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KLN-5: a safe monocationic lipophosphoramide to transfect efficiently haematopoietic cell lines and human CD34$^+$ cells

T. Montier[a,*,1], P. Delépine[a,b,1], K. Le Ny[d], Y. Fichou[a], M. Le Bris[a], E. Hardy[b], E. Picquet[d], J.C. Clément[d], J.J. Yaouanc[d], C. Férec[a,b,c]

[a]*Unité INSERM 613, Institut de Synergie des Sciences et de la Santé, Université de Bretagne Occidentale, avenue Foch, 29609 Brest cedex 2, France*
[b]*Service de Génétique et d'Histocompatibilité, Centre Hospitalier Universitaire de Brest, avenue Foch, 29609 Brest cedex, France*
[c]*Etablissement Français du Sang-Région Bretagne, rue Félix Le Dantec, BP 62025, 29220 Brest cedex 2, France*
[d]*UMR CNRS 6521, Faculté des Sciences et Techniques, Université de Bretagne Occidentale, avenue le Gorgeu, 29200 Brest, France*

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Abstract

The safe and efficient delivery of nucleic acids into haematopoietic stem cells (HSCs) has a wide range of therapeutic applications. Although viruses are being used in most clinical trials owing to their high transfection efficacy, recent results highlight many concerns about their use. Synthetic transfection reagents, in contrast, have the advantage of being safe and easy to manage while their low transfection efficiency remains a hurdle that needs to be addressed before they can be widely used.

Using information on transfection mechanisms, a new family of monocationic lipids called lipophosphoramides was synthesized. Their efficiency to transfer genes into haematopoietic cell lines (K562, Jurkat and Daudi) and CD34$^+$ cells was assessed.

In this study, we report that one of these new compounds, KLN-5, leads to more efficient transfection activity than one of our previously most efficient reagents (EG-308) and the commercially available monocationic lipids (DC-CHOL and DOTAP/DOPE) ($P<0.05$). In addition, only a slight toxicity related to the chemical structure of the new compounds is observed. Moreover, we show that KLN-5 can successfully carry the transgene into haematopoietic progenitor cells (CD34$^+$). These results demonstrate that synthetic transfection reagents represent a viable alternative to viruses and could have potential practical utility in a number of applications.

Keywords: Gene transfer; Non-viral transfection reagent; Monocationic lipophosphoramide; Haematopoietic cell line; CD34$^+$ human progenitor cell

1. Introduction

Among haematopoietic cells, few (~1/100,000) are pluripotent and are able to renew themselves and to differentiate into progeny cells. Haematopoietic stem cells (HSCs) have these abilities and can be easily isolated from peripheral blood, bone marrow and umbilical cord blood. Consequently, and considering their availability for ex vivo manipulation and their wide therapeutic capacity [1], HSCs represent major targets for gene therapy of both inherited and acquired haematological diseases. Additionally, recent results show that gene therapy involving manipulation of HSCs leads to clear clinical benefits [2,3]. These first successes contribute to the evolution of gene therapy from the proof-of-principle established from many in vitro and in vivo studies to a valuable tool for curing or controlling human disorders. However, some clinical trials have shown the limits and the risks of the current viral vector gene therapy method. Viruses have been used in most clinical trials due to their high transfection efficiency [4] but their effectiveness is shadowed by side effects, such as immunogenicity, random integration of the transgene and viral recombination, that can potentially compromise patient safety [5,6].

* Corresponding author. Tel.: +33 2 98 01 80 80; fax: +33 2 98 43 05 55.
E-mail address: Tristan.Montier@univ-brest.fr (T. Montier).
[1] The first two authors contributed equally to this work.

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As an alternative to the viruses, synthetic reagents offer some promising features but their relatively low transfection efficacy needs to be addressed before they can be widely used [4,7]. Additionally, among numerous non-viral transfection delivery systems generated, few demonstrate concrete efficiency, especially in vivo or ex vivo.

In this context, over the last decade, our team has developed a new family of synthetic delivery systems, the monocationic phosphonolipids, and has demonstrated previously their efficiency in vitro [8,9], in vivo [10,11] and ex vivo [12]. Since they were first developed, the transfection efficacy of these compounds has been continuously improved through cation substitution in the polar domain (N^+<P^+<As^+) or through linker elongation (n=1<n=2). Although these modifications lead to lower toxicity and higher efficiency, their transfection capacity is not comparable to that of viruses and increasing transfection rates remains a major goal in this field. To improve transfection efficiency, it is necessary to precisely define the main barriers restricting the lipoplex-mediated DNA transfer before adapting their chemical structures to the biological environment [13]. From biodistribution and in situ hybridisation studies, following transfection with monocationic phosphonolipids, insights were obtained about the mechanism of distribution and cellular penetration, particularly concerning the release of plasmid DNA into the cytoplasm and the DNA's kinetics within the cell, as well as the number of DNA copies that finally migrate to the nucleus [14,15]. Given the results of these studies, contrary to our initial idea of protecting and increasing the stability of the monocationic phosphonolipids, a pH-sensitive site was introduced between the fatty acyl chains and the polar head group to facilitate the release of the transgene from the endosomal vesicles before lysosomal enzymatic degradation occurs, while conserving lipoplex stability in the extracellular environment. Accordingly, the cationic phosphonolipid structure was modified and the phosphorus-to-carbon link was changed into a phosphoramide link. Transfection capacity of the resulting compounds was then successfully assessed in various adherent cells lines and epithelial primary cultures [16].

