in circulation and during liposome/cell interaction; ii) the ability of lipid derivatives to the direct transport across membrane can change the mechanism of endocytosis and intracellular drug traffic, as well as facilitate the unloading of liposomes (to promote drug release from the inner volume of pegylated liposomes resided in pH-lowered endosomal compartment of target cells, particular molecular triggers have been developed [6]). Lipid derivatives of Ara-C [7, 8], 5-fluorouridine [9], gemcitabine [10], mitomycin C [11] applied in liposomal formulations surpassed the initial drugs in pharmacokinetics and antitumor activity.

Melphalan (Mlph, *Figure 1*) and its racemate sarcolysine (DL-melphalan), being cell-cycle non-specific alkylating cytotoxic agents of the nitrogen mustard family, have been used as anticancer medicines for almost 50 years. The low stability at physiological pH conditions and rapid removal from the circulation require administration of high doses of these drugs, which cause multiple side effects (see, e.g. [12]). To improve therapeutic efficacy, some of their long-chain diglyceride derivatives have been suggested as prodrugs. Esterification with 1,3-dipalmitoyl glycerol decreased toxicity of melphalan and imparted lymphotropic targeting to it [13]. 1,2-Dimiristoyl and 1,2-dipalmitoyl glycerol esters of melphalan exhibited reduced toxicity and superiority in survival over intact drug when administered i.p. (obviously, as dispersions) in mice with grafted leucosis P-388 [14]. We previously synthesized 1,2-dioleoyl glycerol ester of sarcolysine with the aim of including it in liposome membrane [15]. The conjugate incorporated into the lipid bilayer well, and revealed moderate cytotoxicity (in the range of 10^{-5} M) in liposomal formulations in a number of cell cultures [16, 17]. Testing in the mouse model of leucosis P-388 showed an increase in the median survival time by a factor of 1.4 as compared to initial sarcolysine (i.p. administration) [16].

The important requirement for the designed amphiphilic molecule is availability of reliable membrane anchor, which should also introduce minimum disturbances in the package of lipid bilayer to provide higher loading with the prodrug. From this point, the preferable are anchors bearing two long aliphatic chains, and especially 1,2-diglycerides. However, in the case of such a small molecule as sarcolysine even single chain anchors fitted well: octadecyl ester of sarcolysine in liposomal formulations caused a sharp increase in the survival of mice with transplanted mammary cancer (IV administration) [18].

Cytostatic agent methotrexate (MTX, folic acid antimetabolite, *Figure 1*) is widely used in clinical practice for the treatment of cancers and autoimmune diseases such as rheumatoid arthritis, where it retains a position of first-choice drug [19]. Recently, a lot of studies have been carried out on improvement in the MTX biodistribution by means of its conjugation with various nanoparticulate carriers, e.g. with polyamide dendrimers [20], dextrane [21], etc. Moreover, therapeutic efficacy of MTX is hampered not only by its systemic toxicity, but also

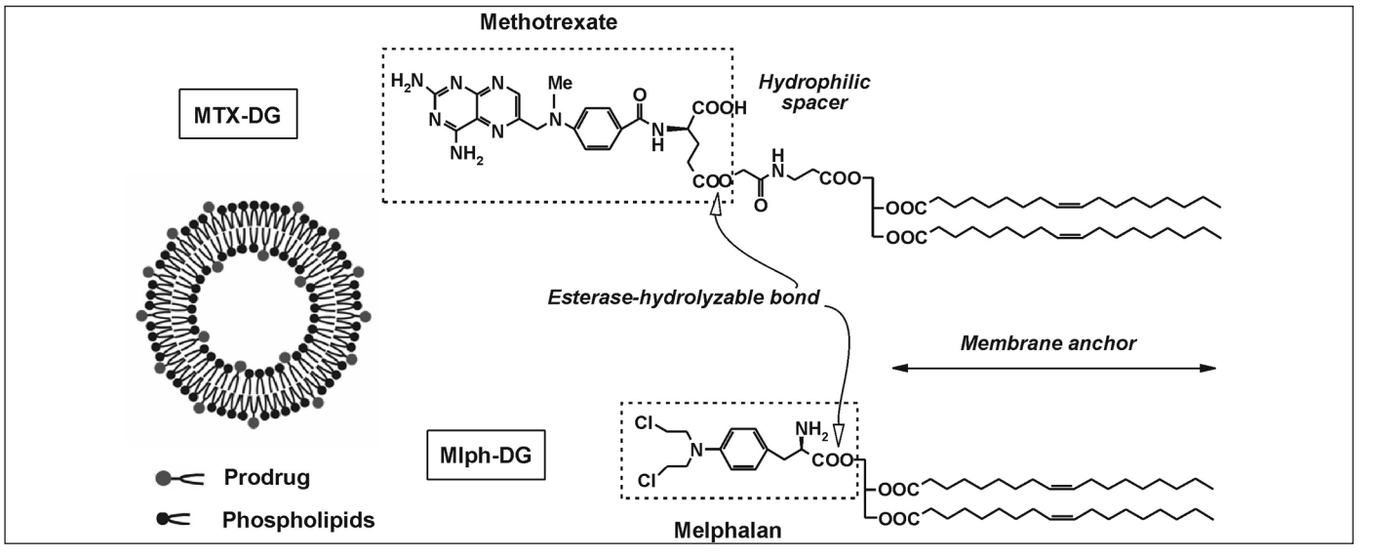

Figure 1 - Molecular structures of the diglyceride ester conjugates of methotrexate and melphalan, and the scheme of the drug-carrying liposome.

by the development of tumor cell resistance (up to 30% of remission failures upon treatment of acute lymphoblastic leukemia [22]) brought about mainly by the deficiency of an active carrier system (reduced folate carrier, RFC) regulating the entrance of folate analogues [19]. Passive transmembrane transport of the MTX molecule is hampered by its polarity. A number of lipophilic antifolates lacking in glutamate residue have been developed, which are capable of diffusing into cells omitting active transport (see, e.g., [23] and cited references); few of them have been used in clinics. Another approach consists in lipophilic modification of glutamate carboxylic groups, with γ-COOH being preferable since lipid substituent at this position decreases the affinity to a target enzyme dihydrofolate reductase (EC 1.5.1.3) to a lesser degree [24]. Both aliphatic γ-esters [24], and a series of lipoamino acid amides [25] of MTX inhibited *in vitro* the growth of human leukemia cells CEM/MTX resistant to MTX owing to defective RFC. Also, phospholipid derivative of MTX, where γ-COOH is esterified by lysophosphatidylcholine, and α-COOH – by dodecanol have been reported to be active against CEM/MTX cells [26]. As concerns liposomal formulations, dimyristoylphosphatidylethanolamine conjugate of MTX (γ-amide) was successfully used in liposomes for the inhibition of rat collagene-induced arthritis [27].

