

Liposomal formulation of 5-fluorocytosine in suicide gene therapy with cytosine deaminase – for colorectal cancer

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Abstract

It is generally accepted that successful gene therapy depends on two major factors: tumor-specific expression of a therapeutic gene and the efficient transfer of a therapeutic gene to tumor cells. For gene-directed enzyme prodrug therapy (GDEPT) involving *Escherichia coli* cytosine deaminase (CD) and 5-fluorocytosine (5-FC), several tumor-specific promoters and virus-based vectors were used. No attention whatsoever was paid to the way of 5-FC delivery to solid tumors, despite the fact that the delivery of drugs to such tumors is generally low because of their insufficient transfer from the blood. To compare the effectiveness of GDEPT with free and liposomal 5-FC, the prodrug was encapsulated in liposomes composed of dipalmitoylphosphatidylcholine (DPPC) and cholesterol (1:1). When the liposomal form of 5-FC was administered i.v., mice treated with a dose of 5 mg of liposomal 5-FC/kg body weight for 10 days, showed complete regression of transplanted tumors and complete cure was observed, whereas in animals treated with the same amounts of the free prodrug, 50% tumor regression and only insignificantly prolonged median survival were found. In summary, these results showed a remarkable enhancement of the antitumor effects of the liposomal form of 5-FC in comparison with the free prodrug. Therapy with liposomal 5-FC thus represents a new approach to achieving a high local concentration of the prodrug for suicide gene therapy using *E. coli* CD.

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1. Introduction

Colon carcinoma is one of the leading causes of mortality due to malignancy in Europe and North America [1,2]. The currently used methods to treat colon cancer, including surgical operation, chemotherapy, and radiotherapy, are not satisfactory, with

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a large number of patients dying because of metastases and recurrent disease. For these reason there is an urgent need for the development of new therapeutic approaches. One is gene-directed enzyme prodrug therapy (GDEPT), which seems to be a promising approach in treating colon carcinoma [3]. In this system of somatic gene therapy, an enzyme of non-vertebrate origin, introduced into the cancer cell, converts an essentially non-toxic drug into a cytotoxic product. *Escherichia coli* cytosine deaminase (CD) converting 5-fluorocytosine (5-FC) to 5-fluorouracil [4,5] is one of the most intensively studied examples of so-called suicide genes.

It is generally accepted that successful gene therapy based on suicide genes depends on two major factors: tumor-specific expression of the therapeutic gene to avoid adverse effects in normal tissues and an efficient transfer of suicide genes into the tumor cells [3]. For gene-directed enzyme prodrug therapy involving *E. coli* CD and 5-FC several tumor-specific promoters and virus-based vectors have been used. In the majority of studies, the CD gene under the control of a carcinoembryonic antigen (CEA) promoter and carried by adenovirus or retrovirus vectors were utilized, respectively, in the treatment of gastric [6,7] and colorectal carcinoma [8].

In the case of CD/5-FC therapy, high doses of the prodrug combined with a prolonged time of its administration can cause side effects: gastrointestinal intolerance such as nausea, vomiting, and diarrhea, as well as bone marrow depression associated with neutropenia, leucopenia, and pancytopenia. As was shown for other chemotherapeutics, one way to limit the side effects would be to encapsulate the highly concentrated prodrug solution in liposomes. Liposomes, spherules (20 nm to several μm in diameter) composed of a polar lipid bilayer entrapping a central aqueous space, are increasingly used for drug delivery to treat a variety of diseases, including cancer [9,10]. The main benefits of liposome usage are: limited side effects, increased drug circulation time (“stealth” liposomes) [11], passive (due to larger fenestration of the endothelium of capillary blood vessels surrounding tumors) [12,13] or active (immunoliposomes) [14] drug targeting, and the possibility to release the drug at specific sites of the body at a specific time (pH-sensitive, thermosensitive-, or magneto-liposomes) [15]. Several liposomal drugs have been present on the market for several years, e.g. Doxil[®], a long-circulating liposomal formulation

of doxorubicin, and AmBisome[®], a liposomal formulation of amphotericin B. Doxil[®] was reported to show less cardiotoxicity [16] and AmBisome[®] – less nephrotoxicity [17] than the free drug.

