Antiviral Activity of Liposomal Preparations of Antibiotic Geliomycin

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A liposomal preparation with maximally possible content of incorporated geliomycin was obtained. Its cytotoxicity was studied in a culture of human embryonal diploid fibroblasts. Antiviral activity was studied on a model of cytomegaloviral infection *in vitro* by the capacity to plaque formation. Liposomal geliomycin was 10-fold less toxic for human cells than the solution of the antibiotic in DMSO and exhibited antiviral activity towards cytomegaloviral infection at a concentration of 0.042 μg/ml.

Key Words: *geliomycin (resistomycin); liposomes; cytotoxicity; antiviral activity; cytomegalovirus*

The use of liposomes as drug carriers attracts considerable recent attention. Liposomes are nontoxic and undergo complete biodegradation in the body. The use of liposomes ensures prolonged release of the drug and reduces its toxicity [1].

Geliomycin (GM) antibiotic is an inhibitor of RNA-polymerase [2] and HIV-1 protease [3]. This antibiotic was regarded as a prospective antiviral drug for a long time, but was never widely used in medicine because it is very poorly dissolved in water and is highly toxic. Incorporation of GA in liposomes improves drug quality and helps to create an injection form with low toxicity.

We studied liposomal GM preparation; evaluated its toxicity for human embryo fibroblasts (HEF) and its antiviral activity towards human cytomegalovirus (CMV).

MATERIALS AND METHODS

Monolamellar vesicles (liposomes) were obtained by extrusion and separation of GM crystals from liposomes using a LiposoFast Basic laboratory extruder (Avestin Inc.) with nuclear membranes with 100-nm pores (the same firm) and nuclear membranes with 200- and 400-nm pores (Tensor). Optical density was measured on a Beckman DU-7 spectrophotometer.

The liposomes consisted of the following lipids: yolk phosphatidylcholine (yPC), cattle heart cardiolipin and cholesterol (Biolek), soybean phosphatidylcholine (sPC; Lipoid), and stearilamine (Sigma). GM was a kind gift from G. F. Gauze Institute for Search of New Antibiotics, Russian Academy of Medical Sciences.

Isotonic solution was prepared from extrapure sodium chloride (Khimmed). All solvents were of "chemically pure" grade (Khimmed).

Multilamellar vesicles with GM were prepared by evaporation of yPC ethanol solution or yPC/cholesterol mixture (7/3), or sPC/cardiolipin (8/2), or sPC/stearilamine (9/1; molar ratios in all cases) to a constant weight. GM solution in a chloroform/methanol (1/1) mixture was added to the lipid film in a volume sufficient to prepare a mixture of lipids and GM with 5, 10, 20, and 30 molar percentage of GM *vs.* lipids. The solutions were evaporated on a rotor evaporator, after which 2 ml saline was added; the lipid concen-

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tration in the final sample was 10-20 mg/ml. The samples were frozen in liquid nitrogen and shaken at ambient temperature. The freezing-defrosting cycle was repeated 5 times.

Monolamellar vesicles (MLV) were prepared by extrusion. Multilamellar vesicles were pressed (with an extruder) once through a nuclear polycarbonate filter with 200-nm pores, thus separating GM not incorporated into liposomes. The suspension was then passed 17 times through 2 membranes with 100-nm pores. The concentration of GM was measured in the resultant MLV, for which aliquots were selected from each sample and a chloroform/methanol (1/1) mixture was added to prepare a homogeneous solution. Optical density of each sample was measured in maximum values of GM absorption (320, 337, 366, 457 nm). Using these data, GM concentration in the liposomal suspension was calculated.

Cell culture was presented by diploid HEF from cell culture collection of D. I. Ivanovskii Institute of Virology. The cells at passages 14-17 were grown in a mixture of Eagle MEM and 199 media in Hanks' solution (1:1) with 10% fetal calf serum and 2 mM glutamine at 5% CO_2. For experiments, HEF were put into 24-well plates (2×10^5 cells/well in 1 ml culture medium) and cultured until the formation of a monolayer.

The cytotoxicity was evaluated by changes in cell morphology and viability in the presence of GM solution in DMSO and/or its liposomal form (MLV composition: 10 M% GM from lipids and 10 mg/ml yPC/cholesterol, 7/3). GM was used in concentrations of 0.005, 0.010, 0.030, 0.040, 0.100, 1.000, and 10.0000 μg/ml. The morphology was evaluated visually. Viability was evaluated by changes in the cytoplasmatic membrane permeability for 0.04% vital stain (Trypan Blue), staining dead cells and not staining viable ones. The concentration causing 50% cell death (CC_{50}) was calculated.