In this study, three monocationic lipophosphoramides were formulated in several ways and their transfection efficiency into the haematopoietic cell lines was then compared with the commercial transfection reagents (DC-CHOL and DOTAP-DOPE) and one of our most efficient reagents (EG-308), generated by previous phosphonolipid experiments. We show that KLN-5, one of the new lipophosphoramide reagents, is more efficient than all the other synthetic reagents tested and leads to 60–65% LacZ-positive ($LacZ^+$) living K562 cells vs. 50–55% following transfection with DC-CHOL. Moreover, through chemiluminescent results, we report here that the efficiency and cytotoxicity of the reagents are strongly correlated to their formulation (volume, co-lipid and charge ratio) and to the targeted cells (Daudi<Jurkat<K562).

Since KLN-5 showed a significant fain of efficiency, human haematopoietic progenitor cells ($CD34^+$) were then transfected with KLN-5 and with DC-CHOL, and the transfection efficiencies were compared. Under these conditions, a significant signal was detected (P<0.004), KLN-5 giving a two fold increase compared to DC-CHOL, showing the efficiency of monocationic lipophosphoramides and the possibility to transfect efficiently haematopoietic progenitor cells with non-viral reagents.

2. Materials and methods

2.1. Cell lines cultures and $CD34^+$ purification

In vitro experiments were performed on K562, Jurkat and Daudi cell lines. These human haematopoietic cell lines were obtained from the American Type Culture Collection (respectively, n°ccl 243 ATCC, n°tib-152 ATCC, n°ccl 213 ATCC, Rockville, MD, USA). They were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 0.2 mM glutamine, 100 U/ml penicillin, 100 μg/ml of streptomycin and 1% fungizone (Gibco-BRL, Cergy-Pontoise, France).

Human progenitor cells ($CD34^+$) were isolated by aphaeresis harvest using a MACS direct $CD34^+$ progenitor cell isolation kit and MidiMACS columns (Miltenyi Biotec, Gladbach, Germany). $CD34^+$ purification was performed as described by the manufacturer and other research groups [17,18]. After purification, $CD34^+$ cells were maintained in a special medium (IMDM, 4 mM L-glutamine, 1% HSA, Insulin, 5% Rh Transferrin, 10^{-4} M β mercaptoethanol, 100 U/ml penicillin, 100 U of streptomycin and 1% fungizone) (Gibco-BRL) supplemented with several growth factors (Flt3 ligand rh, Stem cell factor rh, Il3 rh and Il6 rh) (Stem cell, Vancouver, Canada). Cellular density was maintained between 10^5 and 10^6 cells/ml. All cells were maintained in 5% CO_2 at 37 °C.

2.2. Monocationic phosphonolipids and lipophosphoramides

Four cationic lipids synthesized by our group (KLN-5, KLN-9, KLN-35 and EG-308) and two commercial transfection reagents, DC-CHOL and DOTAP-DOPE (Avanti Polar Lipids, Alabaster, AL, USA), were used in this study. DC-CHOL and DOTAP-DOPE have been described in many previous reports and are used as controls [19,20]. The monocationic lipophosphoramides (KLN) were synthesized according to the method used previously for the synthesis of cationic phosphonolipids such as EG-308 [21,22]. The structures of all phosphonolipids and lipophosphoramides were confirmed by ^1H, ^{31}P and ^{13}C NMR spectroscopy and are presented in Fig. 1. All compounds generated in both phosphonolipid and lipophosphoramide generations were patented (respectively n°60/175342 and n° FR03/50116).

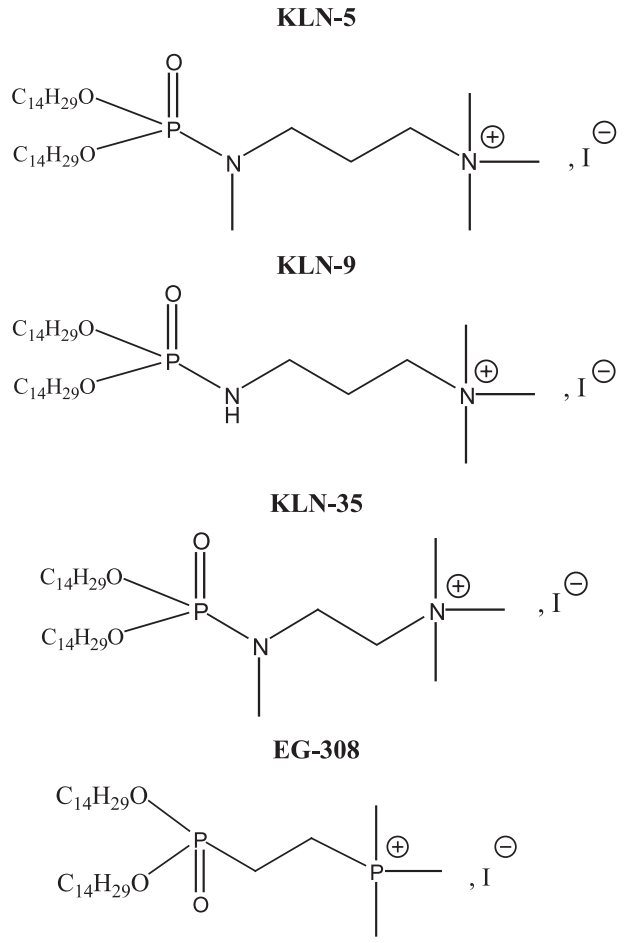

Fig. 1. Structure of monocationic lipophosphoramides (KLN) and phosphonolipids (EG) molecules.