We have developed 1,2-dioleoyl glycerol γ-ester of MTX (MTX-DG, *Figure 1*) designed for incorporation into the liposome bilayer [28, 29]. The bulky MTX moiety is connected to diglyceride membrane anchor by short hydrophilic spacer, which should be located in the polar region near phospholipid head groups to enable a reduction of disturbances in the membrane structure, and loading of a greater quantity of the prodrug into liposomes. As for bond linking the drug moiety with the rest of the prodrug molecule, it should easily be hydrolyzed within the cell. The use of esters as prodrugs clearly benefits from facile enzymatic hydrolysis in the target cell since typically esterases are widely distributed in the body and have wide substrate specificity as compared to amidases [30]. Liposomal MTX-DG displayed moderate cytotoxic activity (IC_{50} about 10^{-6} M) in a melanoma M3 cell culture [29]; therefore this conjugate is a prodrug which liberates MTX by means of intracellular esterases or may directly inhibit dihydrofolate reductase.

The objectives of this study were to characterize physicochemical stability of liposomal formulations containing Mlph-DG and MTX-DG in concentrations relevant for systemic injections in animals, from the point of size and composition, as well as to research into the accessibility of long-term storage liposomal preparations. Also, examination of firmness of the drug ester conjugates in liposomes towards plasma esterases was undertaken using the example of liposomal MTX-DG. Finally, the ability of liposomal MTX-DG to overcome the resistance of tumor cells caused by impaired transmembrane MTX transport was tested.

I. MATERIALS AND METHODS

1. Materials

Diglyceride conjugates of Mlph (L-sarcolysine) [15] and MTX [28, 29] were synthesized as described elsewhere. Phosphatidylcholine (PC) from egg yolk and phosphatidylinositol (PI) from *S. cerevisiae* were purchased from Reakhim (Russia), Sepharose CL-4B from Pharmacia (Sweden), N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES) from Flow Laboratories (UK), ethylenediaminetetraacetic acid (EDTA) and MTX from Sigma (USA). Solvents were purified and dried by standard procedures; all the evaporation processes were performed in vacuo at temperatures below 40°C. Buffer solutions contained 1 mM EDTA: phosphate buffered saline (PBS), pH 7.0 (KH_2PO_4, 0.2 g/L; $NaH_2PO_4 \cdot 2H_2O$, 0.15 g/L; Na_2HPO_4, 1.0 g/L; KCl, 0.2 g/L; NaCl, 8.0 g/L); HBS, HEPES buffered saline, pH 7.2 (25 mM HEPES-Na, 140 mM NaCl).

2. Preparation of liposomes

Lipid films were obtained by co-evaporation of aliquots of stock solutions in chloroform-methanol (1:1) in a round-bottomed flask on a rotary evaporator, with subsequent drying for 30 min at 5 Pa. The resulting compositions were PC-PI-MTX-DG or Mlph-DG, 8:1:1 (mol/mol), and typically contained 12.5 mg PC, 1.6 mg PI and 2.4 mg (2 μmol) MTX-DG or 1.8 mg (2 μmol) Mlph-DG. The films were hydrated for 2 h at room temperature in 0.5 mL of PBS to obtain liposomes for experiments with cell culture or HBS in all other cases. After shaking, the suspensions were treated by five cycles of freeze-thawing (liquid nitrogen + 40ºC), and then extruded 20 times through polycarbonate membrane filters (Nucleopore, USA) with a pore size of 100 nm on a Mini-extruder (Avanti Polar Lipids, USA) to yield translucent liposome dispersions. Prodrug concentrations in liposomal preparations were measured after liposome solubilization in a 20-fold volume of ethanol by registration of UV-spectra with spectrophotometer Ultrospec II 4050 (LKB, Sweden), and calculations at the absorption maximum (MTX-DG: λ_{max} 302 nm, ε ~25000; Mlph-DG: λ_{max} 258 nm, ε ~19700). The waste of prodrugs on filters was determined after maceration of membranes in ethanol and measuring concentrations in obtained solutions.

The dimensions of liposomes were determined on a Coulter Model N4 MD Sub-Micron Particle Analyzer (Coulter Electronics, USA).

3. Determination of liposome composition

Typically, aliquots of liposome dispersions (0.5 mL) were subjected to gel chromatography on the Sepharose CL-4B column (1.3 × 38 cm) equilibrated with HBS containing 0.02% NaN_3. The void volume (16 mL) was discharged, and then 400- to 500-μL fractions were collected. Some liposomal samples (250-300 μL) were frozen in liquid nitrogen (- 196°C), stored for several days at - 70°C, then unfrozen at 40°C in a water bath, treated in an ultrasonic bath at 22 kHz (4 × 5 min), and applied to a CL-4B Sepharose column (1.2 × 26 cm) equilibrated with HBS/0.02% NaN_3; after discharging the void volume (10 mL), 250- to 300-μL fractions were collected. To decompose liposomal dispersions, the fractions were diluted fivefold with ethanol and shaken. The content of prodrugs was evaluated by measuring the UV spectra of these solutions and determining the corresponding optical density (see above). Phospholipid content in the fractions was measured by a phosphate assay [31] using 40 μL-aliquots of aqueous ethanol solutions; blank samples contained 40 μL of ethanol-HBS, 4:1, mixture. Each sample was treated with 70 μL of incinerating acid mixture (60% $HClO_4$-conc. H_2SO_4, 1: 1) by heating for 1 h in a silumin block at a temperature gradually increased up to 300°C. After cooling to room temperature, each sample was mixed with 0.4 mL of freshly prepared 1% aqueous ammonium molybdate solution, and 0.03 mL of the Fiske-Subbarow reagent, and then heated in a boiling water bath for 10 min. The optical density of final solutions was measured at 820 nm, and the amount of phosphorus was determined using as reference a calibration graph obtained for standard KH_2PO_4 solutions. The sensitivity threshold of this procedure was 2 nmol of inorganic phosphorus.

