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Systemic administration of cationic phosphonolipids/DNA complexes and the relationship between formulation and lung transfection efficiency

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Abstract

Performances of cationic lipid formulations for intravenous gene delivery to mouse lungs have been previously reported. We report in this study that cationic phosphonolipids, when appropriately formulated, can be good synthetic vectors for gene delivery to lung after intravenous administration. One of our reagents, GLB43, was capable of mediating a 500-fold higher expression in the lungs of mice than could be obtained with free pDNA alone ($P = 0.018$). We demonstrate that the most important parameters for cationic phosphonolipid transfection activity after systemic administration are the chemical structure of the cationic phosphonolipid, the lipid to DNA charge ratio and the inclusion of co-lipid in the formulation. We report using a luciferase reporter gene that transfection activity in vivo 24 h after cationic phosphonolipid systemic administration could not be predicted from in vitro analysis. In contrast to in vitro studies, cationic phosphonolipids including the oleyl acyl chains (GLB43) were more effective than its analogue with the myristyl acyl chains (GLB73). Using pathological analysis of animal livers, we demonstrate that the toxicity level was correlated with the lipoplex formulation and the lipid to DNA ratio.

Keywords: Gene transfer; Cationic lipid; Formulation; Intravenous administration

1. Introduction

Cationic lipids and recombinant DNA complexes (lipoplexes) have proved useful in many in vitro and in vivo animal model applications and recently have been optimised with the results of the first cationic lipid gene therapy trials [1–4]. A large number of families of cationic vectors have now been synthesised. Whilst the concept of transfecting with lipid/DNA complexes is simple, we still need to improve their transfection efficiency, increase their targeted delivery and improve their transient gene expression; this is particularly true for their use in in vivo protocols for gene transfer. Intravenous administration of lipoplexes has been previously described as efficient to transfect lung tissue [5,6]. Two recent studies [7,8] reported transgene expression in pulmonary epithelial cells (type II alveolar epithelial cells) after systemic delivery of cationic lipid/DNA complexes.

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These data led us to think that intravenous delivery could be a potential mode of administration for the gene therapy of lung diseases, particularly cystic fibrosis (CF). Moreover, Griesenbach et al. [7] reported that it has been suggested that the CF transmembrane regulator (CFTR) is expressed in alveolar type II cells [9], so these cells might be suitable target cells for CF gene therapy. Previously, we have synthesised a novel class of cationic lipids called cationic phosphonolipids [10], and have described the ability of these vectors to transfer DNA into an immortalised CF airway epithelial cell line (CFT1 cells) [11]. Recently Guillaume et al. [12] reported significant data about the transfection efficiency in mouse lungs after intratracheal administration of the cationic phosphonolipids known as GLB73 and 43, their good transfection efficiency was demonstrated in comparison with DC-Chol. Here, we report that cationic phosphonolipids are also effective in transfecting lung cells in vivo after systemic administration. Using two types of cationic phosphonolipids differing only in their aliphatic chain length (GLB73 C14:0 and GLB43 C18:1), we demonstrate that there is a relationship between the chemical structure of cationic phosphonolipids and their transfection efficiency in vivo. Using lipoplexes which include DOPE or cholesterol as a co-lipid, we show that transfection efficiency in vivo is dependent on the neutral lipid included in the cationic phosphonolipid/DNA complex formulation and lipid to DNA ratio. In our experiments, transfection activity in mouse lungs 24 h after cationic phosphonolipid systemic administration could not be predicted from in vitro analysis using the human CFT1 cell line. In a recent study, Song et al. [13] reported that high transfection activity after intravenous administration of DOTMA lipoplexes was correlated with a high toxicity. They reported that liver necrosis may be the main reason for such toxicity. These data led us to evaluate hepatic toxicity of our compounds after systemic administration. Using pathological analysis of animal livers, we demonstrate that the toxicity level was correlated with the lipoplex formulation and the lipid to DNA ratio. We suggest that an optimal set of conditions which include cholesterol as the co-lipid, resulting in good expression in the lung and no hepatic toxicity, could be proposed for intravenous cationic phosphonolipid-mediated gene delivery.