In this study we report on a liposomal formulation of 5-FC in suicide gene therapy using *E. coli* cytosine deaminase. It was shown that administering 5-FC passively encapsulated in 100-nm diameter liposomes was much more effective against colon carcinoma induced in mice in comparison with the free prodrug.

2. Materials and methods

2.1. Lipids and chemicals

Dipalmitoylphosphatidylcholine (DPPC) and cholesterol (Chol) were purchased from Northern Lipids, Canada. 5-Fluorocytosine was obtained from Sigma, St. Louis, USA.

2.2. Liposome formation and characterization

DPPC and Chol were mixed in chloroform in a 1:1 molar ratio. Then the chloroform was evaporated under a nitrogen stream and the sample was further dried under vacuum for several hours to obtain a lipid film at the bottom of the small round-bottom flask. The lipid film was hydrated in 0.9% NaCl to achieve a final lipid concentration of 10 mg/ml, and then it was heated to the 42 °C and vortexed. To obtain unilamellar liposomes, the suspension of multilamellar liposomes was extruded (10 times) through 400-nm followed by 100-nm polycarbonate filters (Nucleopore-Whatman). Vesicle sizes were determined by dynamic light scattering (ZETASIZER 5000 analyzer, Malvern Instruments, Worcs, UK). The liposomal vesicles were fluorescently labeled by mixing lipids dissolved in a starting chloroform solution with rhodamine-phosphatidylethanolamine (PE) (Molecular Probes, Eugene, Oregon, USA) at 1 mol%.

2.3. 5-Fluorocytosine encapsulation

To encapsulate 5-FC, a liposome suspension was obtained essentially as described above except that the lipid film was hydrated with 5-FC solution in 0.9% NaCl. Uncapsulated 5-FC was separated from the vesicles by ultracentrifugation at 147,000g for 40 min (4 °C). The liposomal 5-FC was prepared the day before administration. The liposomal suspension was also subjected to single extrusion through a 100-nm filter under sterile conditions in order to assure the sterility of the sample. The concentration of the encapsulated drug was assessed by measuring the absorbance at 260 nm after dissolving the liposome sample in 0.1% sodium dodecyl sulfate (SDS) in 0.1 M HCl.

2.4. Cells and cell culture

The murine colon carcinoma MC38 cell line was kindly provided by Dr. Elżbieta Pajtasz-Piasecka (Institute of Immunology and Experimental Therapy Polish Academy of Sciences, Wrocław, Poland) [18]. The cells were cultured in RPMI medium supplemented with 5% fetal calf serum (FCS, Gibco BRL, Gaithersburg, USA), 2 mM glutamine, 1% mercaptoethanol, 1% sodium pyruvate and antibiotics (Complete RPMI medium).

2.5. Transfections

Plasmid pBCMGSNeo/CD/CMV containing the deaminase cytosine gene from *E. coli* under the control of the CMV promoter was kindly provided by Prof. S. Szala (Institute of Oncology, Gliwice, Poland). Murine MC38 cells were transfected using Fugene 6 Reagent (Roche, Mannheim, Germany). The DNA [5 µg in 50 µl of OPTI-MEM (Gibco BRL)] was mixed with 15 µl of transfection reagent diluted with 85 µl of OPTI-MEM. The solution was left for 30 min at room temperature and then further diluted with 5 ml of OPTI-MEM and added to the cell culture (10⁶ cells) grown in 100-nm dishes. After 5 h of incubation, the transfection mixture was replaced with fresh complete RPMI medium containing 1 mg/ml G418 (Gibco BRL). After four weeks, the resulting cell colonies were individually transferred to 24-well culture plates and propagated. The cell clones obtained were maintained in complete RPMI medium in the presence of 0.2 mg/ml G418.

2.6. Drug sensitivity assay (SRB assay)

The sensitivities of the parental and CD-transfected cells to 5-FC were assessed by SRB assay [19]. Cells (5 × 10³/well) were seeded in 96-well plates (Saerstedt, Numbrecht, Germany) in complete RPMI medium. The next day they were incubated with increasing amounts of 5-FC for 72 h. After this time the cells were fixed by cold 0.4% sulforhodamine B (SRB, Sigma) in 1% acetic acid and the protein-bound dye was extracted with 10 mM Tris. The absorbance at 540 nm was measured in a Multiscan RC photometer (Labsystems, Helsinki, Finland). The experiment was performed in triplicate. IC₅₀ of 5-FC was calculated using a curve-fitting parameter. Data were presented as means ± SD from triplicate assays.