4. Electron microscopy of liposomes

The supporting films were prepared according to the standard method from a 0.05% pioloform solution in chloroform. Liposomes were arranged on the supporting films by applying a drop of the liposomal dispersion (containing 1-4 mM total lipids), followed by removal of the excess dispersion with a filter paper. The samples were contrasted with a 1% aqueous solution of uranyl acetate and analyzed in a JEM-100CX11 electron microscope (Jeol, Japan) at an acceleration voltage of 80 kV.

5. Determination of the plasma stability of liposomal methotrexate ester diglyceride

5.1. Chromatography instrumentation and conditions

The following equipment was used: Stayer HPLC system (Aquilon Inc., Russia) consisting of pump series II, Rheodyne injector 7725i, UV/Vis detector UVV 104 equipped with 5 mm/10 μL flow cell and Chom&Spec data acquisition system (Ampersand Ltd., Russia). HPLC column Luna Silica, 5 μm, 4.6 × 150 mm (Phenomenex, USA), was used under isocratic mode with the following mobile phase compositions and flow rates in different experiments: chloroform-methanol-glacial acetic acid, 65:25:10, 1.2 mL/min (A); chloroform-methanol, 75:25, 1.1 mL/min (B); chloroform-methanol, 80:25, 1.0 mL/min (C). The column temperature was 22 ± 3°C. For drug substances, the working sample concentrations were 2.5-12.5 mg/mL for MTX and 5-20 μg/mL for MTX-DG. The injection volume was 20 μL (full loop). UV detection was at 303 nm.

The calibrations were linear ($R^2 \geq 0.97$) over the tested concentration ranges. The stock solutions of MTX and MTX-DG in chloroform–methanol, 65:25 (0.5 mg/mL of MTX, and 1 mg/mL of MTX-DG) were stored at - 20°C, and calibration solutions were prepared on the day of analysis by diluting the stock solution with a mobile phase to proper concentrations: (2.5-5-10-12.5) mg/mL for MTX and (5-10-15-25) μg/mL for MTX-DG.

5.2. Preparation of samples for HPLC analysis

Fresh samples of human plasma prepared from the mixed pools of blood collected from different donors (30-40 persons in each experiment) were obtained from the Institute of Experimental Cardiology, Russian Cardiology Research Center, Moscow.

Aliquots of liposomal preparations with or without MTX-DG (50 μL) were diluted in PBS or plasma (450 μL) to reach the drug concentration of 0.47 mg/mL. Then samples were incubated at 37°C for varying times. After incubation, 20-μL sample aliquots were diluted with 450 μL mixture chloform-methanol, 65:25, then vortexed for 1 min and centrifuged at 4°C in Eppendorf tubes for 10 min at 13,000 g. Supernatants were collected and injected into the HPLC system. Three independent experiments were performed with different samples of plasma.

6. Cells and cytotoxicity assay

Human T-lymphoblastic leukemia cells CEM-CCRF and MTX-resistant subline CEM/MTX were continuously cultured at 37°C in 4% CO_2 atmosphere in an RPMI-1640 medium (ICN Biomedical Inc., USA) supplemented with 0.2% $NaHCO_3$, 2 mM L-glutamate, 50 μg/mL gentamicin G, 100 μg/mL streptomycin, and 10% calf embryo serum inactivated by heating (Gibco BRL, UK), pH 7.4, and passaged twice a week. Cells in logarithmic phase of growth were taken for experiments.

The cells were incubated for 48 h in a culture medium in 24-well plates with liposomal preparations containing MTX-DG (from 0.01 to 10 μM) or with MTX as such (from 1 nM to 100 μM). In control samples, the cells were incubated with a PBS aliquot. Cell viability was determined by the standard trypan blue dye exclusion assay. The percentage of living cells was calculated as: (quantity of living cells in experiment/quantity of living cells in control) × 100. Three independent experiments were carried out, each duplicated.

II. RESULTS

1. Preparation and characterization of liposomes

The liposomes were prepared by extrusion through polycarbonate membrane filters with a preset pore size [32]. Giant multilamellar liposomes are formed as a result of hydration of a lipid film in the corresponding buffer. Being subjected to several cycles of freezing in liquid nitrogen and thawing at a temperature above the temperature of phase transition in the lipid bilayer, such liposomes transform into unilamellar vesicles, which acquire a desired size upon multiple extrusion through calibrated membrane filters [33]. We prepared liposomes from the mixtures of natural phospholipids and prodrugs by extrusion through filters with 100-nm pores. *Figure 1* shows a schematic view of liposome construction.

The lipid bilayer of the Stealth liposomes is formed from phospholipids containing saturated acyl chains, such as distearoylphosphatidylcholine or hydrogenised soybean lecithin, and cholesterol (up to 30%) to obtain rigid membranes, which are subjected less to destruction in circulation and leakage of drug from the interior [5]. As a result, it is necessary to heat the samples to 60°C at the hydrated lipid film thawing and extrusion stages as such, as well as during remote loading of doxorubicin. In our case, lipophilic prodrugs bearing diglyceride membrane anchor participate as components in the lipid bilayer and cannot leave it so easy even upon membrane damage. Moreover, fluid lipid bilayer (characterized by a low temperature of the phase transition) is capable of including more amphiphilic molecules besides matrix phospholipids. It is worth noting that membranes of tumor cells merge with fluid lipid bilayers more readily [34, 35]. For this reason, the main components of bilayer in our liposomes are PC from egg yolk and PI from baker's yeast, which contain about half of the saturated acyl chains (C16 and C18); the remaining fractions are represented by unsaturated oleoyl and, less, linoleoyl residues. Phosphatidylinositol, when comprising about 10 % of bilayer, was shown to prolong the life of liposomes in the circulation due to inositol residues, which form a highly hydrated sterically stabilizing shell on

Table I - Photon correlation spectroscopy analysis of the dimensions of drug-containing liposomes (Lp) using a Coulter Model N4 MD instrument.