In previous reports using nonadherent cell lines [8,9], cationic phosphonolipids with two $C_{14}H_{29}$ fatty acyl chains showed the highest efficiency. The same hydrophobic domain was conserved for the new generation to allow a possible comparison of chemiluminescent results between studies. Nevertheless, small differences, such as linker length (n=2 or 3 carbons) or the nature of the group (H or CH_3) associated with the nitrogen atom contained in the lipophosphoramide link, were introduced to determine the structure that leads to the highest transfection results.

Each of the compounds was then prepared alone or in combination with the neutral lipid DOPE (Sigma, Saint Quentin Fallavier, France). The cationic lipid-to-co-lipid ratio was 1:1 (w/w). The phosphonolipids were formulated by mixing chloroform solutions of the different lipids in glass vials followed by removal of chloroform by rotary evaporation to produce dried lipid films. A total of 1 ml sterile pyrogen-free DI water was added per milligram of lipid and the vials were sealed and stored at +4 °C overnight. Small unilamellar vesicles (SUVs) of each compound were prepared by sonication for 10 min in a sonicator bath (Prolabo, Paris, France) [23]. To prepare the cationic lipid/DNA complexes, plasmid DNA was first diluted in sterile pyrogen-free DI water and added to the lipid solution. The lipoplexes were kept at room temperature for 30 min before being used for in vitro assays.

2.3. Determination of the charge ratio of the lipoplex

The charge ratio was calculated theoretically as the molar ratio of KLN or EG compounds (one positive charge per molecule) to phosphate nucleotide residues (average MW 330). In this study, two different formulation conditions were used: either variation of the volume (12.5 μg lipid/4 μg pDNA and 25 μg lipid/8 μg pDNA) while the charge ratio was maintained at two or variation of the charge ratio (+/−) (two to four) while a constant amount of 8 μg of pDNA was delivered. The formulations (volume vs. charge ratio) were then evaluated for their transfection efficiency and cellular toxicity.

2.4. Plasmids

Two plasmids were used for transfection studies. The first was a pCMV-LUC construction encoding the firefly luciferase protein under the control of the cytomegalovirus immediate early gene 1 (IE-CMV) promoter. The second was a pCMV-βGal encoding the β-galactosidase protein and also under the control of IE-CMV promoter.

The plasmids were amplified by the DH5α strain of *Escherichia coli*, isolated by alkaline lysis and purified by the Qiagen endofree plasmid Giga kit (Qiagen SA, Courtaboeuf, France) according to the manufacturer's protocol. The plasmids were then dissolved in endotoxin-free water and stored at −20 °C. DNA was quantified by spectrophotometry at 260 nm and was free of major protein contamination with an A260/280 ratio between 1.8 and 2.0. The amount of endotoxin was determined by using a chromogenic limulus amoebocyte clotting assay (QCL-1000 kit, Biowhittaker, France) and all values were <10 endotoxin units (EU)/mg DNA.

2.5. Transfection protocol

Haematopoietic cells (K562, Jurkat, Daudi and $CD34^+$) were seeded 1 h before transfection onto a 24-well plate at 500,000 cells per well in 500 μl of RPMI medium (serum-free). The following protocol was adapted from Felgner et al. [7] with a few modifications. The cationic lipid–DNA complexes were prepared by adding an appropriate amount of pDNA and of cationic lipid formulation into a polystyrene tube. The tube was incubated for 30 min at room temperature and the complex was then added to each well. The control DNA was prepared in Optimem medium but no lipid was added. Then, this lipid-free solution is added into each well containing the cells. After a 2.5-h incubation at 37 °C, 2 ml of specific cell supplemented medium was added per well. The cells were then incubated again at 37 °C for 48 h prior to luciferase detection or FACS-gal assays.

2.6. Luminescence detection

Forty-eight hours after transfection, the cells were assayed for luciferase expression using a chemiluminescent assay (Promega, Charbonnière, France). Tests were carried out as described by the manufacturer. After two successive washes with PBS, cells were treated with 200-μl Lysis Buffer (Promega) for 15 min. The supernatant was then distributed into the wells of a 96-well opaque plate. Luciferase activity in the supernatant was quantified by a luminometer (MLX® Microtiter Plate Luminometer-Dynex, Guyancourt, France), measuring light emission over a 15-s reaction period. The results were expressed in total relative light units (Total RLU) per 100,000 cells (Total RLU/100,000 cells). The control values were generated by transfection of the cells with pDNA alone. Quantitative variables were expressed as mean±standard error of the mean (S.E.). Differences in the means between the groups were assessed by using the Student's *t* test. A *P* value of less than 0.05 was considered significant.

2.7. Analysis of βGal expression using FACS-gal assay

To investigate gene expression in the cells, FACS-gal assays were also performed as described by Nolan et al. [24] and Fiering et al. [25]. Briefly, enzyme activity is detected by using a fluorogenic substrate (fluorescein di-β-D-galactopyranoside-FDG) hydrolysed by β-galactosidase inside the cell. For these experiments, the cells were transfected by the most efficient transfection agents (KLN5, EG308 and DC-CHOL) at four different charge ratios (+/−) (from two to five). Cells were suspended in 50 μl of RPMI medium and warmed in a 37 °C water bath. FDG (2 mM in sterile pyrogen-free water) (Sigma) was prewarmed at 37 °C and 50 μl was added to each aliquot of cells. FDG and cells were then mixed and immediately incubated in a 37°C bath for 60 s. The cells were then removed from the bath and 1 ml of chilled RPMI medium was added to each tube. Cells were kept on ice until being analysed on a FACScalibur 3C (Becton-Dickinson, Rungis, France). The percentage of LacZ-positive cells ($LacZ^+$) was evaluated for each lipoplex in comparison with control values (cells transfected by naked DNA). The autofluorescent background was estimated from untransfected cells and cells in contact with DOTAP/DOPE alone.