2.7. Animal studies

C57B1/6 male mice, 8–10 weeks old and weighting 25–32 g, were obtained from the Medical Research Center of the Polish Academy of Sciences (Warsaw, Poland) and kept under pathogen-free conditions in sterile plastic cages with free access to food and water. The animal experiments were performed according to the Interna-

tional Animal Care Convention and all experimental protocols described in this study were approved by the First Local Ethic Committee for Animal Experimentation (Wrocław, Poland). The murine colon cancer MC38 cells were harvested with the use of 0.05% trypsin/0.02% EDTA, washed two times, and resuspended in Hank's balanced salt solution (HBSS) without Mg²⁺/Ca²⁺. The single-cell suspension (1 × 10⁷ cells/200 µl) with a cell viability of over 90%, was inoculated subcutaneously (s.c.) in the flank region. Tumor volume was calculated using the formula $(a^2 \times b)/2$, where *a* is the shorter diameter in mm and *b* is the longer diameter in mm. The inhibition of tumor growth was calculated from the formula: TGI [%] (tumor growth inhibition) = $W_T/W_C \times 100 - 100\%$, where *W_T* is the average tumor volume of the treated mice and *W_C* that of the untreated control animals. Free and liposomal 5-fluorocytosine in 0.9% NaCl were administered intraperitoneally (i.p.) or intravenously (i.v.) when the tumor volume reached approx. 50 mm³ (12 days after inoculation with tumor cells).

To study the distribution of liposomes in different organs, rhodamine-labeled liposomes were administered i.v. 3-, 5-, or 7-times two-day intervals. The mice were sacrificed and tissue slides of tumor, liver, spleen and kidney samples were examined under a Zeiss Axioskope 20 fluorescence microscope with filter 02 and zoom 400.

For histological studies, the resected subcutaneous tumors and internal organs were fixed with 4% buffered formalin, processed routinely, and the preparations were stained with hemotoxylin and eosin.

2.8. Statistical analysis

The Mann–Whitney *U*-test for independent samples was used to compare the weights of tumors. The results were considered statistically significant when *p* < 0.05.

3. Results

3.1. Liposomal encapsulation of 5-FC

The lipid formulation DPPC:Chol (1:1) was chosen on the basis of the lack of toxicity of the empty liposome preparation to murine MC38 cells. The unilamellar liposomes used in this study were about 100 nm in diameter and the amount of encapsulated prodrug was 1.2–1.9 mg 5-FC/ml of liposome suspension, which corresponded to 0.12–0.19 mg 5-FC/mg of lipids.

3.2. In vitro sensitivity of MC38/7 cells transfected with *E. coli* CD to 5-FC and its liposomal formulation

Murine colon cancer MC38 cells were transfected with the BCMGSNeoCD/CMV vector containing the *E. coli* CD gene under the control of the CMV promoter and the resulting clones as well as the parental cells were incu-

bated with increasing concentrations of 5-FC for 72 h. The sensitivities of the parental and CD-transfected cells to 5-FC were assessed by the SRB assay. For further studies, the clone named MC38/7 was selected. This clone was about 10^3 times more sensitive to 5-FC than was the parental cell line (Fig. 1). When MC38/7 cells were grown in the presence of the same concentrations of 5-FC, but encapsulated in liposomes, this form of the prodrug was slightly less cytotoxic up to a concentration of 0.06 mM (Fig. 2), which shows that the DPPC: Chol (1:1) liposomes are not toxic to the cells. The ID_{50} for free 5-FC was 0.012 mM and for the liposomal formulation of 5-FC it was 0.06 mM. This effect is most probably due to the slower release of 5-FC from liposomes compared with the free prodrug. The morphology of the parental and modified cells was the same, although the proliferation rate of the CD-transfected cells was about two times higher than that of the parental line.