2. Materials and methods

2.1. Cell lines and plasmid DNA

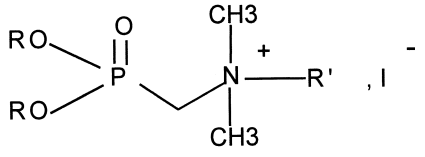
For the in vitro experiments we used the CFT1 cell line. CFT1 cells are SV40 large T-transformed CF tracheal cells obtained from a CF foetus after therapeutic abortion [14]. They were grown in MEM/Ham F-12 (50/50) medium supplemented with 10% of foetal calf serum (FCS), 0.2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% fungizone. The plasmid used was pTG11033 (a gift from TRANSGENE, Strasbourg, France) encoding luciferase protein.

2.2. Preparation of cationic phosphonolipid/DNA complexes

Cationic phosphonolipids used in this report were synthesised by our group. These original molecules were obtained by the Mannich reaction from fatty phosphites followed by quaternarisation of the resulting aminophosphonates [10]. Their structures are presented in Fig. 1. Each of the cationic phosphonolipids was prepared alone or in combination with the neutral lipid DOPE or cholesterol (Sigma, Saint Quentin Fallavier, France) as described elsewhere [15].

2.3. Transfection of CFT1 cells and reporter gene assay

Transfection activity of the cationic lipid/DNA complexes in vitro was assessed using CFT1 cells as described elsewhere [15]. Following a further 48 h at 37°C, the cells were assayed for luciferase expression using a chemiluminescent assay (Promega). Assays were carried out as described by the manufacturer.



R = C14 : 0 $R_1 = CH_3$ GLB 73
R = C18 : 1 $R_1 = CH_3$ GLB 43

Fig. 1. Structure of cationic phosphonolipids.

The results are expressed in TRLU (total relative light units obtained for 16 wells).

2.4. Intravenous gene delivery and luciferase expression in mouse tissues

Cationic phosphonolipid/pTG11033 plasmid DNA complexes were delivered by a single injection of 200 μl in the tail vein of 6-week-old female Swiss mice, each animal received 50 μg of plasmid DNA. Animals were killed 24 h after injection and mouse tissues were immediately frozen on dry ice and stored at −70°C until examined. Luciferase activity was assayed using a chemiluminescent kit (Promega). Extraction of luciferase from mouse tissues was carried out as described by Thierry et al. [5]. The total protein concentration of the tissue extract was determined using the Bio-Rad protein assay. Luciferase activity of each sample was normalised to relative light units (RLU) per mg of extracted protein.

2.5. Determination of charge ratio of lipoplex

The charge ratio was theoretically calculated as molar ratio of GLB compounds (one positive charge per molecule) to nucleotide residue (average MW 330).

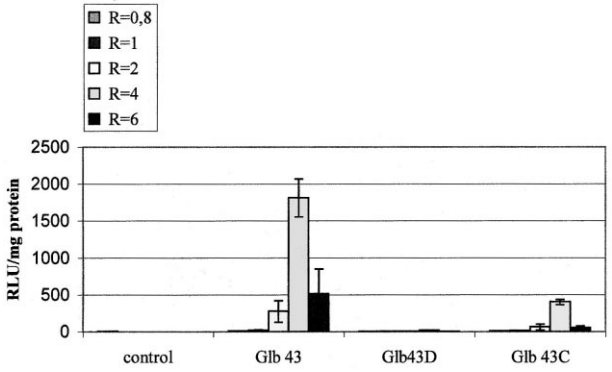


Fig. 2. Influence of cationic phosphonolipid to DNA ratio and cationic phosphonolipid formulation on transfection efficiency in vivo. Twenty-four hours after intravenous administration of 50 μg of pTG11033 DNA plasmid complexed with the indicated amount of the lipoplexes GLB43, GLB43/DOPE (1:1) and GLB43/Chol (1:1), luciferase activity was assayed in mouse lungs. In the control group, mice received only naked DNA. R = calculated charge ratio of the lipoplex. The data are presented as mean ± S.E.M. ($n = 10$).

2.6. Microscopic study of hepatic toxicity

Mice were killed 24 h after intravenous injection. Livers were excised and immediately fixed in Bouin fixative solution for 24–48 h. They were then processed by usual methods of paraffin embedding sections and stained with haematoxylin and eosin. Sections were examined with a photonic microscope.