Lp state	Unimodal analysis		Size distribution processor analysis					
	Mean size (nm)	SD (nm)	Mean size (nm)	SD (nm)	CV (%)	Peaks		
						Size (nm)	SD (nm)	Amount (%)
As prepared	89.7*	33	78.1	45	57	3 95.9	0.5 11	19 81
	57.2**	broad	71	39	55	3 41.2 92.8	0.5 12 11	10 25 65
After freezing/thawing and ultrasonic treatment (2 x 5 min)	72.2	broad	70.1	48	69	3 93.7	0.5 11	26 74
	88.4	broad	182	240	130	38 159 822	12 56 96	26 66 8
After another cycle of ultrasonic treatment	74.8	broad	63.9	49	77	3 90	0.5 13	30 70
	72.8	broad	92.2	63	69	3 37.1 134 Dust	5 7 2	5 37 59 2

*Liposomes loaded with MTX-DG. **Shaded fields refer to liposomes loaded with Mlph-DG.

the liposome surface similarly to poly(ethylene glycol) residues in Stealth liposomes [36, 37].

The initial mixture of lipids contained 10 mol % Mlph-DG or MTX-DG. The volume of buffer solution added to hydrate lipid film was calculated to obtain final concentration of prodrugs in liposomal dispersions about 4 mM. Such concentration is relevant for intravenous injections in terms of dose-volume ratio. In the case of MTX-DG, the buffer contained 1 mM EDTA in order to prevent the aggregation of lipid bilayers, exposing negatively charged MTX residues, in the presence of bivalent metal cations. Previously, we established that these quantities of EDTA are nontoxic upon IV injections in rats (unpublished data).

The liposome dimensions were analyzed within several hours after preparation using photon correlation spectroscopy (*Table I*, upper row). Mean size values of MTX and Mlph liposomal preparations were about 80 and 70 nm, respectively (column: size distribution processor analysis). The presence of more than one population of liposomes, especially perceptible quantities of 3 nm-size fractions in both cases, do not match gel chromatograms (see below, *Figures 4a and 5a*) revealing single peaks eluted after void volumes. It should be noted that the instrument employed (Coulter Model N4 MD) is primarily assigned for determining particle size in turbid media, while the measurements in slightly opalescent liposomal dispersions can lead to artifacts, such as, in our opinion, broad Gaussian distribution indicated for freshly prepared Mlph liposomes. Measurements using a setup capable of analyzing dilute dispersions should be made in future experiments. Anyhow, the unlikely existence of different vesicle populations could hardly influence the homogeneity of prodrug encapsulation because extrusion of the preformed giant liposomes was carried out under conditions of fluid lipid bilayer.

Liposomes were also studied by transmission electron microscopy using a negative contrast technique. The micrographs of the 10-fold diluted liposomal dispersions are presented in *Figures 2a and 3a*. In spite of some collapse of the particles visible on micrographs (more pronounced in the case of the Mlph liposomes), which usually originates in the drying of dispersions on supporting grids, these patterns evidence that both MTX and Mlph liposomes are unilamellar vesicles with dimensions below 100 nm.

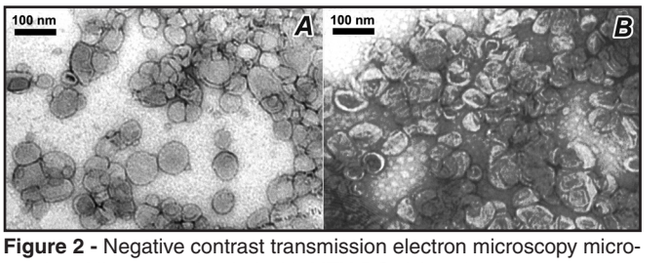

Figure 2 - Negative contrast transmission electron microscopy micrographs of liposomes loaded with MTX-DG: (a) 20 h after preparation, and (b) after deep freezing (- 196°C) and thawing (+ 40°C).

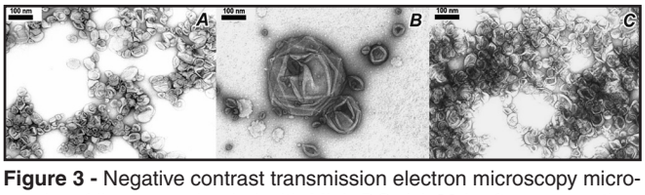

Figure 3 - Negative contrast transmission electron microscopy micrographs of liposomes loaded with Mlph-DG: (a) 20 h after preparation, (b) after deep freezing (- 196°C) and thawing (+ 40°C), and (c) after additional ultrasonic treatment.

2. Determination of liposome composition

The degree of inclusion of the lipophilic prodrugs in liposomes was determined using gel chromatography on a Sepharose column. Fractions were treated with ethanol to decompose liposomes, and then analyzed for the phosphorus by means of colorimetric technique to determine the content of phospholipids [31]. The concentrations of MTX and Mlph conjugates were determined spectrophotometrically, from the optical density of solutions measured at absorption maxima of MTX-DG and Mlph-DG (see Experimental). *Figures 4a and 5a* show gel chromatograms of liposomal preparations containing MTX-DG and Mlph-DG, respectively. Phospholipid and prodrug peaks clearly coincide entirely. Since the contents of prodrugs in liposomal dispersions were only slightly lower than their initial amounts taken for the preparation of lipid films (losses on membrane filters did not exceed 2-5%), it was concluded that conjugates were nearly quantitatively included into liposomes.