2.8. Determination of cell toxicity

The toxicity of the different formulations was determined as the number of cells surviving the transfection experiment as measured by chemiluminometric assay (CytoLite-Packard bioscience, Rungis, France) as described below. In suspension, eukaryotic cells with an intact cell membrane possess a negative surface charge and, as a result, the cationic chemiluminogenic probe (CLP) can interact with the cell surface and the $CLP2^+$ is transformed to a univalent radical state, CLP^+. The reduced co-enzyme added to the cells is then taken up. The reduced co-enzyme is internalized and is required to drive electron-transferring reactions wherein oxygen is the final acceptor. The end result of this pathway is the generation of reactive oxygen species (ROS) which can diffuse freely out of the cell. The ROS react with the CLP^+ on the intact cell membrane and an intermediate is formed which spontaneously decomposes, producing luminescence.

One hour before the assay, the cells were plated in a 24-well plate (500,000 cells per well). Transfections were performed as described above using the various formulations, and the cells were incubated for 48 h at 37 °C. The toxicity assay was then carried out as specified by the manufacturer. The Total RLU/100,000 cells was proportional to the number of viable cells. Untransfected cells were used as a reference. The final results were expressed as a toxicity index, corresponding to the ratio of living cells in the "control well" divided by the number of living cells in the "transfected well". A toxicity index of one equates to no differences between control and transfected cells and implies no cytotoxicity. The toxicity index increases as the toxicity of the tested cationic lipid increases.

3. Results

3.1. Influence of chemical modifications and formulation on transgene expression

The aim of this work was to evaluate the transfection capacity of a new type of compounds, monocationic lipophosphoramides, formulated in several ways and compared with reference transfection reagents on haematopoietic cell lines. Fig. 2A–C, corresponding respectively to K562, Jurkat and Daudi cell lines, shows the influence of chemical modifications and formulations related to the volume of complex delivered (12.5 μg lipid/4 μg pDNA and 25 μg lipid/8 μg pDNA, R=2) on transfection efficiency. The new monocationic lipophosphoramide KLN-5 formulated without DOPE appears to be a better transfection reagent than all the other compounds tested, regardless of the lipoplex formulation or the cell line (P<0.05). Among the commercial transfection reagents, DC-CHOL is the most efficient in the assays performed on haematopoietic cell lines whereas DOTAP/DOPE, which has been previously used successfully with both adherent cell lines and mice models [26,27], was not highly efficient here. However, introducing a phosphorus-to-nitrogen link, as was done in the successful KLN-5, solely is not enough to improve the transfection efficiency of the monocationic lipophosphoramides. KLN-9 and KLN-35, which both include this phosphoramide link, induce as much luminescence as EG-308, a phosphonolipid derived from the previous generation of compounds. In addition, in spite of their fatty acyl chains ($C_{14}H_{29}$) and ammonium polar head groups similar to KLN-5, KLN-9 and KLN-35 give lower transgene expression