2.7. Statistical analysis

Results are reported as means ± S.E.M. The statistical significance of the data was determined with Student's unpaired *t*-test as appropriate. $P < 0.05$ was considered significant.

3. Results

3.1. Influence of cationic phosphonolipid to DNA ratio and formulation on transfection efficiency in vivo

In vitro experiments demonstrate the influence of different parameters on cationic lipid-mediated gene transfer in cell lines [16–18]. Among these, lipid to DNA ratio has been shown to be an important factor. To evaluate the influence of the lipoplex charge ratio on in vivo transfection activity after intravenous administration, 50 μg of pTG11033 plasmid containing firefly luciferase cDNA driven by a CMV promoter was complexed with different quantities of GLB43 cationic phosphonolipid and injected into mice via the tail vein. Different formulations were used, including GLB43/DOPE (1:1, w/w), GLB43/Chol (1:1, w/w) and GLB43 alone, at 0.8:1, 1:1, 2:1, 4:1 and 6:1 (+/−) charge ratio. The luciferase activity detected in the lungs 24 h after intravenous administration is presented in Fig. 2. No reporter gene expression was observed in the liver or spleen regardless of the formulation or the charge ratio used (data not shown). In the lung, luciferase activity increased as the lipoplex charge ratio increased to 4, for all the formulations used. The highest luciferase activity was obtained with a lipid to DNA ratio of 4. With this optimal condition, and using an appropriate formulation, GLB43 was capable of mediating a 500-fold greater expression in the lung than could be obtained with free pDNA alone

($P = 0.018$). With a ratio at 6, a noticeable decrease in activity was observed suggesting that an excess of positive charges may result in a toxic effect.

Although the role of the neutral co-lipid in the cationic lipid/DNA complex has not been well defined, several authors [16,17] have reported that the inclusion of DOPE in the cationic lipid formulation increases transfection activity by enhancing the mixing and fusion of liposomes and cell membranes. DOPE forms inverted hexagonal phase structures at ambient temperature [19,20], and may therefore facilitate the formation of similar structures when formulated with cytofectins [21]. Recent data have demonstrated that including cholesterol in the cationic lipid formulation improves in vivo transfection activity [22,23]. Therefore, we examined the role of the neutral co-lipids DOPE and cholesterol in improving the in vivo transfection efficiency of the cationic phosphonolipid GLB43. Three different formulations (GLB43 alone, GLB43/DOPE and GLB43/Chol) were complexed with 50 μg of pTG11033 plasmid and injected into mice. Five lipid to DNA charge ratios (0.8, 1, 2, 4 and 6) were tested. Results of luciferase activity observed in the lung 24 h after intravenous administration are presented in Fig. 2. For each of the lipid to DNA ratios used, the inclusion of co-lipid (DOPE or cholesterol) in the GLB43 formulation decreased its transfection activity in lungs of mice after intravenous administration and the results of transfection efficiency observed were equivalent whatever the amounts of co-lipid used (data not shown). However, although the maximal level of luciferase expression was obtained using GLB43 alone, the inclusion of cholesterol in this cationic phosphonolipid formulation allowed us to obtain a level of luciferase expression 100-fold greater than with free pDNA alone ($P = 0.006$). No transfection activity was observed using DOPE as co-lipid in the GLB43 formulations.

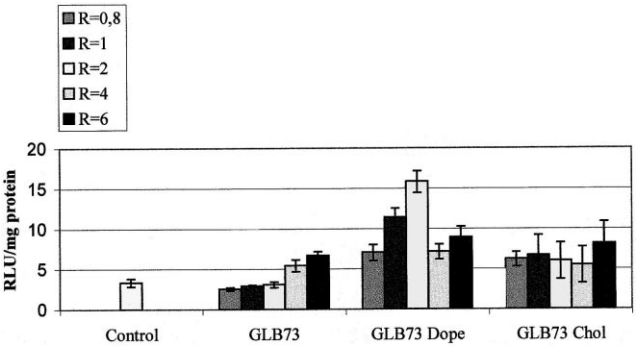


Fig. 3. Effect of di-myristyl fatty acyl chain of cationic phosphonolipid on transfection efficiency in vivo. Luciferase activity was assayed in mouse lungs 24 h after intravenous administration of 50 μg of pTG11033 DNA plasmid complexed with various amounts of lipoplexes composed of GLB73 (C14:0) formulated with or without co-lipid. In the control group, mice received only naked DNA. The data are presented as mean ± S.E.M. ($n = 8$).

