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Folate-associated lipoplexes mediate efficient gene delivery and potent antitumoral activity in vitro and in vivo

Sónia Duarte[a], Henrique Faneca[a], Maria C. Pedroso de Lima[a,b,*]

[a] CNC - Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal
[b] Department of Life Sciences, University of Coimbra, Coimbra, Portugal

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ABSTRACT

The lack of suitable vectors for efficient nucleic acid delivery into target cells represents a major hurdle for the successful application of gene therapy. Cationic liposomes exhibit attractive features for gene delivery, but their efficacy is still unsatisfactory, particularly for in vivo applications, which justifies the drive to further improve their performance by developing novel and efficient formulations. In the present study, we generated a new formulation of lipoplexes through electrostatic association of folate (FA) to 1-palmitoyl-2-oleoyl-sn-glycero-3-ethylphosphocholine (EPOPC):cholesterol (Chol) liposomes, prepared at different lipid/DNA charge ratios, and explored their potential to mediate gene delivery. The optimal FA-lipoplex formulation was evaluated for its efficacy to mediate antitumoral activity upon application of HSV-tk suicide gene therapy, both in vitro and in an animal model of oral cancer. Our results demonstrate that FA-EPOPC:Chol/DNA lipoplexes were able to promote a great enhancement of transfection and high in vitro antitumoral activity compared to plain lipoplexes in two different cancer cell lines. Most importantly, a considerable reduction of tumor growth was achieved with the developed FA-lipoplexes as compared to that observed for control FA-lipoplexes or plain lipoplexes. Overall, our study shows that FA-EPOPC:Chol/DNA lipoplexes constitute a promising system for the successful application of suicide gene therapy aiming at treating solid tumors.

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1. Introduction

Gene therapy holds a great potential as a therapeutic modality for the treatment of neoplastic diseases, appearing as a promising alternative to the current cancer therapies (chemotherapy, radiotherapy and surgery), as reflected by the significant number of clinical trials reported towards this pathological condition (Cavazzana-Calvo et al., 2004; Hughes, 2004; Edelstein et al., 2007). Different approaches have been explored aiming at cancer gene therapy, including correction of cell cycle defects, originated by mutations in the sequence of tumor suppressor genes, their loss or activation of oncogenes; enhancement of the immune response against tumor cells; targeted lysis of tumor cells using selective replicative viruses; anti-angiogenic and suicide gene therapies (Vassaux and Martin-Duque, 2004; Luo et al., 2011; Xiong et al., 2011).

The prodrug/suicide gene approach consists in the introduction of a viral or a bacterial gene into cancer cells, allowing the expression of an enzyme able to convert a non-toxic compound into a lethal drug. Herpes Simplex Virus thymidine kinase gene (HSV-tk) is one of the most promising "suicide" genes and ganciclovir (GCV) is recognized as its respective prodrug. The expression of the HSV-tk gene leads to the production of viral thymidine kinase that metabolizes GCV to ganciclovir monophosphate. Cellular kinases then convert monophosphorylated GCV into ganciclovir triphosphate. As the latter compound is an analogue of deoxyguanosine triphosphate, inhibiton of DNA polymerase and/or incorporation into DNA occur causing chain termination and tumoral cell death (Balfour, 1999; Ketola et al., 2004; Wang et al., 2004; Huang et al., 2010). The HSV-tk/GCV gene therapy system is associated with a crucial element, the so-called "bystander effect", in which a high percentage of tumoral cell death can occur even when only a low percentage of cells have been transfected (Floeth et al., 2001; Faneca et al., 2007; Chang et al., 2010). In an attempt to explain the killing of non-transfected cells, several hypotheses have been proposed (i) passage of the drug through gap junctions (Huang et al., 2010), (ii) endocytosis of apoptotic vesicles (Freeman et al., 1993)

Abbreviations: Chol, cholesterol; EPOPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-ethylphosphocholine; FA, folate; SCC-VII, murine oral squamous cell carcinoma; TSA, mouse mammary adenocarcinoma; VGCV, valganciclovir.

* Corresponding author at: Department of Life Sciences, Faculty of Science and Technology, University of Coimbra, Apartado 3046, 3001-401 Coimbra, Portugal. Tel.: +351 239 104397; fax: +351 239 855789.
E-mail address: mdelima@ci.uc.pt (M.C.P.d. Lima).

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or (iii) stimulation of the immune system in vivo (Ramesh et al., 1996).

Poor efficiency of the delivery systems constitutes one of the major difficulties for the success of cancer gene therapy. Although, viral vectors are still the vectors of choice for efficient gene expression, safety concerns inherent to their application have dampened the enthusiasm for their use in clinical trials. Non-viral vectors, such as cationic liposomes have emerged as promising nucleic acid delivery systems due to their low toxicity and immunogenicity, lack of pathogenicity and versatility. An increasing number of clinical trials using cationic liposomes have been reported, particularly towards cancer diseases (Edelstein et al., 2007), illustrating their potential as viable alternatives to viral vectors in cancer gene therapy. Nevertheless, cationic liposomes suffer from relatively low levels of gene delivery and gene expression as compared to viral vectors, thus the drive to improve these vectors continues. The association of molecules such as proteins, peptides and other ligands to the lipoplexes constitutes one of the different strategies that have been successfully explored aiming at promoting an increase in the biological activity (Faneca et al., 2004; Trabulo et al., 2008, 2010). In our recent work, we have shown that association of folic acid (FA) to DOTAP:Chol/DNA lipoplexes improved significantly transfection in different cancer cell lines even in the presence of serum (Duarte et al., 2011). These promising results and all the features exhibited by FA, like high cell-binding affinity, low immunogenicity, ease of modification, small size, stability during storage and low cost (Reddy and Low, 1998), justify further development of FA-containing lipoplex formulations aiming at their successful application in cancer gene therapy. On the other hand, the application of suicide gene therapy, which benefits from the bystander effect, may help to overcome the low transfection efficiency usually associated with the use of lipoplexes. An additional advantage of HSV-tk/GCV gene therapy is that it selectively targets actively dividing cells (Xi and Grandis, 2003).

In the present study, we investigated the potential of a new lipoplex formulation, generated by electrostactic association of FA to EPOPC:Chol liposomes followed by complexation with plasmid DNA, in the HSV-tk/GCV suicide gene therapy in vitro and in an in vivo model of oral cancer.

Our results demonstrate that electrostatic association of FA to EPOPC:Chol/DNA lipoplexes resulted in a great potentiation of the biological activity in two different cell lines from solid tumors. When applying HSV-tk/GCV suicide gene therapy strategy, we found that FA-associated lipoplexes were highly efficient in promoting cell death and reduction of tumor growth in an animal model of oral cancer.

2. Materials and methods

2.1. Cells

TSA (mouse mammary adenocarcinoma) and SCC-VII cells (murine oral squamous cell carcinoma) were maintained at 37 °C, under 5% CO_2, in Dulbecco's modified Eagle's medium-high glucose (DMEM-HG) (Sigma) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Sigma), penicillin (100 U/ml) and streptomycin (100 μg/ml) and sodium bicarbonate (1.6 g/l).

2.2. Preparation of cationic liposomes and their complexes with DNA

Small unilamellar cationic liposomes (SUV) were prepared from a 1:1 (mol ratio) mixture of 1-palmitoyl-2-oleoyl-sn-glycero-3-ethylphosphocholine (EPOPC) and cholesterol (Chol) or 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP) and Chol, by extrusion of multilamellar liposomes (MLV). Briefly, lipids (Avanti Polar Lipids, Alabaster, AL) dissolved in $CHCl_3$ were mixed at the desired molar ratio and dried under vacuum in a rotatory evaporator. The dried lipid films were hydrated with 1.0 ml high purity water and the resulting MLV were then sonicated, for 3 min, and extruded, 21 times, through two stacked polycarbonate filters of 50 nm pore diameter using a Liposofast device (Avestin, Toronto, Canada). The resulting liposomes (SUV) were then diluted three times with deionized water and filter-sterilized utilizing 0.22 μm pore diameter filters (Schleicher & Schuell). The lipid concentration of the resulting SUVs was determined by quantification of cholesterol using the Liebermann–Burchard reagent.

Lipoplexes were prepared by sequentially mixing a Hepes-buffered saline solution (HBS) (100 mM NaCl, 20 mM HEPES, pH 7.4), with liposomes (volume was dependent on the (+/−) lipid/DNA charge ratio) and with 1 μg of plasmid DNA solution of pCMVluc (VR-1216; a gift of Dr. P. Felgner, Vical, San Diego, CA), pCMVegfp-c1 (Clontech, CA, USA) or pCMVtk (National Gene Vector Laboratory at the University of Michigan (Ann Arbor, MI, USA) in a final volume of 200 μl. The mixture was further incubated for 15 min at room temperature. The lipid/DNA charge ratio was calculated on the basis that 1 μg of DNA corresponds to 3.03 nmol of negative charges. For the preparation of lipoplexes at a charge ratio (+/−) of 1/1, 2.1 μg of DOTAP or 2.5 μg EPOPC were used.

Folate-associated lipoplexes (FA-associated lipoplexes) were prepared by pre-incubating the liposomes (the amount required for achieving the 2/1 or 4/1 lipid/DNA (+/−) charge ratios) with different amounts of FA (0–100 μg of FA/1 μg of DNA) (Sigma) in HBS solution for 15 min, followed by a further 15 min incubation at room temperature, with 1 μg of plasmid DNA solution of pCMVluc, pCMVefgp-c1 or pCMVtk in a final volume of 200 μl.

2.3. Transfection activity

For luciferase activity experiments, 0.5×10^5 TSA cells/well were seeded onto 48-well culture plates, 24 h prior to incubation with lipoplexes. Cells were used at 50–70% confluence and 200 μl of lipoplexes with or without FA containing 1 μg of pCMVluc plasmid DNA were added to cells, which were previously covered with 300 μl DMEM-HG (without serum, unless indicated otherwise). For flow cytometry experiments, 1.2×10^5 of TSA and SCC-VII cells/well were seeded onto 12-well plates and, after 24 h 200 μl of lipoplexes with or without FA containing 3 μg of pCMVegfp-c1, encoding the green fluorescent protein, were added to cells previously covered with 800 μl DMEM-HG (without serum, unless indicated otherwise). After 4 h incubation (in 5% CO_2 at 37 °C), the medium was replaced with DMEM-HG containing 10% FBS, and the cells were further incubated for 48 h. The transfection efficiency mediated by the different lipoplexes was evaluated by analyzing luciferase expression by luminescence measurements and GFP expression by flow cytometry. The quantification of luciferase expression in cell lysates was evaluated by measuring light production by luciferase in a luminometer (Lmax II384; Molecular Devices, CA, USA). Forty-eight hours post-transfection, the cells were washed twice with PBS and lysis buffer [1 mM dithiothreitol; 1 mM ethylenediaminetetraacetic acid; 25 mM Tris-phosphate (pH 7.8); 8 mM $MgCl_2$; 15% glycerol; 1% (v/v) Triton X-100] was added to each well. The protein content of the lysates was measured by the DC Protein Assay reagent (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard. The data were expressed as RLU of luciferase per mg of total cell protein. For flow cytometry analysis of GFP expression, 48 h post-transfection, the cells were washed once with PBS and detached with trypsin (10 min at 37 °C). The cells were then further washed, resuspended in PBS, and immediately analyzed. Flow cytometry analysis was performed

in live cells, using a Becton Dickinson (NJ, USA) FACSCalibur flow cytometer. Live cells were gated by forward/side scattering from a total of 25,000 events and data were analyzed using CellQuest software.