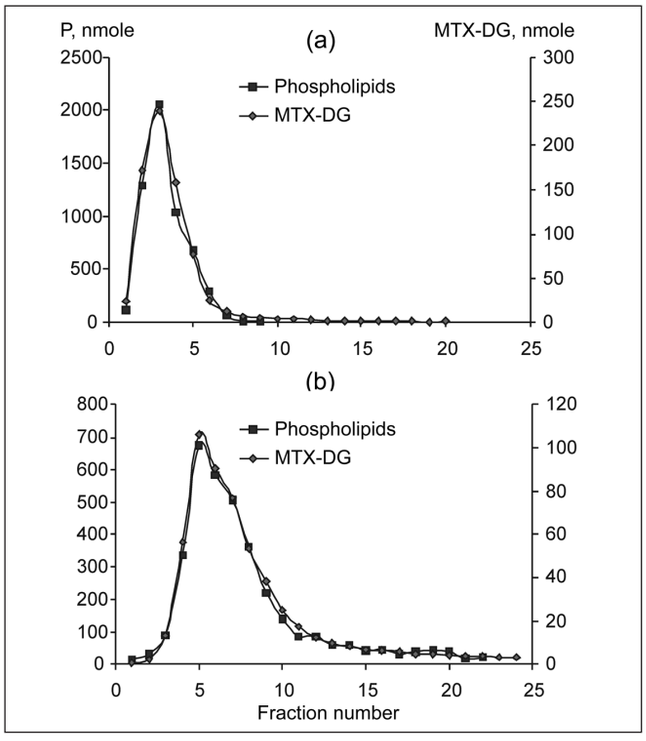

Figure 4 - Chromatograms obtained by gel filtration of liposomes containing MTX-DG: (a) immediately after preparation and (b) after deep freezing (- 196°C) and thawing at + 40°C followed by ultrasonic treatment.

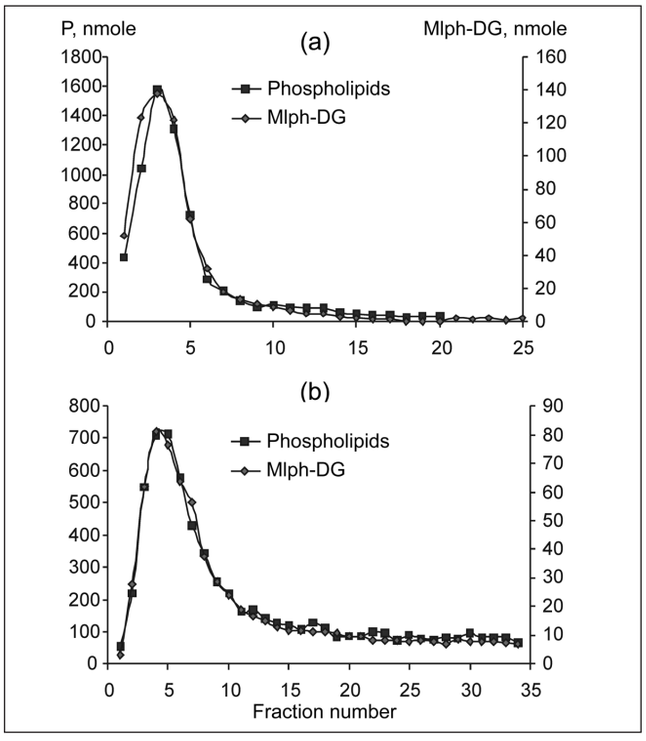

Figure 5 - Chromatograms obtained by gel filtration of liposomes containing Mlph-DG: (a) immediately after preparation and (b) after deep freezing (- 196°C) and thawing at + 40°C followed by ultrasonic treatment.

3. Long-term storage liposomal preparations

Liposomal dispersions can be stored at + 4°C for several days without significant aggregation (data of photon correlation spectroscopy, not shown). However, storing the drugs themselves in aqueous solutions for so long is not recommended. For example, melphalan noticeably degrades after one day in PBS at + 4°C (TLC data). A possible variant of storage for our liposomal preparations could be their freezing. Indeed, for lipophilic prodrugs incorporated into liposome membrane, it is hardly possible that they could be lost to the aqueous phase after a freezing-thawing procedure as is the case with liposomes bearing water soluble drugs in the internal volume.

To validate this hypothesis, dispersions of MTX- and Mlph-liposomes were frozen in liquid nitrogen and then stored for several days at - 70°C. The subsequent defrosting at + 40°C led to the formation of turbid suspensions, which was indicative of the vesicle aggregation. However, following short-term processing of the samples in an ultrasonic bath turned them into slightly opalescent dispersions. Data on the particle size distributions in suspensions measured upon defrosting and ultrasonic treatment are presented in the *Table I* (second and third rows). In the case of the MTX liposomes, treatment in ultrasonic bath leads to a slight decrease in the mean particle diameter as compared to the initial size, accompanied by a broadening of the size distribution and rise of 3-nm fraction. Evidently, relatively half-term ultrasonic treatment could be sufficient for the restoration of MTX liposomal dispersions. As for Mlph liposomes, most of them still remain in partially aggregated state after the first cycle of ultrasonic treatment, revealing also a residual population of 822-nm size. The second cycle of treatment led to further breaking of aggregates to the particles of near initial size; a significance of the appeared dust should be examined in our further experiments.

Figures 2 and 3 present the electron micrographs of liposomal preparations obtained in the course of these studies. Mlph liposomes upon defrosting are seen to aggregate with the formation of coarse dense conglomerates (*Figure 3b*), which can be dispersed to the initial vesicular particles by ultrasonic treatment (*Figure 3c*). In contrast, MTX liposomes do not form dense conglomerates upon defrosting (*Figure 2b*), which is in accordance with data in the Table. Such behavior is apparently explained by the presence of negatively charged MTX α-carboxyl groups on the surface of liposomes.

The composition of liposomes after defrosting and ultrasonic treatment was determined by gel chromatography with subsequent analysis of fractions as described for the initial liposomal preparations. The results shown in *Figure 4b* (for MTX-DG) and *Figure 5b* (for Mlph-DG) reveal that prodrugs are not lost from the lipid bilayer of liposomes into separate aggregates. Both samples were eluted as single peaks, with some gap of fraction numbers pertaining to initial liposomal preparations (*Figures 4a and 5a*) caused by the usage of the Sepharose column of another size, and incomplete coincidence of relative fraction volumes (see section 2.3).