3.2. *Influence of cationic phosphonolipid aliphatic chain length on transfection efficiency in vivo*

The effect of the aliphatic chain length of cationic phosphonolipids on transfection activity 24 h after intravenous administration to mice of 50 μg of pTG11033 plasmid was investigated using two different compounds, GLB43 (C18:1) and GLB73 (C14:0), at different charge ratios and using different formulations (alone, with DOPE (1:1), and with cholesterol (1:1)). The results obtained using the GLB43 formulations were presented above (Fig. 2). Results of transgene expression obtained in mouse lungs us-

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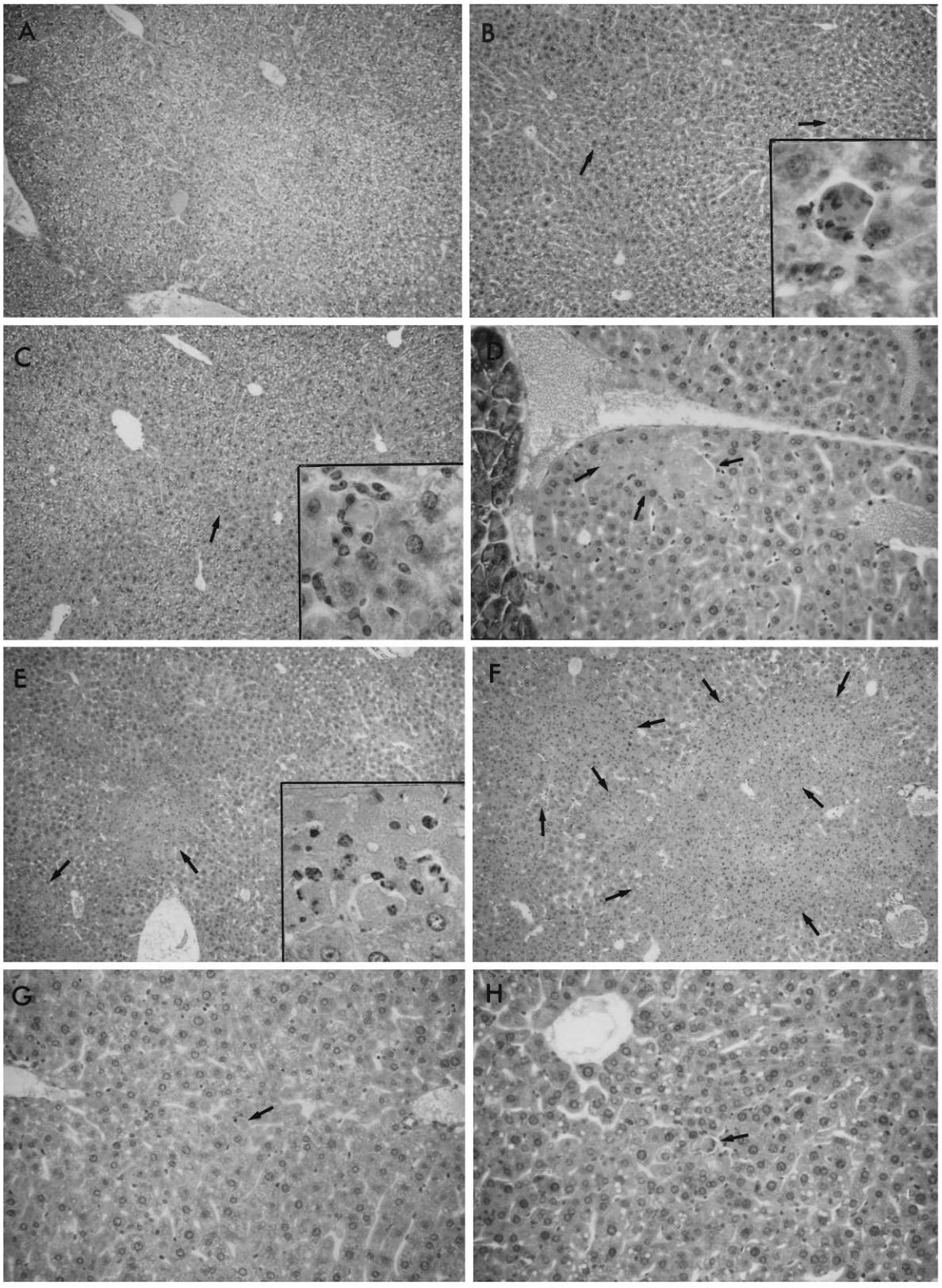
Fig. 4. Effect of cationic phosphonolipid/DNA ratio (R) and lipoplex formulation on in vivo toxicity. Histological analysis of mouse livers 24 h after transfection. Arrows indicate necrotic hepatocytes. Haematoxylin-eosin stain. (A) Injection of 50 μg of pDNA alone (magnification ×18). No abnormality was observed. (B) Injection of GLB43/plasmid DNA, R = 0.8 (magnification ×18). Very rare necrotic hepatocytes scattered in the hepatic lobule. Inset: detail of necrosis (magnification ×115). (C) Injection of GLB43/plasmid DNA, R = 1 (magnification ×18). Rare necrotic hepatocytes scattered in the liver lobule. Inset: detail of