3.3. Effect of 5-FC liposomes on the survival of colon carcinoma-bearing mice

To compare the effectiveness of GDEPT therapy with free and liposomal 5-FC, mice were transplanted subcutaneously with MC38/7 tumor cells. When the tumor-bearing mice were injected i.p. five times every second day with 5 mg/kg body weight (b.w.) of liposomal or free 5-FC, the

growth of CD-expressing MC38/7 cells was significantly suppressed, with 38% and 35% inhibition, respectively, in comparison with untreated animals (Fig. 3). These data shows that there was no difference in antitumor efficacy between these two drug formulations. The treatment of mice with the same amounts of liposomes containing no prodrug (10 mg lipids/ml) did not reduce the growth rate of MC38/7 tumor. The antitumor effects of the liposomal and free forms of 5-FC on CD-expressing tumors were also evaluated in mice receiving the prodrug intravenously (i.v.). When 5 mg/kg b.w. of liposomal 5-FC was administered i.v. every second day for 10 days, complete regression of tumor growth was observed (Fig. 4A). In contrast, mice subjected to treatment with the same concentration of the free prodrug showed 49% inhibition of tumor growth. Again, the treatment of mice with liposomes containing no prodrug (10 mg lipids/ml) did not reduce the growth rate of MC38/7 tumor in comparison with the untreated control. It seems that the limited effectiveness of liposomal 5-FC when administered i.p. is probably due to the low release of liposomes from the peritoneal cavity

To further evaluate the antitumor effectiveness of liposomal 5-FC, mice were treated i.v. with twice lower doses of prodrug (2.5 mg/kg b.w.). After five doses of liposomal 5-FC administered every second day, only mild regression of tumor was seen. Therefore, the therapy was prolonged

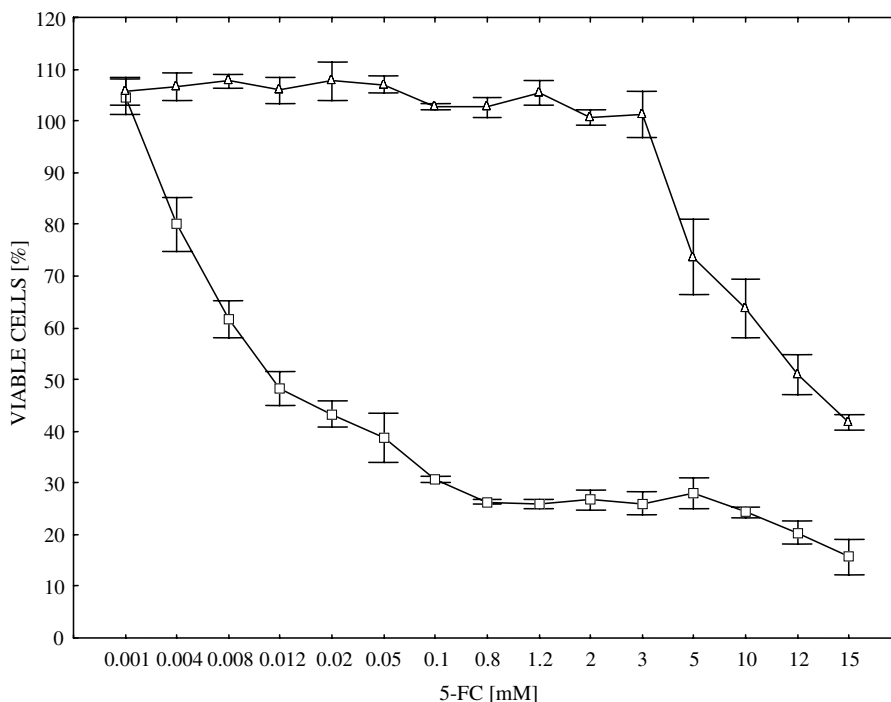


Fig. 1. IC_{50} of murine colon cancer MC38 cells transfected with *E. coli* CD gene. Cancer cells were grown in the presence of increasing concentrations of 5-FC for 72 h. Cell growth was assessed by the SRB assay. \square , MC38/7 cells expressing *E. coli* CD; \triangle , parental MC38 cells.