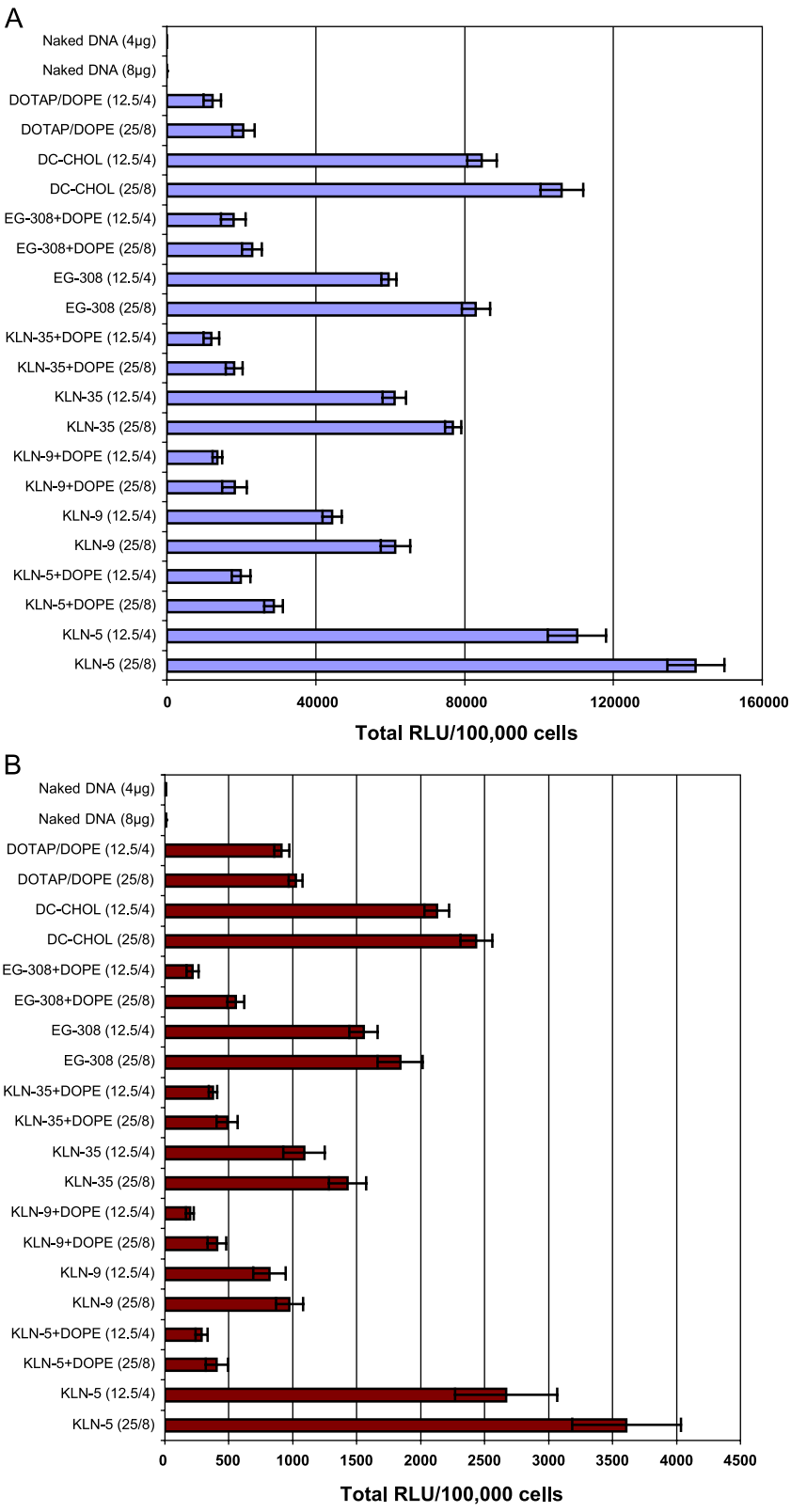

Fig. 2. Influence of formulation on transfection efficiency in vitro. The haematopoietic cell lines (K562, Jurkat and Daudi) were transfected in 24-well plates as described in Materials and methods. Cells transfected with free DNA were used as controls and Luciferase activity was measured using a luminescent assay 2 days after transfection. The values for Total RLU/100,000 cells were obtained by summing the RLU values of eight wells of microtiter plates and were plotted for each formulation. Each data point indicates the mean value of Total RLU/100,000 cells derived from three transfections and the standard deviation of this mean.

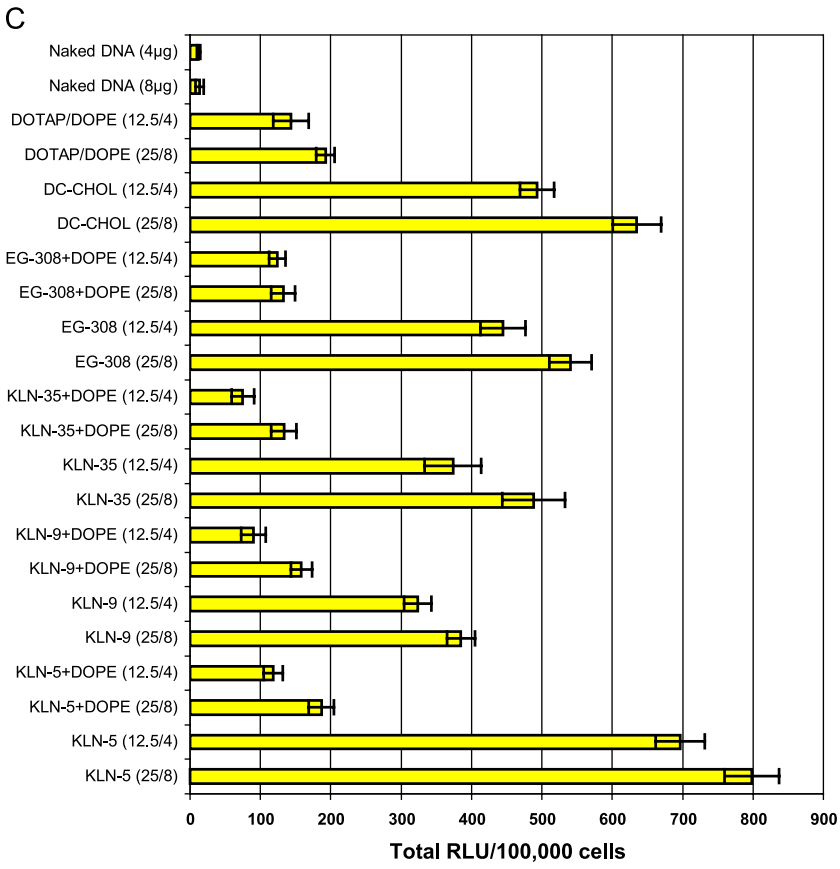

Fig. 2 (*continued*).

than DC-CHOL. Moreover, KLN-9 is the weakest novel transfection molecule despite having both a phosphorus-to-nitrogen link and a spacer composed by three carbons as does KLN-5. In previous work, we described that a distance increase from one to two carbons between the cation and the hydrophobic chains positively influences transfection efficiency [9]. Here, we noted that increasing the length of the linker from two to three carbons seems to increase the transfection efficiency of these new compounds as seen with KLN-5. Nevertheless, this elongation is not the only feature that influences the transfection capacity. The nature of the group (H or CH_3) associated with the nitrogen atom also appears to influence the transfection capacity of these new compounds.

In addition to the chemical structures, our experiments demonstrate that an increase in lipoplex volume also influences transfection regardless of the synthetic agent used. A mixture of 25 µg lipid/8 µg pDNA gives higher luminescence results than 12.5 µg lipid/4 µg pDNA when using the same theoretical charge ratio (+/−) (R=2). Although this difference is significant, it is lower than expected. The toxicity from the additional volume of reagent could be blamed for the small increase in luminescence. To verify this hypothesis, we performed additional charge ratio assays on the non-adherent cell lines using a constant quantity of pDNA (8 µg) delivered. As shown in Fig. 3A–C, KLN-5 formulated without DOPE appears to be the most efficient transfection reagent used (P<0.05) under all tested conditions. These observations confirmed the weak efficiency of KLN-9 and KLN-35 no matter their formulation, in comparison with DC-CHOL and KLN-5. We also noticed that when the charge ratio (+/−) increases from two to four, the luminescence decreases for all the formulations and all the cell lines. These results support the implication that additional volume increases the toxicity.