2.4. Cell viability studies

Cell viability under the different experimental conditions was assessed, in parallel experiments, by a modified Alamar blue assay. Forty-seven hours post-transfection, the cells were incubated with DMEM containing 10% (v/v) Alamar Blue dye. After a 1 h incubation period at 37 °C, the absorbance of the medium was measured at 570 nm and 600 nm. Cell viability was calculated, as a percentage of the non-transfected control cells, according:

$$\text{Cell viability (\% of control)} = [(A_{570} - A_{600}) \text{ of treated cells} \times 100/(A_{570} - A_{600}) \text{ of control cells}]$$

2.5. Intracellular distribution of FA-lipoplexes

TSA cells (4×10^4 cells/well) were plated in 8-well chambered coverslips (Lab-Tek™ II Chamber Slide™ System Nunc™). To observe the intracellular distribution of the complexes, following overnight culture, TSA cells were incubated with the FA (80 μg)-EPOPC:Chol liposomes labeled with 0.1% carboxyfluorescein-PE and complexed with 1 μg of pCMVluc at the (2/1) (+/−) charge ratio in DMEM-HG without serum for 4 h. Following incubation, cells were rinsed with PBS and incubated, during 20 min at 37 °C, with 50 nM LysoTracker Red DND-99 (Molecular Probes), which labels acidic compartments of live cells. Cells were then rinsed with PBS and directly observed in the chambers in 0.3 ml of OptiMEM. Fluorescence distribution inside cells was analyzed under a LSM-510 META, Zeiss confocal microscope without fixation, under the 63× oil immersion objective.

2.6. Competitive inhibition studies and effect of chloroquine on transfection

To elucidate the cellular mechanisms of gene delivery by FA-associated lipoplexes, the following experiments were designed: (1) An excess of FA (2 mM) was added to TSA cells in a final volume of 800 μl and incubated for 30 min at 37 °C before the addition of EPOPC:Chol/DNA lipoplexes or FA (80 μg)-EPOPC:Chol/DNA lipoplexes, which were incubated with the cells for 4 h always in the presence of an excess of FA. The medium was then replaced with DMEM-HG containing 10% FBS and 2 mM of FA, and the cells were further incubated for 48 h before being harvested for luciferase activity measurements. (2) TSA cells were incubated with 800 μl of 0.1 mM chloroquine (CHL), a known endosomolytic agent, during the period of transfection in the presence of EPOPC:Chol/DNA lipoplexes or FA (80 μg)-EPOPC:Chol/DNA lipoplexes, followed by a further 48 h incubation in complete DMEM medium containing 10% FBS and 0.1 mM CHL before evaluation of luciferase activity. The viability of the cells transfected in the presence of CHL was evaluated and compared to that of untreated control cells using the Alamar blue assay.

2.7. In vitro antitumoral activity

The in vitro antitumoral activity mediated by non-viral HSV-tk/GCV gene therapy was evaluated in TSA and SCC-VII cells. Since the production of ganciclovir was discontinued, its prodrug valganciclovir (VGCV), an L-valyl ester of GCV, was used. For these experiments, 0.5×10^5 TSA and 0.4×10^5 SCC-VII cells/well were seeded onto 48-well culture plates for 24 h prior to incubation with lipoplexes. Following 4 h incubation of these cell lines with EPOPC:Chol/DNA lipoplexes or FA-EPOPC:Chol/DNA lipoplexes, containing 1 μg of pCMVtk plasmid DNA encoding the therapeutic gene thymidine kinase, the medium was replaced with normal DMEM-HG. Twenty-four hours after, the medium was replaced with DMEM-HG with or without VGCV at different concentrations (100, 200 or 400 μM) and cells were further incubated for 5 days under culture conditions (5% CO_2 at 37 °C). The medium, with or without VGCV, was replaced at the 3rd day of treatment with VGCV and the cell viability was assessed at days 0, 3 and 5 of treatment with VGCV by the modified Alamar blue assay as referred above.

2.8. Western blot analysis

To determine the basal expression of connexin 43, 2.5×10^5 of TSA and SCC-VII cells/well were seeded onto 6-well culture plates, for 72 h. Protein extracts were obtained from these cells using a lysis buffer containing protease inhibitors (Sigma) and sodium orthovanadate. Protein content was determined using the DC protein assay reagent from Bio-Rad and 20 μg of total protein were resuspended in loading buffer (20% glycerol, 10% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue), incubated for 4 min at 95 °C, and loaded onto a 10% polyacrylamide gel. After electrophoresis the proteins were blotted onto a PVDF membrane according to standard protocols. After blocking in 5% non-fat milk, the membrane was incubated with a mouse anti-connexin 43 antibody (1:200) (Invitrogen) overnight at 4 °C, and with the appropriate secondary antibody (1:20,000) (Amersham, Uppsala, Sweden) for 2 h at room temperature. Equal protein loading was shown by reprobing the membrane with an anti-actin antibody (1:10,000) (Sigma) and with the same secondary antibody. After this incubation period, the blots were washed several times with saline buffer (TBS/T: 25 mM Tris–HCl, 150 mM NaCl, 0.1% Tween and 5 mg/ml non-fat powder milk) and incubated with ECF (alkaline phosphatase substrate; 20 μl of ECF/cm^2 of membrane) for 5 min at room temperature and then submitted to fluorescence detection at 570 nm using a VersaDoc Imaging System Model 3000 (Bio-Rad). For each membrane, the analysis of band intensity was performed using the Quantity One software (Bio-Rad).

2.9. Analysis of the cell cycle

Cell cycle analysis was performed using flow cytometry. In these experiments, 1.2×10^5 of TSA and SCC-VII cells/well were seeded onto 12-well culture plates, and after 24 h, FA-EPOPC:Chol/DNA lipoplexes containing 3 μg of pCMVtk plasmid DNA, were added to cells and incubated for 4 h. After that, the medium was replaced with normal DMEM-HG. Twenty-four hours later, this medium was replaced again with DMEM-HG with or without VGCV (400 μM) and cells were further incubated for 2 days under culture conditions (5% CO_2 at 37 °C). The untransfected (control cells) and transfected TSA and SCC-VII cells were harvested by trypsinization, centrifuged at 1100 rpm for 20 min and fixed with cold 70% ethanol, followed by storage at 4 °C until analysis. The cells were washed twice with PBS, centrifuged at 1100 rpm for 20 min and resuspended in a solution containing 500 μl of PBS, 5 μl of RNaseA (100 μg/ml, Invitrogene) and 5 μl of propidium iodide (1 mg/ml, Invitrogene). Cells were incubated at room temperature in the dark for 1 h, and DNA content was detected by flow cytometry (Becton Dickinson FACSCalibur flow cytometer). The relative proportions of cells in the G0/G1, S, and G2/M phases of the cell cycle were determined by analysis of flow cytometry data in ModFit LT™ software.

2.10. In vivo antitumoral activity: tumor implantation and treatment

After being detached with trypsin and washed two times with PBS, SCC-VII cells were resuspended in PBS saline buffer, to obtain a final cell density of 2×10^6 cells/ml. 50 μl of this cell suspension (1×10^5 cells) were immediately injected subcutaneously in the left flank of female 6-week-old C3H/HeOuJ mice (Charles River Laboratories, Barcelona, Spain). A subcutaneous xenograft tumor was selected for our studies, since it would not face the animal morbidity problems of an orthotopic oral tumor, which interferes with alimentation of the animals and may limit their survival for the expected duration of therapy response evaluation. Since most primary and recurrent lesions in oral cancer are accessible to injection, this type of cancer is a particularly appropriate target for treatment by direct injection (Xi and Grandis, 2003). When the tumor volume reached approximately 0.025 cm^3, usually 8 days after cell injection, the animals were submitted to different treatments (four animals per group). These treatments consisted of four intratumoral administrations of 40 μl of EPOPC:Chol/DNA (+/−) (2/1) or FA-EPOPC:Chol/DNA (+/−) (2/1) lipoplexes (prepared with pCMVtk plasmid and using 40 μg of DNA/administration) at days 0, 2, 4 and 6. In two groups of animals, one treated with the FA-associated lipoplexes and the other treated with plain lipoplexes, mice were submitted to intraperitoneal administrations of VGCV (75 mg/kg) performed from the first day after the first treatment and during 10 consecutive days. Tumor growth was monitored every 3 days by measuring two perpendicular tumor diameters with a calliper. Mice were sacrificed when the tumor volume reached approximately 1.5–2 cm^3.

2.11. Statistical analysis

All data are presented as mean ± SEM. Data were analysed using the Prism software (version 5.0). Statistical significance of differences between data was evaluated by One-way ANOVA using the Tukey test. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Effect of FA on the biological activity and cytotoxicity of EPOPC:Chol/DNA lipoplexes

In our recent work (Duarte et al., 2011) we demonstrated that electrostatic association of FA to DOTAP:Chol/DNA lipoplexes results in a significant potentiation of their transfection activity. Furthermore, results from our laboratory have shown that among several cationic lipid-based formulations, DOTAP:Chol/DNA and EPOPC:Chol/DNA complexes were those exhibiting the highest levels of transfection (Faneca et al., 2004). Based on these findings, we tested the effect of FA association on the biological activity mediated by EPOPC:Chol/DNA lipoplexes, which was compared with that previously obtained for the most active DOTAP:Chol/DNA lipoplexes (FA (40 μg)-DOTAP:Chol/DNA lipoplexes). As shown in Fig. 1A, electrostatic association of FA to EPOPC:Chol/DNA lipoplexes prepared at (2/1) and (4/1) lipid/DNA (+/−) charge ratios promoted a significant increase of transfection activity in TSA cells. As observed, an increase in the amount of FA up to 80 μg resulted in a strong enhancement in the biological activity, the levels of transfection activity mediated by FA-lipoplexes being similar for both charge ratios. Addition of an amount of FA higher than 80 μg did not result in any further improvement in the luciferase expression levels (Fig. 1A). As shown in Fig. 1B, no significant toxicity was observed upon incubation of TSA cells with FA (80 μg)-EPOPC:Chol/DNA lipoplexes, prepared at the two indicated lipid/DNA (+/−) charge ratios.