Therefore, the composition of liposomes loaded with lipophilic prodrugs was completely restored after freezing in liquid nitrogen followed by storage at - 70°C, defrosting and treatment in ultrasonic bath. Restoration of the liposomal size requires more exact tuning of ultrasonic treatment for both types of liposomes. On the whole, deep freezing without cryoprotectors can be used for the long-term storage of our liposomal preparations.

4. Determination of plasma stability of liposomal methotrexate ester diglyceride

To analyze mixtures containing amphiphilic prodrug and initial hydrophilic drug, we applied HPLC. Commonly used reversed-phase chromatography with gradient mobile phase (water, or aqueous buffers-acetonitrile, iso-propanol etc.) did not give satisfactory results in our case, when substances with quite different physicochemical characteristics were analyzed: MTX-DG adsorbed with strong retention on column (C18, C8, and C5 phases have been proved) or, alternatively, raced through it in a micellar form. Methotrexate and MTX-DG were clearly separated with retention times of 9.05 and 3.75 min, respec-

tively (*Figure 6a*), on a silica gel phase with isocratic mobile phase composed of organic solvents (system A). A control chromatogram of liposomes without MTX-DG (after dilution with the mobile phase as described in the experimental part) is given in the inset.

Chemical stability of MTX-DG in liposomal formulation was analyzed by incubation of dispersions in PBS at 37°C. First results are shown in *Figure 6b*. Obviously, MTX-DG did not degrade under these conditions for at least 8 h. Also, according to the data of gel chromatography on a Sepharose column (not shown), the prodrug did not throw over the lipid bilayer of liposomes incubated in such a way for 24 h. Further HPLC analysis (mobile phase C) evidenced the integrity of prodrug molecule (data not shown).

Then, stability of liposomal MTX-DG towards esterases of human plasma was analyzed. The results of two independent experiments are shown in *Figures 7 and 8*. Silica gel is very sensitive to the traces of water introduced, for example, from solvents, as well as from the samples as such. It requires prolonged regeneration after each cycle of chromatographic procedures, which are carried out during several hours' incubation. As a result, we had to vary slightly the ratio of solvents in a mobile phase as well as flow rates in different experiments. Correspondingly, retention times for MTX-DG and MTX were 4.95 and 9.03 min (*Figure 7a*, mobile phase B) in one experiment, and 3.25 and 6.28 min (*Figure 8a*, mobile phase C) in another. As can be seen from *Figures 7 and 8*, MTX-DG is stable in 90% human plasma *in vitro* up to 24 h of incubation at 37°C.

5. Cytotoxicity of liposomal methotrexate ester diglyceride in the cultures of cells with different sensitivity to methotrexate

A comparative study of cytotoxic activity of liposomal preparations bearing MTX-DG was performed *in vitro* using human T-lymphoblastic cells of the CEM-CCRF line and MTX-resistant RFC (see Introduction) deficient CEM/MTX subline. The cytotoxicity was determined using a standard method based on the trypan blue dye exclusion, and accepted for the cytostatic agent MTX [23-25]. The results of these tests are presented in *Figure 9*. The calculated IC_{50} values for MTX with respect to cells of CEM/MTX and CEM-CCRF lines amounted to 16.4 ± 4.9 μM and 0.075 ± 0.005 μM (*Figure 9a*), while corresponding values for liposomal MTX-DG were 1.68 ± 0.05 μM and 0.88 ± 0.07 μM (*Figure 9b*). Thus, the resistance of leukemia cells CEM/MTX (characterized by the ratio of IC50 for CEM/MTX to IC_{50} for CEM-CCRF) decreases from 218 to 1.9 (i.e. by a factor of 114) on the change from MTX per se to liposomal MTX-DG formulation.

III. DISCUSSION

In this work, the structural characteristics of liposomes prepared by extrusion from natural phospholipids and lipophilic prodrugs of the widely used in clinics agents melphalan (L-sarcolysine) and methotrexate synthesized previously [15, 28, 29] were studied. Both drug conjugates are shown to incorporate virtually quantitatively into liposomes composed of egg yolk phosphatidylcholine-yeast phosphatidylinositol (antiopsonizing lipid)-prodrug at a molar ratio of 8:1:1. It was assumed that PI included to protect liposomes in circulation does not cause side effects reported for pegylated liposomes (mucositis, etc.) [5]. The obtained liposomal preparations contain the drug equivalents in concentrations (~ 4 mM) relevant for IV injections to experimental animals in comparative trials with initial drugs. In actual fact, accepted IV doses for Mlph and MTX are 7 and 10 mg/kg (about 23 μmoles per kg of each), respectively, when working with mice. The corresponding volumes of liposome dispersions in our case are therefore slightly more than 0.1 mL per mouse of 20 g weight.

The obtained preparations are stable dispersions of unilamellar vesicles with the mean size not exceeding 100 nm, which provides

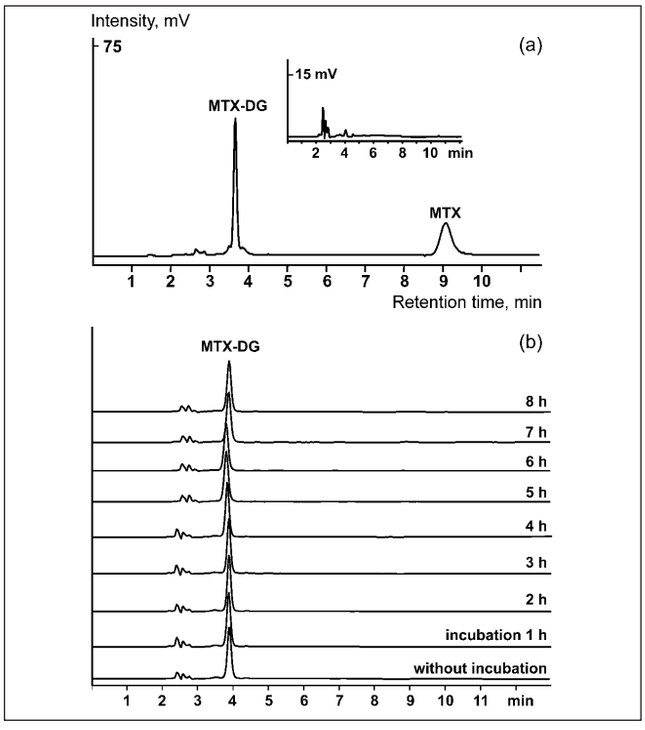

Figure 6 - Chemical stability of liposomal MTX-DG: (a) a representative chromatogram of the calibration solution with MTX-DG and MTX, concentrations 20 and 10 μg/mL, respectively (HPLC with mobile phase A); inset, liposomes without MTX-DG; (b) chromatograms of the liposomal MTX-DG samples after incubation for different times in PBS, pH 7.0, 37°C.