necrotic cells with a slight inflammatory reaction (magnification ×115). (D) Injection of GLB43/plasmid DNA, R = 2 (magnification ×45). Several aggregates of necrotic hepatocytes, arrows delineate the zone of necrosis. (E) Injection of GLB43/plasmid DNA, R = 4 (magnification ×18). Fields of necrotic cells are much more numerous and larger. Inset: detail of aggregate of necrotic cells (magnification ×115). (F) Injection of GLB43/plasmid DNA, R = 6 (magnification ×18). Very large lesions made up of a confluent area of necrotic cells. (G) Injection of GLB43 DOPE/plasmid DNA, R = 4 (magnification ×45). Very rare necrotic cells with very slight lipid accumulation. (H) Injection of GLB43 cholesterol/plasmid DNA, R = 4 (magnification ×45). Isolated necrotic cells with slight lipid accumulation.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ing the GLB73 formulations are presented in Fig. 3. In general, regardless of the formulation used, it appears that GLB73 (C14:0) was less effective than GLB43 (C18:1). Moreover, in contrast to GLB43, GLB73 needed to be used with a co-lipid to present a weak transfection activity in the lungs 24 h after intravenous administration. With this compound, the maximal level of luciferase expression was obtained using DOPE as co-lipid and with a lipid to DNA ratio of 2 ($P = 0.0195$).