Comparison of the transfection activity mediated by the best EPOPC:Chol/DNA lipoplex formulation (FA (80 μg)-EPOPC:Chol/DNA lipoplexes) with that of the most active FA-associated DOTAP:Chol/DNA lipoplexes (Duarte et al., 2011) revealed that the former were significantly more efficient (more than 2-fold) than the latter (Fig. 1C). Based on these results, FA (80 μg)-EPOPC:Chol/DNA (2/1) lipoplexes, which present a size (around 1.5 μm), were selected as the optimal formulation for performing the subsequent studies in TSA cells.

3.2. Transfection activity mediated by FA-lipoplexes in the presence of serum

Aiming at predicting the in vivo behavior of the EPOPC:Chol/DNA lipoplexes, transfection studies were performed in the presence of serum to mimick biological milieu. As observed in Fig. 2, plain lipoplexes prepared at 2/1 and 4/1 (+/−) charge ratios lost their biological activity, while FA (80 μg)-EPOPC:Chol/DNA lipoplexes remained essentially active. Although under these conditions, a decrease in the transfection activity was observed for these lipoplexes, their biological activity is higher than that for FA (40 μg)-DOTAP:Chol/DNA lipoplexes (Duarte et al., 2011).

3.3. Intracellular distribution of FA-lipoplexes

The intracellular distribution of FA (80 μg)-EPOPC:Chol/DNA (+/−) (2/1) lipoplexes was evaluated by confocal microscopy. The intracellular localization of the lipoplexes was visualized by incorporating carboxyfluorescein-PE into the liposomal membrane, while the endolysosomal pathway of TSA cells was labeled with LysoTracker Red, which labels acidic compartments in live cells. As shown in Fig. 3A, FA-EPOPC:Chol/DNA lipoplexes did not accumulate in the endolysosomal pathway, since there was no evidence of co-localization of the labeled lipoplexes with the fluorescent probe LysoTracker Red. These observations suggest that FA-associated lipoplexes were released into the cytoplasm before reaching the lysosomes, thus avoiding DNA degradation in this organelle.

3.4. Effect of excess FA and chloroquine on the transfection activity

To investigate the possible contribution of FA receptor to the uptake of FA-lipoplexes, competitive inhibition studies were performed. TSA cells were preincubated with 2 mM of FA for 30 min at 37 °C, before the incubation with lipoplexes for 4 h. The medium was then replaced with DMEM-HG containing 10% FBS and 2 mM of FA, and the cells were further incubated for 48 h. A large excess of FA in the medium, representing 10 times the amount of FA associated with lipoplexes, had no significant effect on the levels of transfection mediated by FA-lipoplexes, while improved the biological activity of plain lipoplexes (Fig. 3B).

The effect of CHL, an endosomolytical agent that prevents the acidification of the endosome lumen, on the luciferase gene expression mediated by EPOPC:Chol/DNA lipoplexes was assessed. For this purpose, transfection was performed in the presence of 100 μM CHL. As observed in Fig. 3B, in the presence of CHL, the levels of transfection mediated both by plain and FA-associated lipoplexes were decreased. Notably, the reduction in the biological activity for FA-lipoplexes was 2-fold higher than for plain lipoplexes, the levels of gene expression mediated by FA-associated lipoplexes being 5-fold lower in the presence of CHL. This difference cannot be attributed to cytotoxicity caused by CHL, since cell viability was not significantly different from that observed in the absence of this agent (data not shown).

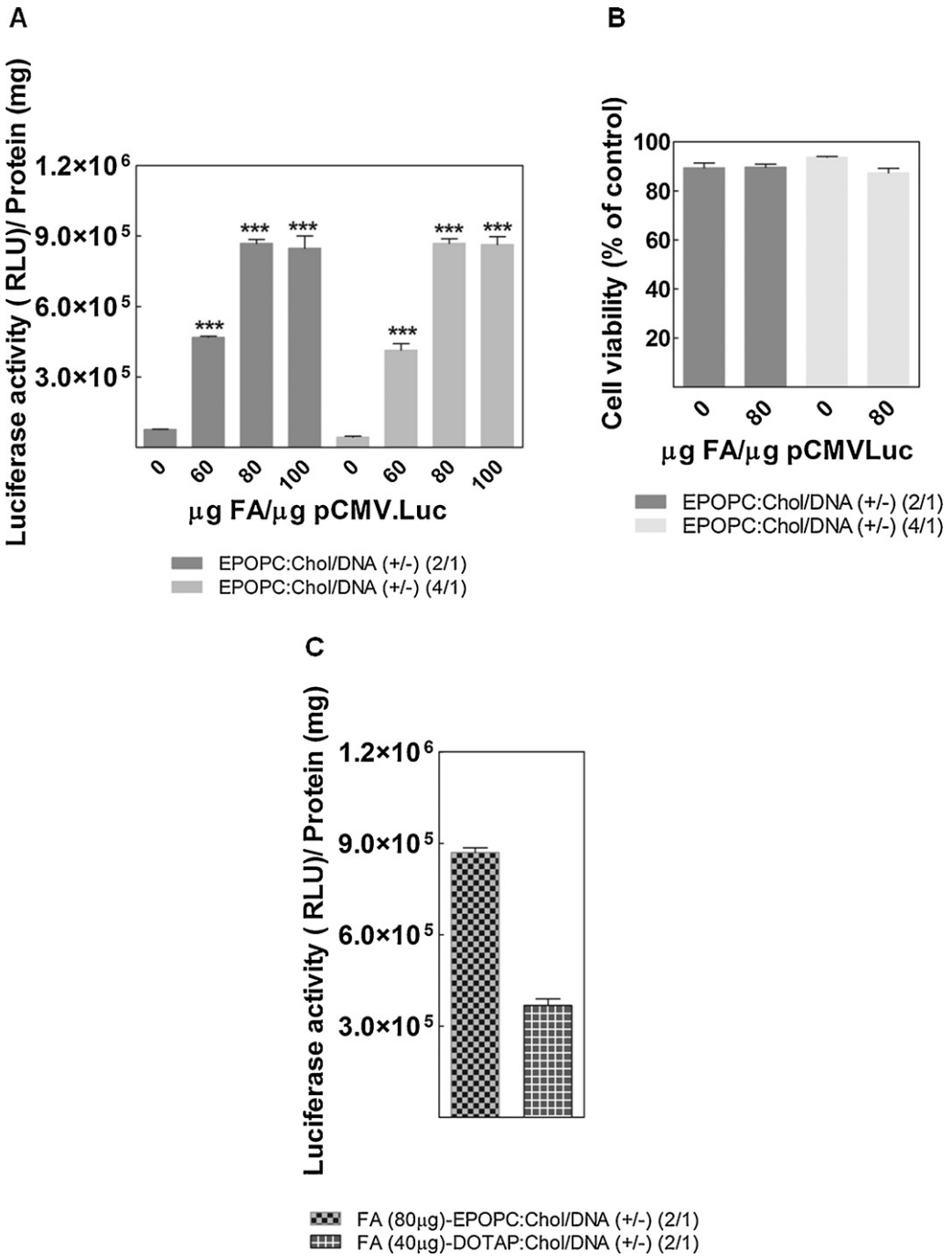

Fig. 1. Effect of electrostatic association of folate (FA) to EPOPC:Chol/DNA lipoplexes on luciferase gene expression (A) and cell viability (B). EPOPC:Chol liposomes, pre-incubated or not with FA (0 up to 100 μg/μg of DNA), were complexed with pCMVluc at the indicated theoretical lipid/DNA charge ratios and then incubated with TSA cells, as described in Section 2. (C) The results observed for the luciferase gene expression levels mediated by FA-EPOPC:Chol/DNA lipoplexes and FA-DOTAP:Chol/DNA lipoplexes in TSA cells. DOTAP:Chol liposomes, pre-incubated with 40 μg of FA, and EPOPC:Chol liposomes, pre-incubated with 80 μg of FA, were complexed with pCMVluc at the (2/1) lipid/DNA charge ratio. The data from luciferase gene expression experiments are expressed as RLU of luciferase per mg of total cell protein (mean ± SEM obtained from triplicates) and values of cell viability measured by the Alamar blue assay are expressed as the percentage of the untreated control cells (mean ± SEM obtained from triplicates). The results are representative of at least three independent experiments. Asterisks (***$p < 0.001$) indicate values that differ significantly from those measured in the absence of FA.

3.5. Antitumoral effect mediated by HSV-tk/GCV suicide gene therapy in vitro

The main goal of our work was to evaluate the therapeutic efficacy of the HSV-tk/GCV suicide gene therapy strategy mediated by FA-associated EPOPC:Chol/DNA lipoplexes in solid tumors. To this regard, we assessed the cytotoxic effect of the HSV-tk/GCV suicide gene approach mediated by FA-associated lipoplexes in TSA (derived from a mammary adenocarcinoma) and SCC-VII cells (derived from an oral carcinoma). Results from transfection studies performed in SCC-VII cells (data not shown) using FA-associated EPOPC:Chol/DNA lipoplexes revealed that FA (40 μg)-EPOPC:Chol/DNA (+/−) (2/1) lipoplex formulation, presenting a size around 1.5 μm, was the most efficient, which was therefore selected for therapeutic activity studies in these cells. Accordingly, transfection was performed with plain lipoplexes or

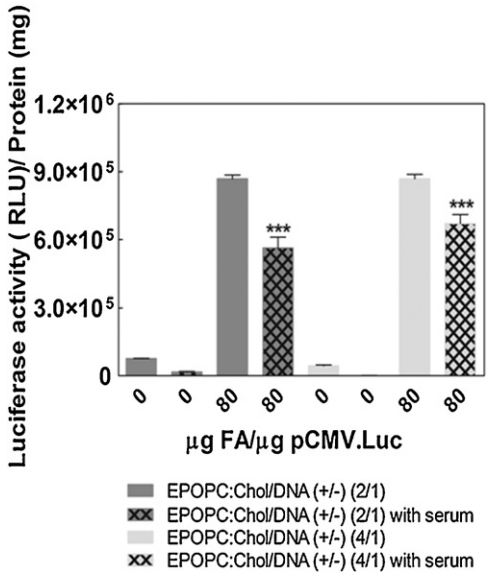

Fig. 2. Influence of serum on the biological activity mediated by FA-EPOPC:Chol/DNA lipoplexes. EPOPC:Chol/DNA lipoplexes prepared at the indicated (+/−) charge ratios, with or without electrostatically associated FA, were incubated with TSA cells in the presence or absence of 10% serum. Levels of luciferase gene expression are presented as RLU of luciferase per mg of total cell protein (mean ± SEM obtained from triplicates), and are representative of at least three independent experiments. ***$p < 0.001$ indicate values that differ significantly from those measured in the same conditions (the same lipid/DNA charge ratios (+/−) and amount of FA), but in the absence of serum.