Another aspect of the formulation concerns the use of DOPE in the mixture. The results show that addition of DOPE in the formulation leads to a decrease in the transfection capacity of the lipoplexes. These observations are confirmed for all the phosphonolipid and lipophosphoramide compounds. In the charge ratio assays, formulations with DOPE also show a similar decrease albeit less dramatic.

Figs. 2 and 3 also show that the luminescence results vary depending on the cell line. Indeed, for a constant number of cells (500,000 cells per well) in contact with the lipoplex formulations, the results vary from 1 to 100 between K562 and Jurkat (T-leukemia cells) and from 1 to 1000 between K562 and Daudi (B-leukemia cells). These results illustrate the variable degree of difficulty in transfecting different haematopoietic-derived cell lines and the requirement to adapt reagents and formulation to the target cells.

3.2. Relationship between toxicity and formulations

Following the transfection capacity studies, the impact of lipoplex formulations on cell viability was investigated. Fig. 4A illustrates that the toxicity index increases when 25 μg lipid/8 μg pDNA is added to the cells in comparison with untransfected cells, but is not significant in comparison with 12.5 μg lipid/4 μg pDNA. As described above, the luminescence results and the corresponding toxicity index are not markedly different. Moreover, we noted that with DOPE-containing formulations, the toxicity is lower and the difference between formulations with and without DOPE is significant ($P<0.05$). This may be a result of the neutral role of DOPE in membrane stability [28].

In parallel, we analysed the influence of charge ratio, in the range of two to five, on cytoxicity. Using the K562 cell line with the most efficient transfection reagents (KLN-5, EG-308 and DC-CHOL), the toxicity ratio was shown to increase in parallel with the charge ratio, especially when it equals four or five (Fig. 4B). This illustrates the dilemma in delivering the highest possible amount of pDNA by the highest possible charge ratio, which increases the cytotoxicity. All these observations underline that the volumes used in this study are appropriate to the new generation of lipophosphoramides and represent an optimal balance between transfection capacity and cellular toxicity.

Since KLN-5, EG-308 and DC-CHOL were the most efficient reagents, their performances were analysed by FACS-scan assays using different charge ratios. Regardless of which reagent is used, the percentage of $LacZ^+$ viable cells decreases when the charge ratio is increased (Fig. 5A). Furthermore, we observe that up to $R=4$, KLN-5 leads to higher percentages of $LacZ^+$ viable cells than EG-308 and

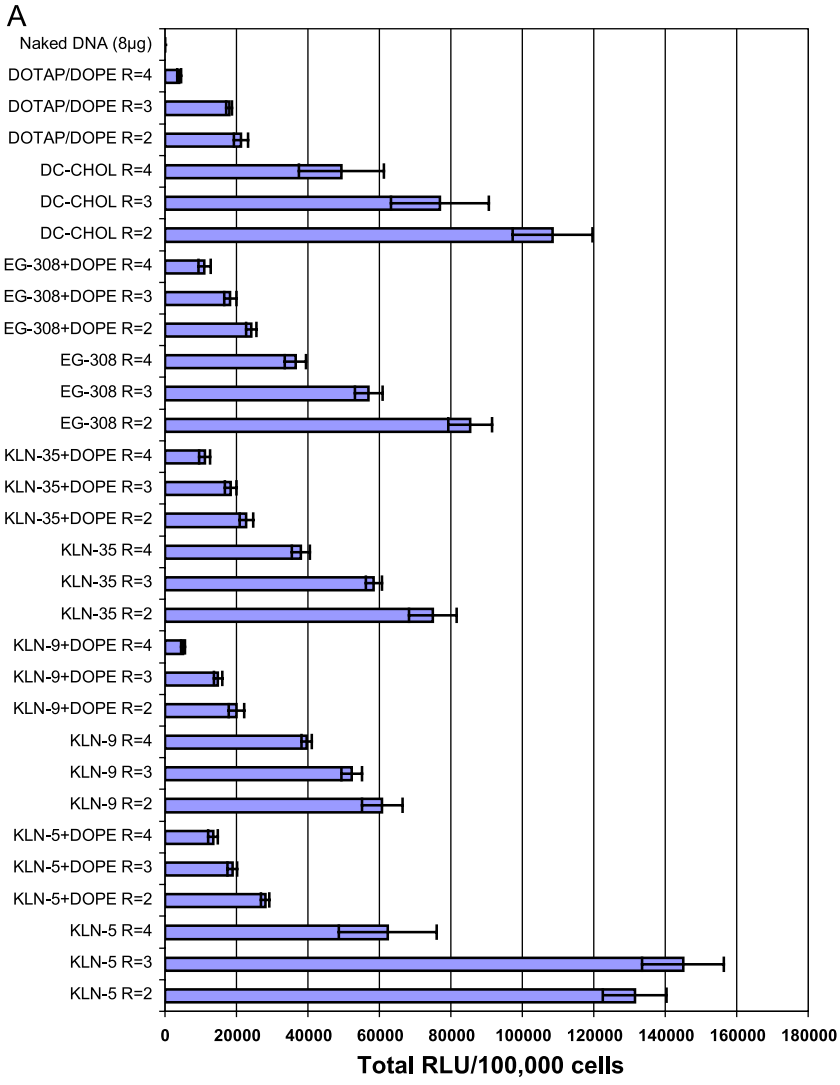

Fig. 3. Charge ratio (+/−) influence on the in vitro transfection efficiency. Transfection was performed as described in Materials and methods. Cells transfected with free DNA were used as controls and Luciferase activity was measured using a luminescent assay 2 days after transfection. The values for Total RLU/100,000 cells were obtained by summing the RLU values of eight wells of microtiter plates and were plotted for each reagent and charge ratio. Each data point indicates the mean value of Total RLU/100,000 cells derived from three transfections and the standard deviation of this mean.