3.3. Effect of cationic lipid/DNA ratio and lipoplex formulation on in vivo toxicity

Hepatic toxicity has been reported after systemic administration of lipoplexes [13]. In order to study the toxic effects of cationic phosphonolipid-mediated gene transfer after intravenous delivery, systematic pathological analysis of the liver was carried out. No toxicity was observed after injection of formulations including DOPE as a co-lipid or using pDNA alone (Fig. 4A,G). When GLB43 was injected without co-lipid, liver necrosis was observed with the severity of lesions increasing according to the cationic phosphonolipid/DNA ratio (Fig. 4B–F). Inclusion of cholesterol in the GLB43 formulations allowed us to decrease the liver toxicity of the formulation (Fig. 4H). These observations suggest that cationic phosphonolipid metabolites may play a key role in the toxicity process. Further experiments injecting these different metabolites are necessary in order to understand this toxicity and to help improve the chemical design of our molecules. No toxicity was observed after intravenous administration of the GLB73 formulations.

3.4. Comparison of cationic phosphonolipid-mediated gene transfer activity in vitro and in vivo

Transfection activity of the different GLB43 and GLB73 formulations/pTG11033 complexes in vitro was assessed using the immortalised CF epithelial cell line CFT1. Results of luciferase activity observed 48 h after transfection are presented in Fig. 5A. As was previously described [11,16,24], when DOPE was absent from the phosphonolipid formulations only weak transfection activity was observed. Inclusion of cholesterol in the formulation improved transfection activity of GLB43 and GLB73 in vitro, but the results obtained were inferior to those observed in

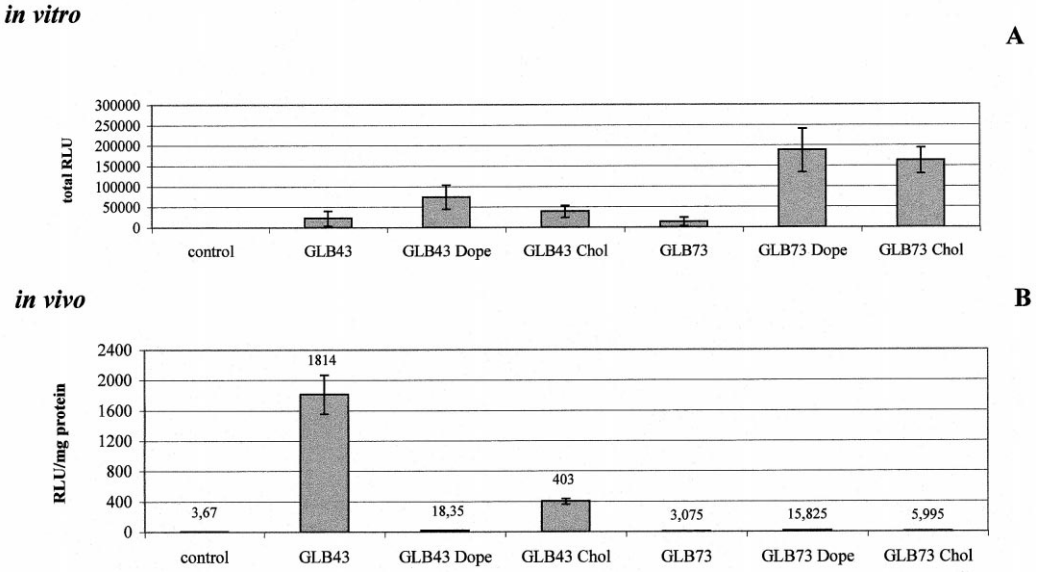

Fig. 5. Comparison of cationic phosphonolipid-mediated gene transfer activity in vitro and in vivo. (A) Comparison of GLB43 and GLB73 formulations in in vitro transfection assays carried out on a CFT1 cell population with pTG11033 plasmid DNA. Two days after transfection, luciferase activity was determined with a luminescent assay. Each data point indicates the mean value of total RLU derived from three transfections and the standard error of this mean. (B) Comparison of GLB43 and GLB73 formulations, complexed with 50 μg of pTG11033 DNA plasmid, in transfection of mouse lungs after intravenous administration. Cationic phosphonolipid to DNA ratios used were 2:1 for GLB73 and 4:1 for GLB43. S.E.M. is calculated from 10 mice for GLB43 and eight mice for GLB73.

the presence of DOPE. In vitro, the phosphonolipid with the di-myristyl fatty acyl chain had the most efficient level of transfection. Results obtained in vivo after intravenous administration of the two phosphonolipid formulations/pTG11033 are presented in Fig. 5BB In this case, in contrast to in vitro studies, the best results were obtained with the GLB43 compound and when co-lipid was absent from the formulation.

4. Discussion

In contrast to in vitro studies, many groups have reported low transfection efficiencies for cationic lipids in vivo especially in the gene therapy of CF [2–4,25]. In most CF studies the lung was directly targeted by local administration of the lipoplex (aerosol, intratracheal and nasal instillation) and the thick mucus accumulation observed in CF patients could decrease lipoplex uptake by the airway epithelium [7]. Performances of cationic lipid formulations for intravenous gene delivery to mouse lungs have been previously reported [5,6]. We report in this study that cationic phosphonolipids, when appropriately formulated, can be good synthetic vectors for gene delivery to lung after intravenous administration. One of our reagents, GLB43, was capable of mediating a 500-fold higher expression in the lungs of mice than could be obtained with free pDNA alone ($P = 0.018$). The good transfection efficiency obtained using GLB43 could be explained by the characteristic chemical structure of this compound. In fact, in order to approach the chemical structure of natural phospholipids, a phosphonate linker was included between the polar head and the hydrophobic moiety of the molecule. Moreover, this type of linker could influence the biodegradability of the compound and in this way may play an important role in transfection activity of this type of cationic lipid.