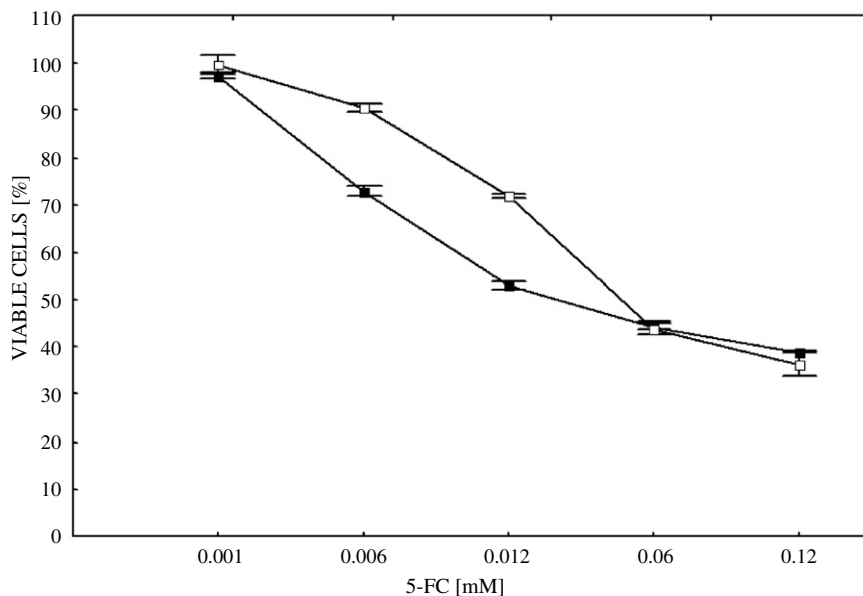


Fig. 2. Cytotoxicity of free and liposomal 5-FC to murine colon cancer MC38/7 cells transfected with *E. coli* CD gene. Cancer cells were grown in the presence of increasing concentrations of free (■) and liposomal formulation (□) of 5-FC for 72 h. Cell growth was assessed by the SRB assay.

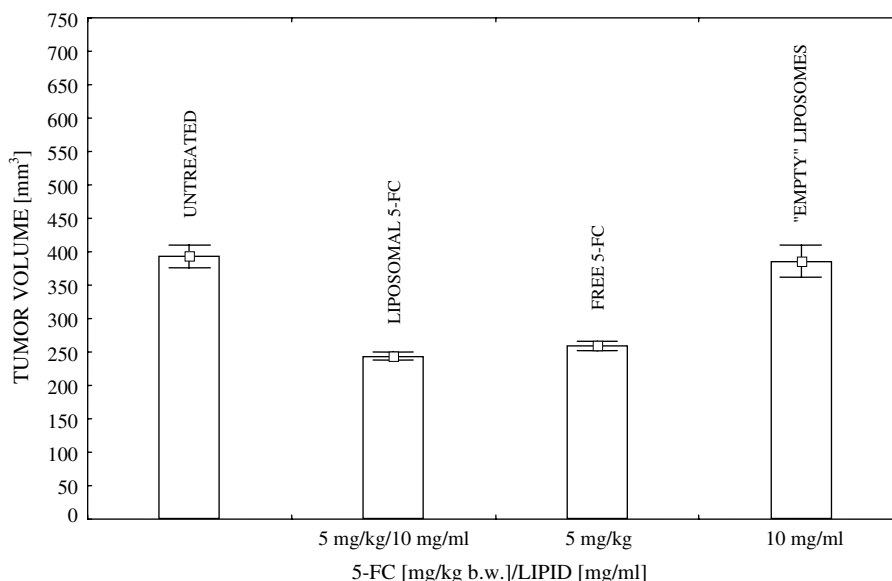


Fig. 3. Therapeutic effect of intraperitoneal administration of the free and liposomal forms of 5-FC on MC38 tumor growth. Mice were transplanted s.c. with colon carcinoma MC38/7 cells expressing *E. coli* CD. When tumor volume was about 50 mm³ (12 days after colon cancer cells transplantation), they were treated i.p. every second day for 10 days (5×) with free or liposomal 5-FC in a dose of 5 mg/kg body weight or with the same amounts of liposomes containing no prodrug (10 mg lipids/ml). At least six animals were in each experimental group and seven in the control group. Tumor volumes were calculated every second day and are presented as means, $n = 6-7$. $p < 0.005$ for free 5-FC and $p < 0.001$ for liposomal 5-FC compared with the untreated group (Mann–Whitney). The data represent tumor volumes two days after the last administration of 5-FC.