the most efficient formulations (FA (80 μg)-EPOPC:Chol/DNA (+/−) (2/1) for TSA cells and FA (40 μg)-EPOPC:Chol/DNA (+/−) (2/1) for SCC-VII cells) containing HSV-tk gene and then incubated for different times with VGCV at various concentrations (up to 400 μM). As illustrated in Fig. 4, a decrease in cell viability was observed in HSV-tk transfected cells upon increasing VGCV concentration, the highest toxicity being observed at 400 μM VGCV for both cell lines (Fig. 4I and II). The observed cytotoxic effect was also found to be dependent on the incubation time of VGCV with transfected cells (Fig. 4IA and B, and IIA and B), the highest cytotoxicity being obtained at day 5 of incubation with VGCV (Fig. 4IC and IIC). After 5 days of treatment with 400 μM VGCV, more than 90% of cytotoxicity was observed in TSA and SCC-VII cells transfected with FA-associated lipoplexes (Fig. 4IC and IIC). The extent of cytotoxicity observed for FA-associated EPOPC:Chol/DNA was much higher than that for plain EPOPC:Chol/DNA (+/−) (2/1) lipoplexes in both cell lines (Fig. 4I and II). No significant toxicity was observed upon transfection of cells in the absence of VGCV, and incubation with VGCV per se did not exert any toxic effect on non-transfected cells. These results confirmed that the high cytotoxicity observed in transfected cells after treatment with VGCV is not due to the transfection process or to the prodrug VGCV, but rather to the therapeutic strategy.

3.6. Analysis of the connexin 43 expression

Given the difficulties to introduce a therapeutic gene in the entire cell population of the tumor, the success of the suicide gene therapy depends on a crucial phenomenon called the "bystander effect", by which the introduced gene can lead to tumoral cell death even in cells where it is absent. Transfer of the toxic metabolites of ganciclovir from the HSV-tk transfected to the non-transfected cells through gap junctions is one of the mechanisms involved in the bystander effect. The occurrence of the bystander effect was confirmed, since a high cytotoxicity (90%) was obtained for both TSA and SCC-VII cells (Fig. 4), although only 35% of TSA and 50% of SCC-VII cells were transfected with FA-associated EPOPC:Chol/DNA lipoplexes (data not shown). This small difference (35% versus 50%) in transfection efficiency was, however, associated to a large difference in the levels of transgene expression (approximately 10-fold higher for TSA cells, data not shown), as assessed by luciferase gene expression. Since the same percentage of cytotoxicity (approximately 90%) was achieved for TSA and SCC-VII cells, we investigated whether different expression levels of Cx 43 gap junctions in these cells would compensate for the higher levels of transgene expression observed in TSA cells, thus leading to a similar antitumoral effect. Fig. 5A shows a pronounced Cx 43 expression in both cell lines, this being slightly higher in SCC-VII than in TSA cells, as assessed by protein quantification analysis (Fig. 5B). Such higher connexin 43 expression associated with the more efficient transfection observed for SCC-VII cells may explain, at least partially, the same cell death effect of the therapeutic strategy in both cell lines, despite the higher biological activity found in TSA cells.

3.7. Cell cycle analysis

HSV-tk/GCV suicide gene therapy may induce alterations in the cell cycle through the incorporation of GCV into cellular DNA (Wei et al., 1998). In this work, we evaluated, by flow cytometry analysis, how HSV-tk/ganciclovir therapy would affect the cell cycle of TSA and SCC-VII cells. The percentage of cells in each phase of the cell cycle is shown in Fig. 6 for TSA cells (A and B) and for SCC-VII cells (C and D). In the absence of VGCV, both transfected and non-transfected cells exhibit a similar percentage of cells in each phase of the cell cycle (Fig. 6A and C). However, under these conditions, the percentage of G1/G0 TSA cells was lower (66%) than for SCC-VII cells (81%), while the corresponding percentage of cells in the S-phase and G2/M-phase was higher in TSA cells (21% and 13%, respectively) than in SCC-VII cells (12% and 7%, respectively). Treatment with 400 μM VGCV affected not only the proliferation of HSV-tk-transfected cells, but also that of non-transfected cells (Fig. 6A and C). In non-transfected TSA and SCC-VII cells, VGCV alone caused a reduction of the percentage of cells in G1-phase (39% and 15%, respectively), and an increase of the percentage of cells in S-phase (51% and 73%, respectively) and G2-phase (166% and 62%, respectively). A more drastic inhibition of TSA and SCC-VII cell growth was obtained when these cells were submitted to the therapeutic strategy (transfection with HSV-tk followed by treatment with VGCV). In this case, a decline of G1- and G2-cells and a large accumulation of cells in S-phase were observed in both cell lines, compared to what was found in the absence of VGCV. In TSA cells there was 54% reduction of G1/G0 cells, 2.7-fold increase of S-phase cells and 46% decrease of G2/M-phase cells, while in SCC-VII cells there was 33% reduction of G1/G0 cells, 3.7-fold increase of S-phase cells and 73% decrease of G2/M-phase cells. Although the application of the HSV-tk/VGCV suicide gene therapy resulted in different percentages of TSA and SCC-VII cells in cell cycle phases (G0/G1, S and G2/M phases), a similar change in the cell cycle phase profile was observed for both cell lines.

3.8. In vivo antitumoral effect of HSV-tk/GCV suicide gene therapy mediated by FA-lipoplexes

Following the demonstration of the in vitro efficacy of the HSV-tk/GCV suicide gene therapy mediated by FA-associated lipoplexes in TSA and SCC-VII cancer cell lines, we evaluated the therapeutic potential of this strategy in vivo using our optimal formulation (FA-EPOPC:Chol/HSV-tk (2/1) lipoplexes). For this purpose, we selected SCC-VII cells to generate a murine model for oral cancer, since being a model with a higher tumor growth rate, it would allow evaluate the efficacy of our therapeutic strategy in extreme conditions of solid tumors. This model was generated

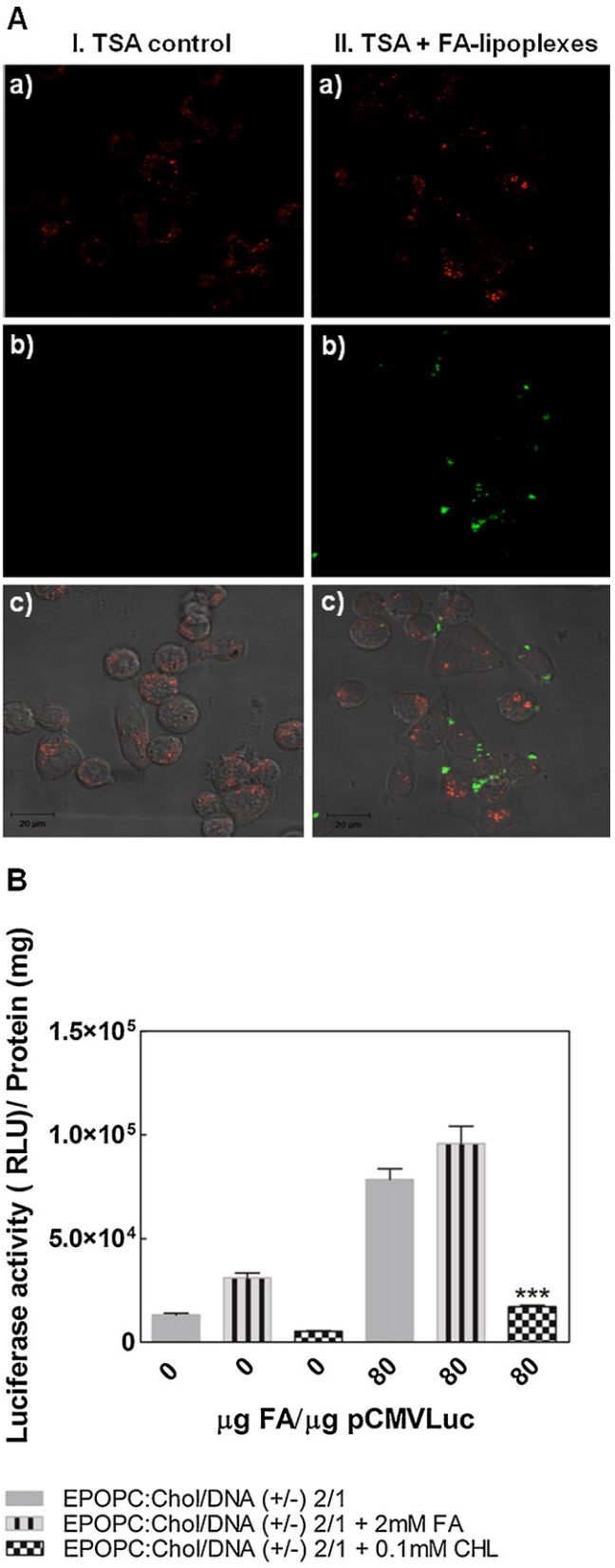

Fig. 3. Intracellular distribution of FA-EPOPC:Chol/DNA lipoplexes (A) and competitive inhibition studies and effect of chloroquine on transfection (B). Representative confocal microscopy images (63× oil immersion objective) of control TSA cells (I) and intracellular distribution of FA (80 μg)-EPOPC:Chol/DNA (+/−) (2/1) lipoplexes in TSA cells (II). TSA cells were incubated, for 4 h, with the FA-liposomes labeled with 0.1% carboxyfluorescein-PE and complexed with 1 μg of pCMVluc at the (2/1) (+/−) charge ratio in DMEM-HG without serum. Cells were then treated with 50 nM LysoTracker Red DND-99; (a) endolysosomal pathway labeled with LysoTracker Red DND-99; (b) FA-lipoplexes labeled with carboxyfluorescein-PE; (c) merging of the FA-lipoplexes and endolysosomal pathway in bright field. TSA cells were incubated with an excess of FA for 30 min at 37 °C before the incubation with EPOPC:Chol/DNA lipoplexes or FA-EPOPC:Chol/DNA lipoplexes for 4 h. The medium was then replaced with DMEM-HG containing 10% FBS and 2 mM of FA, and the cells were further incubated for 48 h. Alternatively, TSA cells were incubated with 0.1 mM chloroquine (CHL), for the 4 h of transfection in the presence of EPOPC:Chol/DNA lipoplexes or FA-EPOPC:Chol/DNA lipoplexes, followed by a further 48 h incubation in complete DMEM medium containing 10% FCS and 0.1 mM CHL. In both experiments, luciferase activity was evaluated 48 h post-transfection. Results are expressed as relative light units (RLU) per mg of protein (mean ± SEM) and are representative of three independent experiments. ***$p < 0.001$ indicate values that differ significantly from those measured in the same conditions (the same lipid/DNA charge ratios (+/−) and amount of FA), but in the absence of CHL.