Antiviral activity of GM encapsulated in MLV was studied by infecting HEF cells with CMV (reference strain AD 169, a kind gift from Prof. D. Emanuel). Infective activity of the virus inoculate was estimated by the formula A=(a×b)/V, where A is the number of plaque-forming units/ml (PFU/ml), is the mean number of plaques per well, b is virus dilution, and V is the volume of virus-containing material added (0.2 ml in our experiments). HEF cells were infected at different infection multiplicity: 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} PFU/cell. After CMV adsorption for 1 h at 37°C the virus was washed and culture medium containing GM liposomal form in concentrations of 0.005, 0.010, 0.030, 0.040, and 0.100 μg/ml was added into the wells. Antiviral activity of the preparation was evaluated by the plaque reduction method (evaluation of GM concentration suppressing the plaque-forming activity of the virus by 50% (IC_{50}).

RESULTS

One of the main characteristics of liposomal preparations is the content of drug substance incorporated into liposomes. At stage I of the study we evaluated the relationship between the lipid composition of liposomes and degree of GM incorporation. To this end, antibiotic incorporation into liposomes consisting of the following lipids were studied: yPC, yPC/cholesterol (7/3), sPC/cardiolipin (8/2) and sPC/stearilamine (9:1). The presence of cholesterol decreased GM solubility in the membrane and thus decreases the degree of its incorporation (Fig. 1). The maximum incorporation of GM in MLV without cholesterol is 97% GM of the initial content (20 M% GM of lipids), while in MLV with cholesterol the same amount of GM is incorporated in the presence of 15 M% GM of lipids.

The study of the antibiotic incorporation into liposomes containing negatively charged phospholipid (cardiolipin) in the sPC/cardiolipin (8/2) mixture showed that the maximum incorporation of GM was similar to that with yPC liposomes (97% or 20 M%; Fig. 1). Addition of a negatively charged lipid did not improve GM binding to liposomes.

Incorporation of GM in positively charged liposomes from the sPC/stearilamine (9/1) lipid mixture was much worse (Fig. 1). After addition of 20 M% GM to this lipid mixture only half of GM (48%, or 10 M%) incorporated into liposomes.

If the initial content of GM is increased, its incorporation in the liposomes decreases, because of formation of precipitate (hard particles not pressed through the nuclear filter with 100-nm pores). The most stable

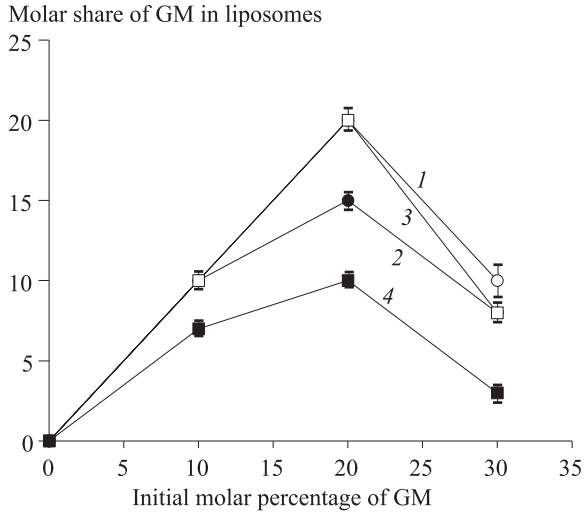

Fig. 1. Encapsulation of geliomycin (GM) in monolamellar vesicles, depending on lipid composition and initial concentration of GM. *1*) yolk phosphatidylcholine (yPC); *2*) yPC/cholesterol, 7/3; *3*) soybean phosphatidylcholine (sPC)/cardiolipin, 8/2; *4*) sPC/stearilamine, 9/1, lipid concentration 20 mg/ml.