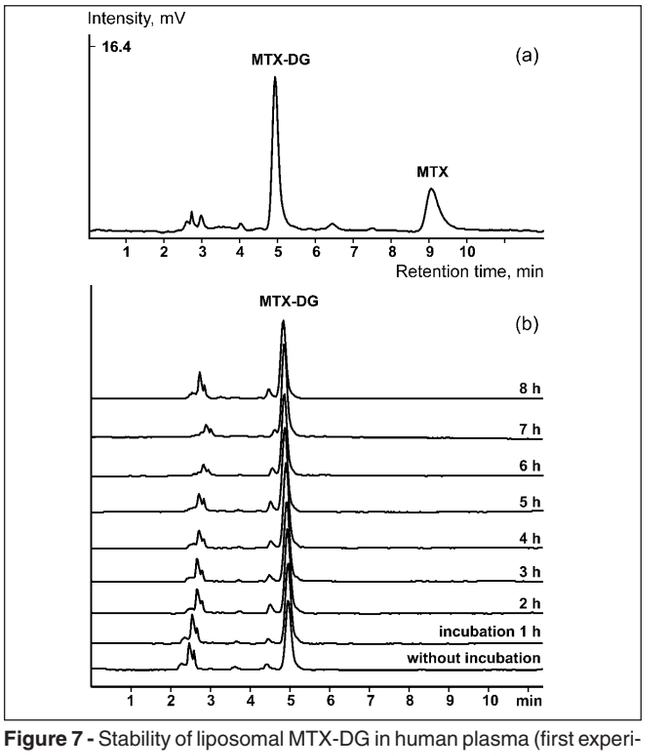

Figure 7 - Stability of liposomal MTX-DG in human plasma (first experiment): (a) a representative chromatogram of the calibration solution with MTX-DG and MTX, concentrations 2.5 and 5 μg/mL, respectively (HPLC with mobile phase B); (b) chromatograms of the liposomal MTX-DG samples after incubation for different times in 90% plasma at 37°C.

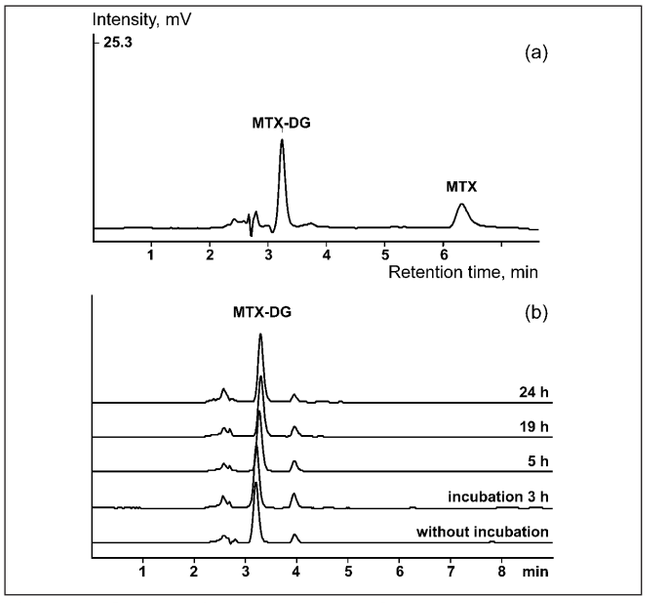

Figure 8 - Stability of liposomal MTX-DG in human plasma (second experiment): (a) a representative chromatogram of the calibration solution with MTX-DG and MTX, concentrations 1.25 and 2.5 μg/mL, respectively (mobile phase C); (b) chromatograms of the liposomal MTX-DG samples after incubation for different times in 90% plasma at 37°C.

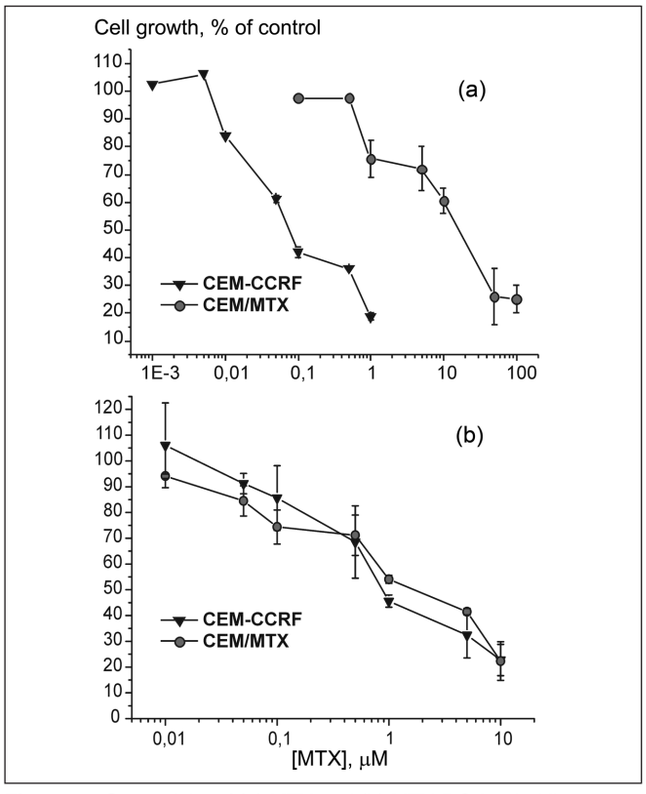

Figure 9 - Cytotoxicity of (a) MTX and (b) MTX-DG-loaded liposomes in the cultures of human leukemia cells possessing different sensitivity to MTX: CEM-CCRF (T-lymphoblastic cell line) and CEM/MTX (RFC-deficient MTX-resistant subline). After 48-h incubation, the cell viability was evaluated by the standard trypan blue dye test. Results depicted are means ± SE values from three independent experiments, each was performed in duplicate; the results were processed using Origin 6.0 (MicroCal Software Inc., USA).