by subcutaneous injection of SCC-VII cells into the mouse flank (C3H/HeOuJ). In this study we investigated whether the intratumoral delivery of HSV-tk gene mediated by FA-EPOPC:Chol/DNA lipoplexes would result in tumor cell killing and size reduction upon VGCV treatment. The applied therapy consisted of four intratumoral administrations of 40 μl of FA-EPOPC:Chol/HSV-tk (2/1) lipoplexes or HBS, performed on days 0, 2, 4 and 6 after tumor implantation. The group of animals treated with HSV-tk gene/VGCV system were submitted to ten intraperitoneal administrations of VGCV (75 mg/kg), performed from day 1 (after tumor implantation) during 10 consecutive days. Injection of FA-EPOPC:Chol/HSV-tk lipoplexes followed by VGCV treatment was significantly more effective in inhibiting tumor progression than FA-EPOPC:Chol/HSV-tk lipoplexes (in the absence of VGCV) (Fig. 7). As observed, at day 12 after initiating the treatment, a reduction of 50%, 64% and 83% in tumor size was observed in animals treated with FA-EPOPC:Chol/HSV-tk/VGCV, as compared to mice injected with plain lipolexes, FA-EPOPC:Chol/HSV-tk lipoplexes or HBS, respectively. These findings demonstrate that the suicide gene therapy strategy mediated by FA-EPOPC:Chol/HSV-tk lipoplexes resulted in a potent antitumoral effect, while showing the superiority of this formulation in the reduction of tumor size with respect to plain lipoplexes (Fig. 7).

4. Discussion

The main goal of our work was to evaluate the therapeutic efficacy of the HSV-tk/GCV suicide gene therapy strategy when mediated by a new lipid-based formulation, consisting of FA-associated EPOPC:Chol/DNA lipoplexes, in solid tumors. For this purpose, studies were performed to test the biological activity of EPOPC:Chol/DNA lipoplexes containing different amounts of FA in order to find the optimal formulation that could mediate the most efficient therapeutic effect. Similarly to what was observed in our previous studies for FA-DOTAP:Chol/DNA lipoplexes, FA association to EPOPC:Chol/DNA lipoplexes resulted in a significant increase in the transfection activity (Fig. 1A). The larger size (1.5 μm) of the most active FA-associated EPOPC:Chol/DNA lipoplexes as compared to that of plain lipoplexes can partially explain their high efficiency of transfection, as due to a more efficient cell internalization (presumably favored by a more extensive deposition of the large complexes over the cell surface), and/or to the fact that more copies of the plasmid may be carried in the larger complexes. Importantly, the new lipoplex formulation (FA-EPOPC:Chol/DNA) was considerably more efficient than that previously studied (Fig. 1C).

The inhibitory effect of serum on transfection efficiency is considered to be one of the limitations to the use of lipoplexes in vivo. Lipoplex composition and physicochemical properties are important parameters known to influence the effect of serum. The increase of cholesterol content and lipid/DNA charge ratio (+/−),

for instance, can enhance the stability of lipoplexes in the presence of serum (Faneca et al., 2002; Zhang and Anchordoquy, 2004). Although the biological activity of plain lipoplexes was significantly reduced in the presence of serum, irrespectively of the lipid/DNA charge ratio tested, the FA-associated EPOPC:Chol/DNA lipoplexes remained active in the presence of serum, thus providing evidence of their potential for in vivo applications (Fig. 2).

Endocytosis was reported to be the major pathway of cellular entry of lipoplexes (Farhood et al., 1995; Zabner et al., 1995). FA is known to interact with human cells through specific

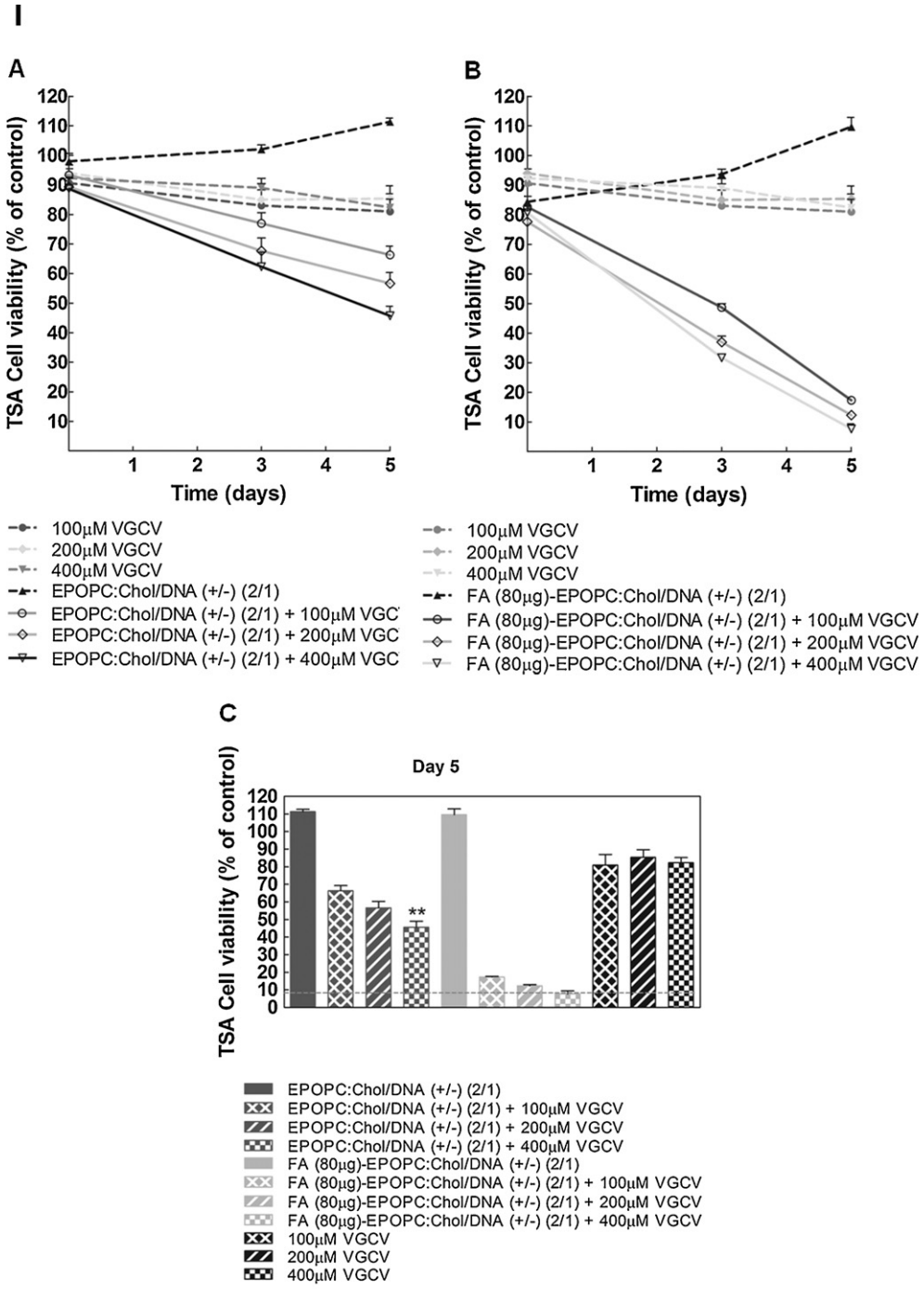

Fig. 4. Effect of valganciclovir concentration and incubation time on cell viability. TSA cells were incubated with EPOPC:Chol/DNA (+/−) (2/1) (panel IA) or FA (80 μg)-EPOPC:Chol/DNA (+/−) (2/1) lipoplexes (IB) and SCC-VII cells were incubated with EPOPC:Chol/DNA (+/−) (2/1) (panel IIA) or FA (40 μg)-EPOPC:Chol/DNA (+/−) (2/1) lipoplexes (panel IIB), containing 1 μg of pCMVtk plasmid DNA. Following 24 h, the medium was replaced with DMEM-HG or DMEM-HG containing different concentrations of valganciclovir (VGCV) (100, 200 or 400 μM). Medium was replaced again at day 3 of the treatment with VGCV. Cell viability was measured by the Alamar blue assay at days 0, 3 and 5 of treatment with VGCV. The results illustrated in panels (IC) and (IIC) correspond to the cell viability measured at the fifth day of treatment with VGCV for TSA and SCC-VII cells, respectively, following incubation with EPOPC:Chol/DNA (+/−) (2/1) lipoplexes or FA-EPOPC:Chol/DNA (+/−) (2/1) lipoplexes. The data are expressed as the percentage of the untreated control cells (mean ± SEM obtained from triplicates) and are representative of at least four independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ indicate values that differ significantly from those measured in the same conditions (the same lipoplexes), but in the presence of 100 μM of VGCV.