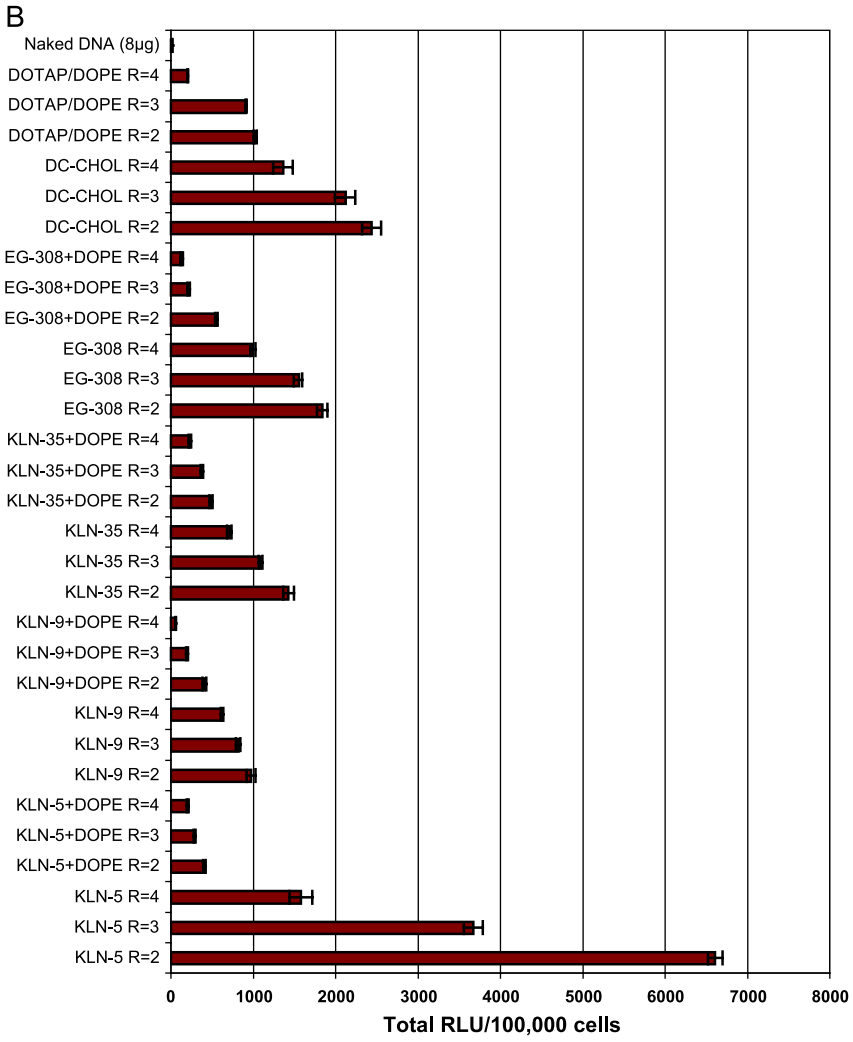

Fig. 3 (*continued*).

DC-CHOL under similar conditions ($P<0.05$). FACS–gal assays show that 62.35% of the global K562 cell population are positive and alive following transfection by KLN-5 with a charge ratio of two. In contrast, using the same charge ratio, EG-308 and DC-CHOL return respectively 49.52% and 55.38% of $LacZ^+$ viable cells (Fig. 5B). At the same time, the final expression level of the transgene is improved when the charge ratio increases for a constant amount of pDNA delivered, even though the percentage of viable cells is markedly reduced (data not shown). However, at a charge ratio of five, the percentages of $LacZ^+$ viable cells obtained following transfection with each of the reagents are very similar. These experiments show the significant progress made in transfection capacity, using KLN-5 compared with EG-308, while conserving relative safety. Indeed, these reagents' transfection efficiency stems from two parameters: the chemical structure itself and the charge ratio. In addition, the cytotoxicity resulting from these parameters needs to be balanced with the increased transgene expression produced by the alterations to the chemical structures.

3.3. Formulation and $CD34^+$ results

As demonstrated, KLN-5 shows higher transfection efficiency than the previous generation of compounds, illustrated by EG-308, in haematopoietic cell lines. Previously, our group tried to use the GLB compounds to transfect cells of interest such as $CD34^+$ cells [29]. The results were disappointing in that no signal was detected. As KLN-5 exhibits a significant increase in phosphonolipid transfection capacity, we tested it on human progenitor haematopoietic $CD34^+$ cells and compared its performance to that of DC-CHOL. In Fig. 6, the luminescence results show that these cells were significantly transfected using KLN-5 ($P<0.05$), in comparison with results obtained with pDNA alone (8 µg), even if the expression level remained low. The best results were obtained using a charge ratio of four no matter the compound, KLN-5 leading to higher luminescent results than DC-CHOL ($P<0.05$). However, the charge ratio of four previously showed the greatest cytotoxicity on cell lines. In addition to the difference previously described between cell lines, these results