Among the parameters that influence gene transfer activity of cationic lipids, including a neutral lipid in the formulation has been described as playing a key role. In most commercially available cationic lipid formulations used for gene delivery DOPE is the neutral component. Until recently, DOPE was described as more efficient than several other co-lipids when included in cationic lipid formulations [16,17,26–28]. Recent data have shown that gene transfer mediated by the cationic lipid DOTAP was more efficient in vivo when cholesterol was used as the co-lipid [22,23]. Moreover, recently Crook et al. [29] reported that including cholesterol in DOTAP transfection complexes increased the delivery of DNA to cells in vitro in the presence of serum. Several reasons have been suggested to explain the effect of cholesterol as a co-lipid. Crook et al. [29] suggest that cholesterol maintains the transfection in the presence of serum by improving cell binding and uptake whilst Semple et al. [30] observed that the half-life of liposomes in the circulation of mice can be increased by including cholesterol in the formulation. Lasic [31] and Papahadjopoulos [32] have also discussed the role of cholesterol. Using GLB43 we observed higher luciferase expression in mouse lungs with cholesterol used as the co-lipid than with DOPE. But, using our in vivo conditions, the best results were still obtained using GLB43 alone. These results are in agreement with those of Song et al. [13] who reported that including helper lipid in DOTMA liposomes decreased the level of in vivo gene expression. The decrease in transfection efficiency of cationic lipids in the presence of serum could be one explanation of their low activity in vivo, which consequently limits their in vivo applications. However, the inhibitory effect of serum on lipofection observed in vitro can be overcome by increasing the charge ratio of the cationic/lipid DNA complex [33]. We observed this in vivo when, using GLB43 alone or formulated with cholesterol, the optimal lipid to DNA charge ratio was shown to be 4. No transfection activity was obtained with neutral or negatively charged complexes. Mahato et al. [34] and Song et al. [13] also reported that an excess of positive charges was required for optimal transfection in vivo. In our experiments only theoretically calculated charge ratios were reported, therefore these observations need to be confirmed by determination of the ζ potential of the lipoplexes.

In general, in vitro studies have shown that cationic lipids exhibit significant toxicity. Thus, at higher concentrations of cationic lipid, specific transfection is high but cells are lost, whilst at lower lipid concentrations both toxicity and transfection are minimal [35,36]. In our experiments, pathological analyses of transfected mouse livers were carried out. We

observed that higher lipid to DNA charge ratios resulted in serious toxic effects which correlated with increasing charge ratio, in agreement with the findings of a previous study [13]. Hepatic necrosis was particularly prevalent when cationic phosphonolipid GLB43 was used without co-lipid. Inclusion of cholesterol in the GLB43 formulation allowed us to obtain both good transfection efficiency and less toxicity. No toxicity was observed after injection of GLB43 or plasmid DNA alone. These results suggest that cholesterol might decrease the toxic effect of cationic lipid and allow us to obtain good transfection efficiency after intravenous administration.

Our results suggest that the activity of cationic phosphonolipids for in vivo intravenous delivery could not be predicted from the in vitro analysis. They are consistent with those of Lee et al. [24] who reported that the most effective cationic lipid/pDNA for in vitro transfection of CFT1 cells was in general not similar to those for in vivo delivery to the lung. Even though including co-lipid in the cationic lipid formulation improved transfection activity in vitro [16], with our experimental conditions the opposite effect was observed in vivo. This observation was in agreement with those of previous studies [13], which suggested that the role of DOPE or cholesterol in cationic lipid formulations was different in vivo from in vitro. Our results describing the influence of the structure of the hydrophobic moiety of the phosphonolipid on transfection activity were not consistent with the conclusions from previous studies [37].

In summary, we have demonstrated that efficient transfection activity in mouse lungs can be achieved after intravenous administration of cationic phosphonolipids. Further experimentation of immunohistochemistry will be necessary to confirm the exact localisation of the transgene expression in pulmonary cells. The results obtained highlight that the activity of cationic phosphonolipids for in vivo intravenous delivery could not be predicted from the in vitro analysis. Pathological analysis suggests that optimisation of cationic phosphonolipid formulations is necessary to obtain efficient activity and less toxicity.

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