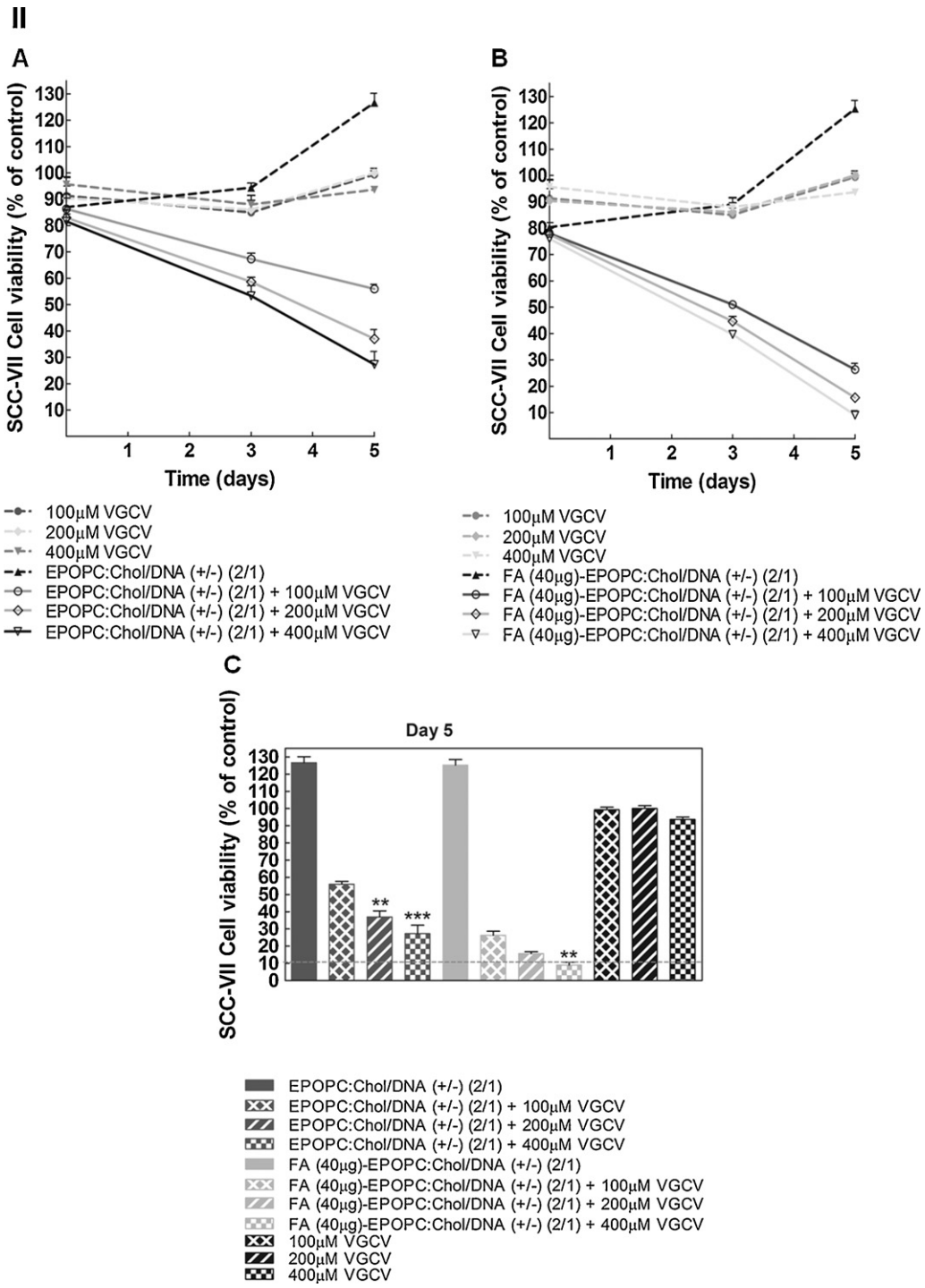

Fig. 4. (*Continued*).

receptors, which bind this molecule with high affinity (Antony, 1996). The mechanisms of transfection mediated by FA complexed to lipoplexes have not been studied. In this work we investigated whether the enhancement of gene expression mediated by FA-lipoplexes occurred via binding to a specific receptor. Our experiments showing that excess FA did not inhibit transfection provided evidence that FA receptor-mediated endocytosis was not the main mechanism contributing to the uptake of FA-EPOPC:Chol/DNA lipoplexes (Fig. 3B). These results are in agreement with those obtained from the work developed by Guo and Lee (2001), in which the authors have demonstrated that the transfection increase of PEI/DNA polyplexes promoted by folic acid is not mediated by the FA receptor. Moreover, the fact that the optimal folic acid concentration (0.2 mM), corresponding to 80 μg of FA, for transfection enhancement obtained in our studies was significantly higher than the Kd (10^{-6} mM) of folic acid for the FA receptor provided additional evidence that FA receptor is not likely involved in mediating the observed folic acid effect, as suggested for PEI/DNA polyplexes by the same authors (Guo and Lee, 2001). Therefore, although FA is not acting as a receptor ligand, it still facilitates transfection by lipoplexes, possibly by mediating endocytosis by a non-specific receptor similar to that previously observed by transferrin

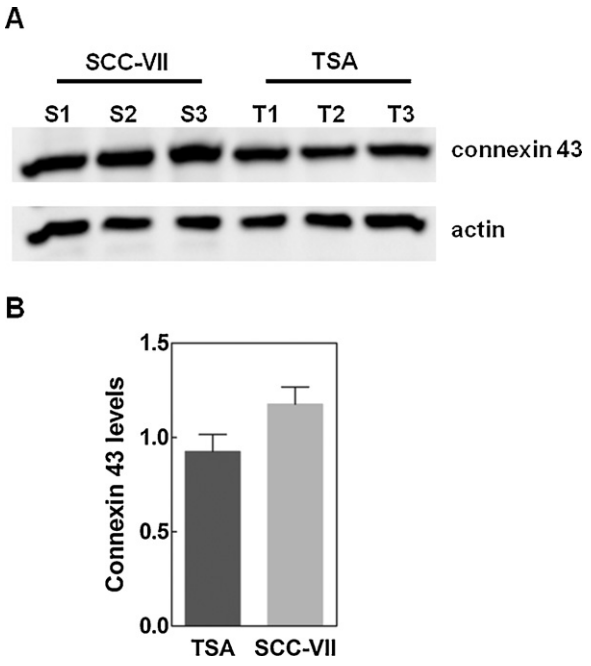

Fig. 5. Western blot quantification of connexin 43 in TSA and SCC-VII cells. TSA and SCC-VII cells were seeded onto 6-well culture plates, for 72 h. After this period, connexin 43 levels were quantified after immunoblotting of protein extracts obtained from these two cell lines. (A) Representative gel showing connexin 43 protein in TSA and SCC-VII cells and (B) levels of connexin 43 obtained by quantification of the bands shown in panel (A) corrected for individual actin signal intensity.

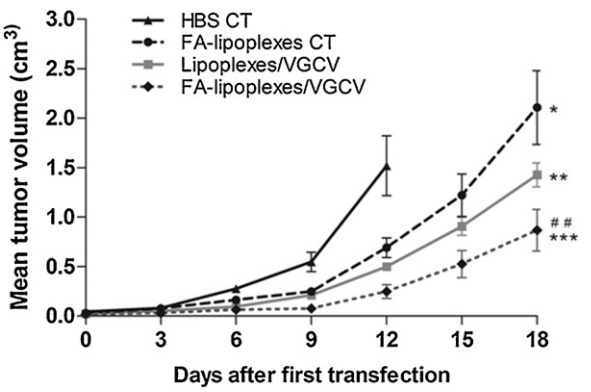

Fig. 7. Antitumor effect of HSV-tk expression followed by treatment with VGCV in a subcutaneous mouse model of oral carcinoma. Eight days after SCC-VII cell implantation, tumors were submitted to four intratumoral administrations of FA-EPOPC:Chol/DNA (+/−) 2/1 lipoplexes or plain lipoplexes (EPOPC:Chol/DNA (+/−) 2/1), both containing 40 μg of plasmid DNA coding for HSV-tk, at days 0, 2, 4 and 6. In two groups of animals, one treated with the FA-lipoplexes and the other treated with plain lipoplexes, mice were submitted to ten (75 mg/kg) intraperitoneal administrations of VGCV. Control (CT) mice were injected with HBS or with FA-lipoplexes in the absence of VGCV. Tumor growth was monitored every 3 days with callipers and mice were sacrificed when the tumor volume reached approximately 1.5–2 cm^3. Results represent the tumor size after treatment (mean ± SEM) of different experimental groups ($n = 4$). Statistical significance between experimental groups was determined by one-way ANOVA analysis. $^{***}p < 0.001$, $^{**}p < 0.01$ and $^{*}p < 0.05$ indicate values that differ significantly from those measured in the HBS control group; $^{\#\#}p < 0.01$ indicate values that differ significantly from those measured in the FA-lipoplexes control group.

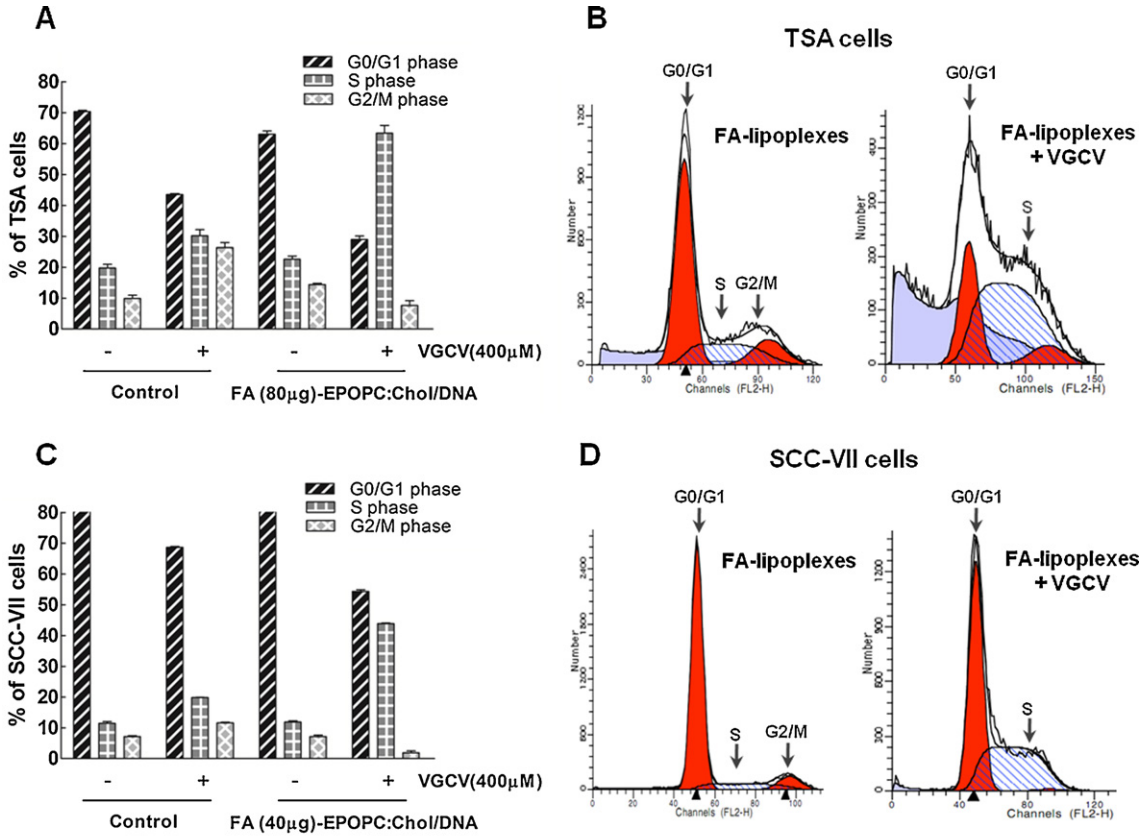

Fig. 6. Cell cycle analysis of VGCV-treated TSA and SCC-VII cells expressing HSV-tk gene. TSA and SCC-VII cells were incubated with FA-EPOPC:Chol/DNA lipoplexes (transfected cells) or HBS (control cells). After 24 h, control and transfected cells were incubated with 400 μM of VGCV for 2 days before cell cycle analysis. Results from analysis of the TSA (A) and SCC-VII (C) cell cycle distribution and representative histograms of flow cytometry of the cellular DNA content in TSA (B) and SCC-VII (D) cells. Apoptotic cells that appear in the left of G0/G1 peak are not apparent for SCC-VII cells but are very marked for TSA cells (histograms in panels B and D).