10 more days with the same doses of liposomal 5-FC. With such an extended period of treatment, tumor volume was reduced by 50% (Fig. 4B). With the same dose of free

5-FC administered i.v. and with a similar treatment regime, no visible effect on tumor growth was observed. In a control experiment, when parental MC38 tumor

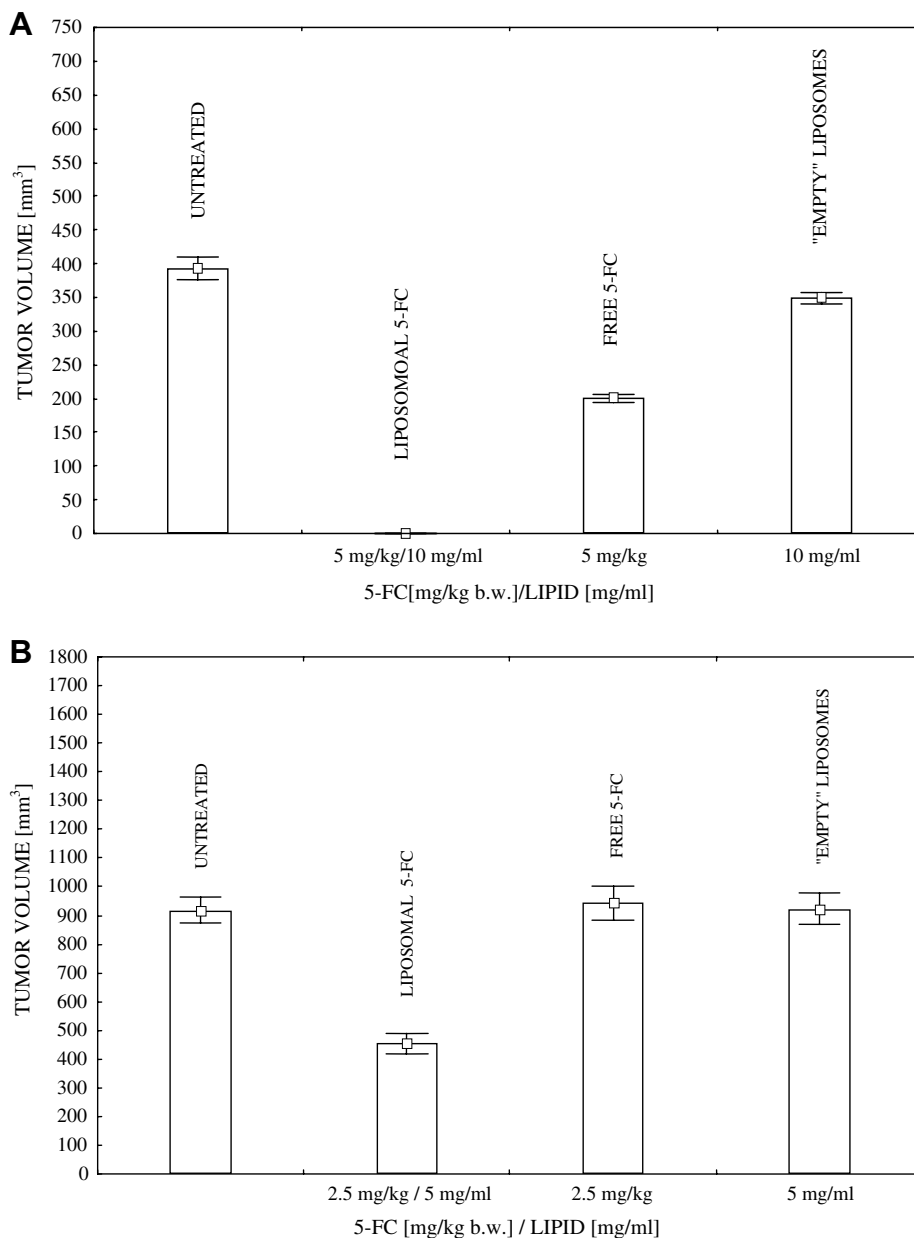


Fig. 4. Therapeutic effect of intravenous administration of the free and liposomal forms of 5-FC on MC38 tumor growth. Mice were transplanted s.c. with colon carcinoma MC38/7 cells expressing *E. coli* CD. When the tumor volume was about 50 mm³ (12 days after colon cancer cells transplantation), they were treated i.v.: (A) with free or liposomal 5-FC in a dose of 5 mg/kg body weight or with the same amounts of liposomes containing no prodrug (10 mg lipids/ml) every second day for 10 days (5×), (B) with free and liposomal 5-FC in a dose of 2.5 mg/kg body weight or with the same amounts of no prodrug-containing liposomes (10 mg lipid/ml) every second day for 20 days (10×). At least six animals were in each experimental group and seven in the control group. Tumor volumes were calculated every second day and are presented as means, $n = 6-7$. $p < 0.005$ for free and liposomal 5-FC (A) and $p < 0.01$ for liposomal 5-FC (B) compared with the untreated group (Mann–Whitney). The data represent tumor volumes two days after the last administration of 5-FC.

not-expressing CD was subjected to therapy with liposomal or free 5-FC administered i.v., no visible effect on tumor growth was found (data not shown).

The therapeutic efficacy of the liposomal prodrug formula was assessed by observation of the survival times of mice bearing MC38/7 tumors (Fig. 5). Complete cure

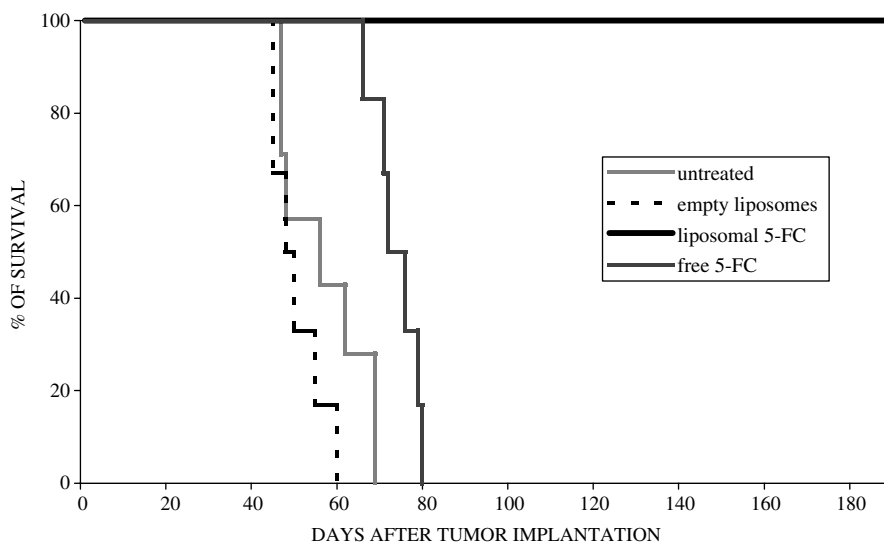