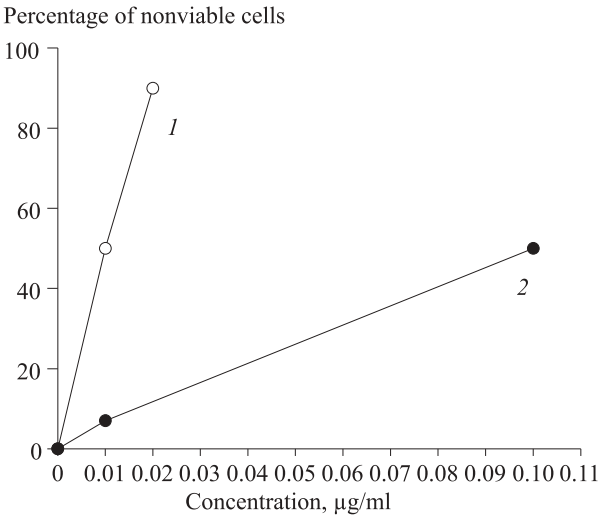

Fig. 2. Cytotoxic effect of GM on human embryo diploid fibroblasts. *1*) GM solution in DMSO; *2*) liposomal form of GM.

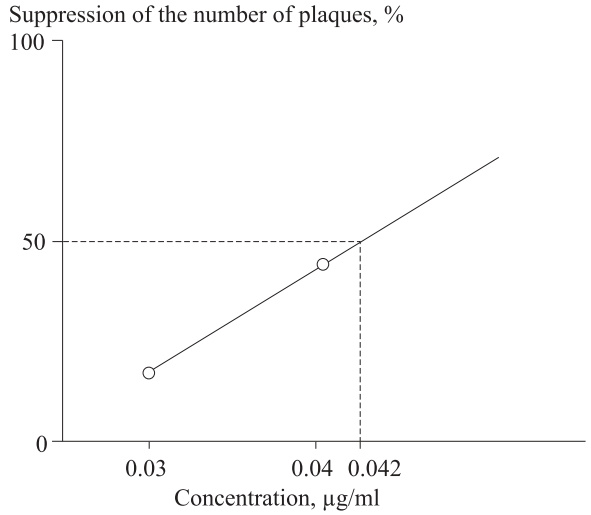

Fig. 3. Suppression of cytomegalovirus activity by GM encapsulated in monolamellar liposomes.

of all samples were liposomes consisting of yPC/cholesterol (7:3), containing 10 M% GM of the lipid content. These MLV retained a stable size (100 nm) and GM content for a long time. We investigated the toxicity of this preparation for human cells and antiviral activity for human CMV.

The cytotoxicity of two forms of GM was studied: water solution containing no more than 1% DMSO and GM encapsulated in MLV. GM solution (0.1 μg/ml) caused a 100% cytotoxic effect (caused death of all cells in the population) after 24 h. GM in concentrations of 0.005 μg/ml and lower exhibited no cytotoxic effect after 3-day exposure, CC_{50}=0.01 μg/ml (Fig. 2).

The study of the liposome form of GM showed that 100% cytotoxicity manifested at the antibiotic concentration of 1.0 μg/ml and higher; 50% cytotoxicity was observed at a concentration of 0.1 μg/ml (Fig. 2). The liposomal form of GM was by an order of magnitude less toxic for HEF cells.

GM did not suppress the plaque-forming activity of CMV after infection of HEF cells at infective multiplicity of 0.100, 0.010, and 0.001 PFU/cell. GM suppressed the viral activity at a lower concentration of the virus (infective multiplicity 10^{-4} PFU/cell). In a concentration of 0.03 μg/ml GM, encapsulated in liposomes, decreased the infective activity of CMV by 17%, in a concentration of 0.04 μg/ml by 44%; 50% suppression of plaque formation under the effect of CMV at infection multiplicity of 0.0001 PFU/cell was observed in the presence of GM liposomal form in a concentration of 0.042 μg/ml (Fig. 3).

Hence, encapsulation of GM in liposomes led to solubilization of the antibiotic in normal saline. The antibiotic incorporation depends on the lipid composition of liposomes, the maximum incorporation being 20 M% GM of the lipid content. Studies of the cytotoxicity and antiviral activity showed that the liposomal preparation of GM was 10-fold less toxic for human cells than the antibiotic solution in DMSO; the preparation exhibited antiviral activity towards CMV at a concentration of 0.042 μg/ml.

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REFERENCES

1. A. S. Dudnichenko, Yu. M. Krasnopol'skii, and V. I. Shvets, *Liposomal drug preparations in Experimental Studies and Clinical Practice* [in Russian], Kharkov (2001).
2. K. A. Vinogradova, N. P. Kirillova, Z. G. Sokolova, *et al.*, *Antibiot. Khimioter.*, **36**, No. 3, 28-37 (1991).
3. B. E. Roggo, F. Petersen, R. Delmendo, *et al.*, *J. Antibiot.* (Tokyo), **47**, 136-142 (1994).