(Simoes et al., 1999). Based on our previous results (Duarte et al., 2011), we have also evidence that the internalization of FA-EPOPC:Chol/DNA lipoplexes, prepared at the 2/1 (+/−) charge ratio, is not due to the establishment of electrostatic interactions with the cell plasma membrane. Plain lipoplexes containing EPOPC prepared at a certain charge ratio exhibit the same surface charge density as DOTAP-based systems (Duarte et al., 2011), since the difference between the two systems lies on the cationic lipid which presents the same charge density. As FA confers a negative charge to the lipoplexes, as previously demonstrated by the lower zeta potential of FA-associated lipoplexes when compared to that obtained with the corresponding plain lipoplexes (Duarte et al., 2011), FA-EPOPC:Chol/DNA lipoplexes prepared at the 2/1 (+/−) charge ratio will exhibit a negative surface charge and therefore are not able to directly interact with the negatively charged cell membrane.

After internalization via endocytosis, the lipoplexes are localized in endosomes, which can either fuse with lysosomes for degradation or recycle their contents back to the cell surface. Therefore, escape from endosomes is vital for efficient transfection (Khalil et al., 2006). Our results revealed no evidence of localization of the FA-associated lipoplexes in acidic organelles, suggesting that they were released into the cytoplasm before reaching the lysosomes, thus avoiding DNA degradation in this organelle (Fig. 3A). Interference of the endocytic pathway with a lysosomotropic reagent such as chloroquine was found to decrease gene expression (Fig. 3B). These results are in agreement with those previously reported showing that this agent may decrease transfection, by inhibiting endosome acidification, which is required for the release of DNA from endosomes (Farhood et al., 1995; Khalil et al., 2006; Maitani et al., 2007). Our experiments with chloroquine indicated that acidification of endosomes plays an important role in transfection mediated by both plain and FA-lipoplexes, although the observed effect of chloroquine was more drastic for the latter than for the former. Based on the results obtained by other authors showing that cationic lipids can play a role in destabilizing the endosomal membrane, which leads to the release of lipoplexes to the cytoplasm (Hafez et al., 2001; Khalil et al., 2006), it can be suggested that transfection mediated by plain and FA-lipoplexes results from the contribution of the cationic lipid EPOPC and is highly dependent on the endosome acidification. The higher decrease in the biological activity obtained for FA-lipoplexes in the presence of chloroquine shows that not only the cationic lipid but also FA contributed to the destabilization of the endosomal membrane and subsequent release of lipoplexes and/or DNA from the endosomes. This may be attributed to the amphiphilic properties of the folic acid molecule, which bears two hydrophilic carboxyls and a hydrophobic pterine ring structure (Guo and Lee, 2001).

Similarly to what was observed in vitro using a reporter gene, the studies on the evaluation of the cytotoxic effect of the HSV-tk/GCV suicide gene approach demonstrated that FA-EPOPC:Chol/DNA (+/−)(2/1) lipoplexes were significantly more efficient in mediating an antitumoral effect than plain lipoplexes, in both TSA and SCC-VII cells (Fig. 4I and II). Such large extent of cell death is highly dependent on time of incubation and concentration of VGCV (Fig. 4IA and B, and IIA and B). After 5 days of treatment with 400 μM VGCV more than 90% of death was observed in both types of cells, showing the therapeutic potential of this strategy when mediated by FA-EPOPC:Chol/DNA lipoplexes (Fig. 4IC and IIC). HSV-tk/GCV gene therapy approach benefits from a crucial element, the so-called "bystander effect", which promotes an amplification of the cell death effect (Floeth et al., 2001; Faneca et al., 2007; Chang et al., 2010). The occurrence of this effect was confirmed since cytotoxicity was found to be close to 100% for both TSA and SCC-VII cells, thus being much higher than the percentage of transfected cells (approximately 35% for TSA and 50% for SCC-VII cells). This is due to the transfer of toxic GCV metabolites produced in transfected cells to non-transfected cells through gap junctions and/or apoptotic vesicles (Freeman et al., 1993; Colombo et al., 1995; Elshami et al., 1996; Denning and Pitts, 1997; Touraine et al., 1998), leading to death not only of transfected cells but also of neighboring non-transfected cells. Recent studies reported an enhanced bystander effect of HSV-tk/GCV suicide gene therapy upon introduction of bone marrow-derived stem cells (BMSCs) expressing HSV-tk combined with overexpression of the gap junction connexin 43 (Cx 43) into glioma cells (Huang et al., 2010). Evaluation of the basal expression of the gap junction Cx 43 showed a pronounced Cx 43 expression in both cell lines, although slightly higher expression levels were observed in SCC-VII cells than in TSA cells (Fig. 5). Our finding of the same percentage of death in both TSA cells and SCC-VII cells, despite the higher transfection activity mediated by FA-lipoplexes observed in TSA cells, may be explained by both the slightly higher percentage of transfected SCC-VII cells and the more extensive Cx 43 expression found in these cells.

In this study, we investigated the effect of the HSV-tk/GCV strategy mediated by FA-EPOPC:Chol/DNA lipoplexes on the TSA and SCC-VII cell cycle. A drastic inhibition of TSA and SCC-VII cell growth was achieved, as clearly shown by the decline of G1- and G2-cells and the large accumulation of cells in S-phase observed in both cell lines (Fig. 6), which is most likely attributed to inhibition of cellular DNA polymerases and erroneous ganciclovir triphosphate incorporation into DNA (Selby and Sancar, 1993; Sancar, 1994; Kazantsev and Sancar, 1995). Our results are in accordance with those reported by Halloram et al., in which the authors show that cell treatment with the HSV-tk/GCV system caused the cell entry into S phase, being followed by an irreversible arrest in late S/G2 phase that accounted for the eventual death of treated cells (Halloran and Fenton, 1998).

The efficacy of the optimal FA-associated lipoplex formulation in mediating an antitumoral effect upon application of the HSV-tk/GCV suicide gene therapy strategy was tested in a mouse model generated by subcutaneous implantation of SCC-VII cells, which exhibit a high proliferation capacity and lead to an aggressive squamous cell carcinoma (Yu et al., 2005; Miura et al., 2006). Our results clearly show a considerable reduction of tumor growth upon intratumoral delivery of the suicide gene mediated by FA-EPOPC:Chol/DNA lipoplexes, followed by intraperitoneal administration of VGCV, as compared to that observed with intratumoral administration of HBS, control FA-lipoplexes or plain lipoplexes (Fig. 7). FA-lipoplexes (in the absence of VGCV) induced, by itself, an antitumoral effect, since tumors from animals treated with these lipoplexes were slightly smaller than those from animals treated with HBS. This was most probably due to immunostimulation provoked by CpG motifs, present in the plasmid DNA and/or lipoplexes per se. It has been reported that the cytosine–phosphate–guanine (CpG) motifs, present in the DNA plasmid vectors, are responsible for most of the immunostimulatory effects of cationic liposome/DNA complexes, since the methylation or elimination of these motifs resulted in a strong reduction in cytokine production (Tan et al., 1999; Scheule, 2000; Yew et al., 2000). Furthermore, it was reported that after intravenous administration of either DOTAP/cholesterol or DOTMA/cholesterol liposomes, serum TNFα and ALT levels were normal, suggesting that liver injury as well as cytokine production was caused by lipoplexes and not by cationic liposomes, per se. In addition, enhanced levels of serum TNFα and ALT activity were observed upon increase of pDNA dose or charge ratio of the lipoplexes (Ito et al., 2009).

Regarding the therapeutic effect observed with the HSV-tk/GCV mediated by FA-EPOPC:Chol/DNA lipoplexes and plain lipoplexes, it may be concluded that, besides the

immunostimulation provoked by lipoplexes-CpG motifs, the major contribution for the antitumoral action results from the therapeutic strategy. As previously referred, the suicide gene therapy strategy benefits from the bystander effect, which is due to the transfer of toxic VGCV metabolites from HSV-tk transfected to non-transfected tumor cells. However, in vivo, the bystander effect also includes activation of the immune system probably induced by the release of soluble factors, like TNF-α and IL-1, from HSV-tk transfected tumor cells and/or the release of cellular debris resulting from the necrosis process (Floeth et al., 2001). This immune system response may involve activation of NK and T cells, mainly cytotoxic CD8-positive T cells, which modifies the tumor microenvironment, thus contributing for the enhanced therapeutic effect (Faneca et al., 2007; Neves et al., 2009).

5. Conclusions

Our results show that the developed FA-EPOPC:Chol/DNA lipoplexes were able to mediate a great enhancement of transfection and a potent antitumoral effect. Importantly, the procedure used in the preparation of FA-associated lipoplexes is based on a non-covalent association, which is simpler and less expensive than a covalent coupling, facilitating large-scale production and thus constituting an additional advantage of this formulation.

Overall, the present study shows that the new FA-EPOPC:Chol/DNA lipoplexes constitute a promising system for the successful application of suicide gene therapy approach aiming at treating solid tumors, including oral carcinomas.