Fig. 5. Long-term survival of tumor-bearing mice after intravenous treatment with the free and liposomal forms of 5-FC. Tumor-bearing mice were treated with free and liposomal 5-FC as described in Fig. 3A. At least six animals were in each experimental group and seven in the control group.

from tumor was observed in mice treated with 5 mg/kg b.w. of the liposomal form of 5-FC administered i.v. No tumors were found in such animals after eight months of observation. Tumor-bearing mice treated with the same doses of free 5-FC had survival prolongation which was not very different (74 days) from those of mice treated with “empty” liposomes (51 days) or untreated tumor-bearing control animals (57 days).

3.4. Distribution of liposomes in different organs

The distribution of rhodamine liposomes in different organs after i.v. administration was analyzed using fluorescence microscopy. Accumulation of liposomes was found in each of the studied organs (liver, kidney, and spleen) as well as in the tumor tissues (data not shown), as observed by others [20]. However, in contrast to tumor, there was a decrease in the fluorescence intensity after the fifth dose of liposomes. This is probably a result of saturation of RES (reticular endothelial system) macrophages with liposomes in these organs and the slow degradation of the lipids. In addition, liposomes which are not trapped by the RES are free to pass out of the liver, spleen and kidney, in contrast to tumor tissue, where they are effectively arrested [20].

3.5. Toxicity of liposomal 5-FC

The toxicity of liposomal 5-FC was evaluated by histochemical staining of tissue sections from several organs, using hematoxylin and eosin. Microscopic examination

of the livers, spleens, hearts and kidneys revealed no apparent differences between control mice and animals treated with the liposomal 5-FC.