passive transport of active agents to the tumor and inflammation zones in the course of systemic administration. The most developed method for long-term storage of liposomal preparations is lyophilization of dispersions in the presence of various carbohydrate cryoprotectants, such as trehalose, saccharose etc., which retain liposomes intact in the course of freezing, drying, and rehydration [38, 39]. However, this procedure is known to be accompanied by undesirable enlargement of liposomes. We have shown that long-term storage of our liposomal preparations can be carried out without cryoprotectors, by using low-temperature freezing, and storage at frozen state (at - 70°C) with subsequent short-term treatment in ultrasonic bath after defrosting. The conditions of ultrasonic treatment should clearly be examined in greater detail to provide liposome disaggregation without breaking of vesicular membranes. Apparently several months' storage of the frosted dispersions does not require temperatures lower than - 20°C (which is more attractive from the standpoint of technology). Nevertheless, this issue should be particularly studied.

The stability of methotrexate conjugate in liposomes towards chemical degradation was verified by HPLC analysis after 24-h incubations in PBS. In addition, liposomes retained MTX-DG under these conditions, which also implies chemical stability of the prodrug. We then demonstrated that MTX-DG applied in liposomal formulation did not degrade in 90% human plasma at least during 24-h incubation *in vitro*. Therefore, liposomes protect methotrexate diglyceride ester conjugate from premature hydrolysis by plasma esterases, thus prolonging time of clearance of the prodrug from circulation and improving pharmacokinetics of initial methotrexate. In contrast, lipoamino acid esters of idebenone (CNS-active agent, structural analog of ubiquinone) were shown to have hydrolysis half-lives of about 80 min when incubated in 80% human serum [40].

Development of the HPLC analysis procedure for the mixtures of melphalan with Mlph-DG was hampered by low stability of Mlph (as such, and derivatives) under incubation in neutral aqueous medium at physiological temperature. This caused the appearance of numerous contradictory data concerning its pharmacokinetics, as well as antitumor activity (see e.g., rfs in [41]). Being an active alkylating agent, melphalan undergoes hydrolysis of bis(β-chloroethyl) group and yields more than 70% of bis(β-hydroxyethyl) derivative in 1 h of incubation at 41.5°C in a buffer, pH 7.4; the product of hydrolysis has significantly lower retention time under reversed-phase HPLC as compared to Mlph [41]. Our studies gave the following results: Mlph degraded by 20 % in 1 h, by 40% – in 2 h, and completely – in 18 h, when incubated at 37°C in PBS, pH 7.0 (data not shown). We separated mixtures of Mlph and Mlph-DG by reversed-phase HPLC (C18 and C5) using ion-pair reagent sodium hexyl sulfonate in the mobile phase, since silica gel HPLC of Mlph-DG was complicated by ion effects of free amino group forming inner salt in initial Mlph. However, we failed to get individual peaks of Mlph-DG, although its molecular structure was fully confirmed by NMR and MS data. Probable reasons could be insufficient compensating for ion effects by ion-pair reagent, as well as display of different chromatographic mobilities of diastereomers, since Mlph-DG contains racemic moiety of 1,2-diolein. Nevertheless, a comparison of chromatograms obtained in the course of incubation of liposomal Mlph-DG in PBS or human plasma at 37°C for 24 h did not reveal any difference. Both peaks of initial Mlph-DG gradually and proportionally decreased, without any new peaks. Therefore, we could conclude that plasma esterases did not split Mlph-DG with release of melphalan or products of its hydrolysis.

The results of experiments on the cultures of human leukemia cells with different sensitivity to methotrexate showed that MTX-diglyceride conjugate in liposomal formulation is capable of overcoming the tumor cell resistance to the drug, related to impaired transmembrane transport

caused by decreased RFC activity. Besides that, the development of resistance to MTX can involve proteins eliminating xenobiotics from the cells and accounting for the multiple drug resistance (MDR). These include integral proteins of the ABC family, which participate in the development of resistance to lipophilic antifolates (see, e.g. [23], and references therein). In this respect, it should be noted that the use of drugs in liposomal formulations also overcomes the MDR of tumor cells related to the drug efflux mediated by P-glycoprotein (ABCB1, a protein of the ABC family) [42]. It is quite probable that liposomal drug delivery systems are capable of overcoming MDR caused by the entire spectrum of ABC proteins since the mechanism of drug endocytosis is changed from the active or passive transport across the membrane to the pinocytosis of nanoparticles.

The mechanism of the intracellular traffic and fate of MTX-DG and Mlph-DG has not yet been studied. Most probably, esterases first cleave the diglyceride residues, and then the drugs accomplish their specific action. The intracellular hydrolysis of prodrugs is apparently one of the factors reducing manifestation of cytotoxic activity of initial drug, along with the process of liposome unloading. Usually, the differences between cytotoxicity levels of a drug per se and the drug encapsulated in rigid vesicles (including pegylated liposomes) in *in vitro* assays are expressed by a factor of several tens or even more. The IC50 value of liposomal MTX-DG in the culture of sensitive leukemia cells CEM-CCRF is only about tenfold of that revealed for initial methotrexate. Taking into account the physicochemical stability of liposomal preparation, this fact could testify both to the facilitated unloading of liposomes in the cells and to the rapid hydrolysis of MTX-DG by intracellular esterases. Previously, we received similar results when cytotoxicity of liposomal Mlph-DG was tested in a number of cell cultures [16,17]. In addition, another possible explanation should not be excluded: that of gradual decomposition of liposomes during incubation in the cultural medium with subsequent passive diffusion of the prodrugs into the cells.

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In conclusion, the results of the studies on physicochemical properties, plasma stability, and cytotoxicity demonstrate that liposomal formulations loaded with diglyceride esters of methotrexate and melphalan represent potent drug delivery vehicles. The choice of optimal doses and regimen for the treatment with these liposomal preparations clearly requires further investigations of their pharmacokinetics and biodistribution.

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