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References

Antony, A.C., 1996. Folate receptors. Annu. Rev. Nutr. 16, 501–521.
Balfour Jr., H.H., 1999. Antiviral drugs. N. Engl. J. Med. 340, 1255–1268.
Cavazzana-Calvo, M., Thrasher, A., Mavilio, F., 2004. The future of gene therapy. Nature 427, 779–781.
Chang, D.Y., Yoo, S.W., Hong, Y., Kim, S., Kim, S.J., Yoon, S.H., Cho, K.G., Paek, S.H., Lee, Y.D., Kim, S.S., Suh-Kim, H., 2010. The growth of brain tumors can be suppressed by multiple transplantation of mesenchymal stem cells expressing cytosine deaminase. Int. J. Cancer 127, 1975–1983.
Colombo, B.M., Benedetti, S., Ottolenghi, S., Mora, M., Pollo, B., Poli, G., Finocchiaro, G., 1995. The bystander effect: association of U-87 cell death with ganciclovir-mediated apoptosis of nearby cells and lack of effect in athymic mice. Hum. Gene Ther. 6, 763–772.
Denning, C., Pitts, J.D., 1997. Bystander effects of different enzyme-prodrug systems for cancer gene therapy depend on different pathways for intercellular transfer of toxic metabolites, a factor that will govern clinical choice of appropriate regimes. Hum. Gene Ther. 8, 1825–1835.
Duarte, S., Faneca, H., de Lima, M.C., 2011. Non-covalent association of folate to lipoplexes: a promising strategy to improve gene delivery in the presence of serum. J. Control. Release 149, 264–272.
Edelstein, M.L., Abedi, M.R., Wixon, J., 2007. Gene therapy clinical trials worldwide to 2007—an update. J. Gene Med. 9, 833–842.
Elshami, A.A., Saavedra, A., Zhang, H., Kucharczuk, J.C., Spray, D.C., Fishman, G.I., Amin, K.M., Kaiser, L.R., Albelda, S.M., 1996. Gap junctions play a role in the 'bystander effect' of the herpes simplex virus thymidine kinase/ganciclovir system in vitro. Gene Ther. 3, 85–92.
Faneca, H., Cabrita, A.S., Simoes, S., Pedroso de Lima, M.C., 2007. Evaluation of the antitumoral effect mediated by IL-12 and HSV-tk genes when delivered by a novel lipid-based system. Biochim. Biophys. Acta 1768, 1093–1102.
Faneca, H., Simoes, S., de Lima, M.C., 2002. Evaluation of lipid-based reagents to mediate intracellular gene delivery. Biochim. Biophys. Acta 1567, 23–33.
Faneca, H., Simoes, S., Pedroso de Lima, M.C., 2004. Association of albumin or protamine to lipoplexes: enhancement of transfection and resistance to serum. J. Gene Med. 6, 681–692.
Farhood, H., Serbina, N., Huang, L., 1995. The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. Biochim. Biophys. Acta 1235, 289–295.
Floeth, F.W., Shand, N., Bojar, H., Prisack, H.B., Felsberg, J., Neuen-Jacob, E., Aulich, A., Burger, K.J., Bock, W.J., Weber, F., 2001. Local inflammation and devascularization—in vivo mechanisms of the bystander effect in VPC-mediated HSV-Tk/GCV gene therapy for human malignant glioma. Cancer Gene Ther. 8, 843–851.
Freeman, S.M., Abboud, C.N., Whartenby, K.A., Packman, C.H., Koeplin, D.S., Moolten, F.L., Abraham, G.N., 1993. The bystander effect: tumor regression when a fraction of the tumor mass is genetically modified. Cancer Res. 53, 5274–5283.
Guo, W., Lee, R.J., 2001. Efficient gene delivery via non-covalent complexes of folic acid and polyethylenimine. J. Control. Release 77, 131–138.
Hafez, I.M., Maurer, N., Cullis, P.R., 2001. On the mechanism whereby cationic lipids promote intracellular delivery of polynucleic acids. Gene Ther. 8, 1188–1196.
Halloran, P.J., Fenton, R.G., 1998. Irreversible G2-M arrest and cytoskeletal reorganization induced by cytotoxic nucleoside analogues. Cancer Res. 58, 3855–3865.
Huang, Q., Liu, X.Z., Kang, C.S., Wang, G.X., Zhong, Y., Pu, P.Y., 2010. The anti-glioma effect of suicide gene therapy using BMSC expressing HSV/TK combined with overexpression of Cx43 in glioma cells. Cancer Gene Ther. 17, 192–202.
Hughes, R.M., 2004. Strategies for cancer gene therapy. J. Surg. Oncol. 85, 28–35.
Ito, Y., Kawakami, S., Charoensit, P., Higuchi, Y., Hashida, M., 2009. Evaluation of proinflammatory cytokine production and liver injury induced by plasmid DNA/cationic liposome complexes with various mixing ratios in mice. Eur. J. Pharm. Biopharm. 71, 303–309.
Kazantsev, A., Sancar, A., 1995. Does the p53 up-regulated Gadd45 protein have a role in excision repair? Science 270, 1003–1004 [author reply 1005–6].
Ketola, A., Maatta, A.M., Pasanen, T., Tulimaki, K., Wahlfors, J., 2004. Osteosarcoma and chondrosarcoma as targets for virus vectors and herpes simplex virus thymidine kinase/ganciclovir gene therapy. Int. J. Mol. Med. 13, 705–710.
Khalil, I.A., Kogure, K., Akita, H., Harashima, H., 2006. Uptake pathways and subsequent intracellular trafficking in nonviral gene delivery. Pharmacol. Rev. 58, 32–45.
Luo, W.Y., Shih, Y.S., Lo, W.H., Chen, H.R., Wang, S.C., Wang, C.H., Chien, C.H., Chiang, C.S., Chuang, Y.J., Hu, Y.C., 2011. Baculovirus vectors for antiangiogenesis-based cancer gene therapy. Cancer Gene Ther. 18, 637–645.
Maitani, Y., Igarashi, S., Sato, M., Hattori, Y., 2007. Cationic liposome (DC-Chol/DOPE = 1:2) and a modified ethanol injection method to prepare liposomes, increased gene expression. Int. J. Pharm. 342, 33–39.
Miura, M., Blattmann, H., Brauer-Krisch, E., Bravin, A., Hanson, A.L., Nawrocky, M.M., Micca, P.L., Slatkin, D.N., Laissue, J.A., 2006. Radiosurgical palliation of aggressive murine SCCVII squamous cell carcinomas using synchrotron-generated X-ray microbeams. Br. J. Radiol. 79, 71–75.
Neves, S., Faneca, H., Bertin, S., Konopka, K., Duzgunes, N., Pierrefite-Carle, V., Simoes, S., Pedroso de Lima, M.C., 2009. Transferrin lipoplex-mediated suicide gene therapy of oral squamous cell carcinoma in an immunocompetent murine model and mechanisms involved in the antitumoral response. Cancer Gene Ther. 16, 91–101.
Ramesh, R., Munshi, A., Abboud, C.N., Marrogi, A.J., Freeman, S.M., 1996. Expression of costimulatory molecules: B7 and ICAM up-regulation after treatment with a suicide gene. Cancer Gene Ther. 3, 373–384.
Reddy, J.A., Low, P.S., 1998. Folate-mediated targeting of therapeutic and imaging agents to cancers. Crit. Rev. Ther. Drug Carrier Syst. 15, 587–627.
Sancar, A., 1994. Mechanisms of DNA excision repair. Science 266, 1954–1956.
Scheule, R.K., 2000. The role of CpG motifs in immunostimulation and gene therapy. Adv. Drug Deliv. Rev. 44, 119–134.
Selby, C.P., Sancar, A., 1993. Molecular mechanism of transcription-repair coupling. Science 260, 53–58.
Simoes, S., Slepushkin, V., Pires, P., Gaspar, R., de Lima, M.P., Duzgunes, N., 1999. Mechanisms of gene transfer mediated by lipoplexes associated with targeting ligands or pH-sensitive peptides. Gene Ther. 6, 1798–1807.
Tan, Y., Li, S., Pitt, B.R., Huang, L., 1999. The inhibitory role of CpG immunostimulatory motifs in cationic lipid vector-mediated transgene expression in vivo. Hum. Gene Ther. 10, 2153–2161.
Touraine, R.L., Ishii-Morita, H., Ramsey, W.J., Blaese, R.M., 1998. The bystander effect in the HSVtk/ganciclovir system and its relationship to gap junctional communication. Gene Ther. 5, 1705–1711.
Trabulo, S., Mano, M., Faneca, H., Cardoso, A.L., Duarte, S., Henriques, A., Paiva, A., Gomes, P., Simoes, S., de Lima, M.C., 2008. S4(13)-PV cell penetrating peptide and cationic liposomes act synergistically to mediate intracellular delivery of plasmid DNA. J. Gene Med. 10, 1210–1222.
Trabulo, S., Resina, S., Simoes, S., Lebleu, B., Pedroso de Lima, M.C., 2010. A non-covalent strategy combining cationic lipids and CPPs to enhance the delivery of splice correcting oligonucleotides. J. Control. Release 145, 149–158.
Vassaux, G., Martin-Duque, P., 2004. Use of suicide genes for cancer gene therapy: study of the different approaches. Expert Opin. Biol. Ther. 4, 519–530.
Wang, J., Lu, X.X., Chen, D.Z., Li, S.F., Zhang, L.S., 2004. Herpes simplex virus thymidine kinase and ganciclovir suicide gene therapy for human pancreatic cancer. World J. Gastroenterol. 10, 400–403.
Wei, S.J., Chao, Y., Hung, Y.M., Lin, W.C., Yang, D.M., Shih, Y.L., Ch'ang, L.Y., Whang-Peng, J., Yang, W.K., 1998. S- and G2-phase cell cycle arrests and apoptosis induced by ganciclovir in murine melanoma cells

transduced with herpes simplex virus thymidine kinase. Exp. Cell Res. 241, 66–75.
Xi, S., Grandis, J.R., 2003. Gene therapy for the treatment of oral squamous cell carcinoma. J. Dent. Res. 82, 11–16.
Xiong, J., Sun, W.J., Wang, W.F., Liao, Z.K., Zhou, F.X., Kong, H.Y., Xu, Y., Xie, C.H., Zhou, Y.F., 2011. Novel, chimeric, cancer-specific, and radiation-inducible gene promoters for suicide gene therapy of cancer. Cancer, doi:10.1002/cncr.26289 [Epub ahead of print].
Yew, N.S., Zhao, H., Wu, I.H., Song, A., Tousignant, J.D., Przybylska, M., Cheng, S.H., 2000. Reduced inflammatory response to plasmid DNA vectors by elimination and inhibition of immunostimulatory CpG motifs. Mol. Ther. 1, 255–262.
Yu, Z., Chan, M.K., O.c., P., Eisenberg, D.P., Shah, J.P., Singh, B., Fong, Y., Wong, R.J., 2005. Enhanced nectin-1 expression and herpes oncolytic sensitivity in highly migratory and invasive carcinoma. Clin. Cancer Res. 11, 4889–4897.
Zabner, J., Fasbender, A.J., Moninger, T., Poellinger, K.A., Welsh, M.J., 1995. Cellular and molecular barriers to gene transfer by a cationic lipid. J. Biol. Chem. 270, 18997–19007.
Zhang, Y., Anchordoquy, T.J., 2004. The role of lipid charge density in the serum stability of cationic lipid/DNA complexes. Biochim. Biophys. Acta 1663, 143–157.