4. Discussion

Successful tumor gene therapy, including of colorectal cancer, is dependent on two major factors: (i) effective transfer and (ii) cell-specific expression of therapeutic genes [3,21]. Based on these assumptions, many efforts have been undertaken in CD/5-FC therapy on the use of cell-specific promoters to express *E. coli* CD only in cancer cells and the development of highly effective gene carriers [6–8]. However, no attention has been paid to the way of 5-FC delivery to tumor sites despite the fact that drug delivery to solid tumors is generally low because of their insufficient transfer from the blood. The high local concentration of 5-FC seems to be an important issue because many transfected cells express *E. coli* CD at low or very low levels when the suicide gene is under the control of a widely used, tumor-specific CEA promoter [22]. To overcome such problems, high doses of antitumor agent could be used, but relatively non-toxic 5-FC is known for its side effects, especially when used for a long time [23]. One way to limit the side effects would be to encapsulate the highly concentrated prodrug solution in liposomes.

To compare the effectiveness of GDEPT therapy with free and liposomal 5-FC, the prodrug was encapsulated in liposomes composed of DPPC and cholesterol (1:1). This formulation was chosen because it was not toxic to murine colon cancer MC38 cells and encapsulated relatively high amounts of the prodrug in comparison with other formulas. To evaluate the *in vivo* antitumor effects of 5-FC, mice were injected s.c. with colon carcinoma MC38/7 cells expressing *E. coli* CD and then subjected to treatment with the prodrug. When 5-FC was administered i.v. mice treated with a dose of 5 mg of liposomal 5-FC/kg b.w. for 10 days showed complete regression of transplanted tumors, whereas animals treated with the same amounts of free prodrug showed only 50% tumor regression. To study the *in vivo* therapeutic efficacy of the liposomal prodrug formula, prolongation of survival was analyzed. When the liposomal form of 5-FC was administered i.v. complete cure was observed, but only an insignificantly prolonged median survival of 74 days was found when the free prodrug was inoculated i.v.

It should be noted that the application of the DPPC/Chol liposome as a potential carrier of 5-FC is first approach to carrier construction. Surface-unmodified liposomes, in particular MLV's (multilamellar vesicles), are used rather seldom as drug carriers as they are short-lived in the circulation being subject to RES (reticular endothelial system)-macrophage attack. They have also been reported to have side effects [24]. On the other hand, one of them, Ambisome[®], a single-bilayer liposome-based commercial antifungal drug less than 100 nm in diameter, was reported to be used in hematology patients with invasive fungal infections and was found to be effective and well tolerated [25]. Surface-modified (stealth) liposomes are usually used to deliver antitumor drugs, primarily to achieve increased stability and prolonged blood circulation with limited side effects of the drug [11]. An example of a widely used commercial preparation is Doxil[®], which, in addition to extended stability in the circulation, is characterized by decreased cardiotoxicity [16]. Others report limited hepatotoxicity of drugs encapsulated in surface-modified liposomes (e.g. [26]). Further modifications with either specific antibodies [14] or specific ligands for cellular receptors, e.g. transferrin or RGD peptides [27,28], could be employed. Therefore, further studies on liposomal formulation(s) of 5FC will be necessary to further optimize the appropriate drug carrier.

The highly increased therapeutic efficacy of the liposomal 5-FC formula is most probably associated with the unusual permeability of tumor vessels. Functional pore sizes as large as 2 µm were found in tumor vessels using colloidal carbon and stealth liposomes [12,13,29]. It was further shown by light and electron microscopy that the unusual leakiness of tumor vessels can be explained by the abnormal shapes and loose interconnections of endothelial cells with intercellular openings of a mean diameter of 1.7 [30]. Our results showing the accumulation of rhodamine-labeled liposomes in tumor tissues are in accordance with the data obtained by the last-cited authors. It should be mentioned that the accumulation of liposomes not only in tumor tissues, but also in other organs, e.g. liver, spleen, and kidney, was not associated with side effects or apparent damage to normal tissues.

In summary, our results showed remarkable enhancement of antitumor effects by the liposomal form of 5-FC in comparison with the free prodrug. Therapy with liposomal 5-FC represents a new approach to achieving a high local concentration of prodrug for suicide gene therapy using *E. coli* deaminase cytosine.

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