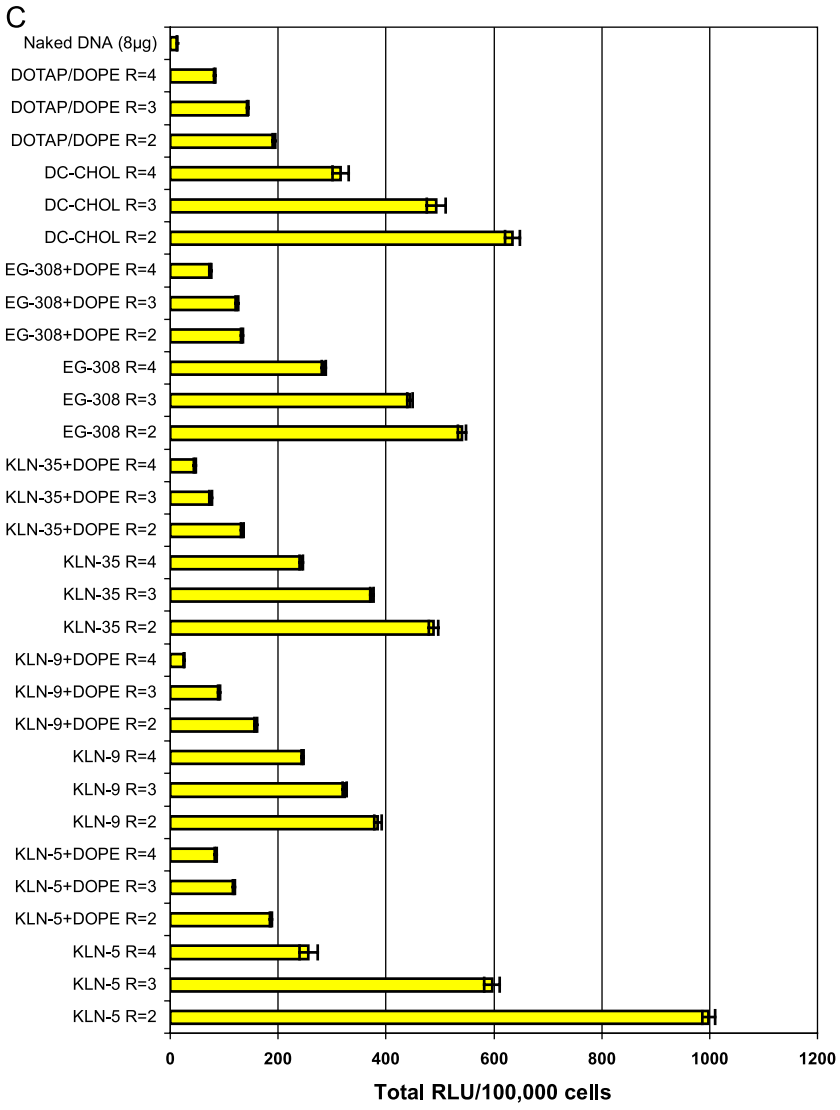

Fig. 3 (*continued*).

illustrate the variation between in vitro cell lines and ex vivo stable cells and the importance of the formulation in each case.

4. Discussion

Despite their low transfection efficiency in comparison with viruses, synthetic reagents exhibit several advantages as potential agents for gene therapy applications, especially directed towards haematopoietic cells. Their lack of immunogeneticity, low toxicity and episomic nature of the DNA are essential criteria for the development of a safe system for clinical use. However, their transfection efficiency is the most important issue in their use as a possible therapeutic agent.

Since the first cationic phosphonolipids were synthesized, numerous modifications have been introduced into the hydrophobic domain, the polar head group and the linker length [9], leading to a progressive and significant increase of the transfection capacity of these compounds both in vitro and in vivo. Recently, following insights about biodistribution and the intracellular pathways of the lipoplexes, we considered that introducing a phosphoramide site, which would enhance a fast separation of the hydrophobic domain from the initial structure of the lipoplexes, could be beneficial.

In the present work, some compounds from the resulting new generation of monocationic lipids, called lipophosphoramides, are described and their transfection efficiency to haematopoietic cell lines is measured in comparison with either reagents from the previous generation (EG-308) or standard monocationic transfection reagents, such as DOTAP/DOPE and DC-CHOL. For each reagent, several formulations were prepared and tested. From this study, major findings are highlighted.

First, the introduction of a phosphoramide link coupled with a methyl group (KLN-5) into the initial phosphonolipid structure leads to a significant increase in the transfection efficiency of the non-adherent cell lines tested, without any additional toxicity induced by changing the structure. Additionally, KLN-5 allows a significant expression in $CD34^+$ progenitor haematopoietic cells, even if the transgene expression is still low. However, introducing a phosphoramide pattern into the phosphonolipid structure alone is not sufficient to improve transfection capacity. Additional changes are required before a significant increase in efficiency is observed. Indeed, KLN-9 and KLN-35, despite their phosphoramide link, did not show any increase in transgene expression when compared with EG-308. Removing the polar head group from the fatty acyl chains influences the luminescence results, but the nature of the group associated with the nitrogen atom seems to be more critical. There are a number of possible explanations for this effect. The first is a steric cluttering phenomenon. If the phosphoramide link is present as a specific enzyme and/or as a pH-related cleavage site, the chemical groups closest to it may have an influence. Consequently, a methyl group at this position could block access to the cleavage site, whereas a hydrogen atom at the same position may not and, thus, fast separation of the hydrophobic domain should occur. However, we observe that the results for KLN-35, which has a methyl group, do not differ significantly from those obtained with EG-308, which has no phosphoramide link. This cannot be explained by the presence of a methyl group such as in KLN-5, but may be a result of the linker length or the composition of the polar head group, since N^+ leads to lower transfection rates than P^+, as demonstrated in previous studies [9]. The performance of KLN-9 differs significantly from EG-308 no matter the formulation or the cell line

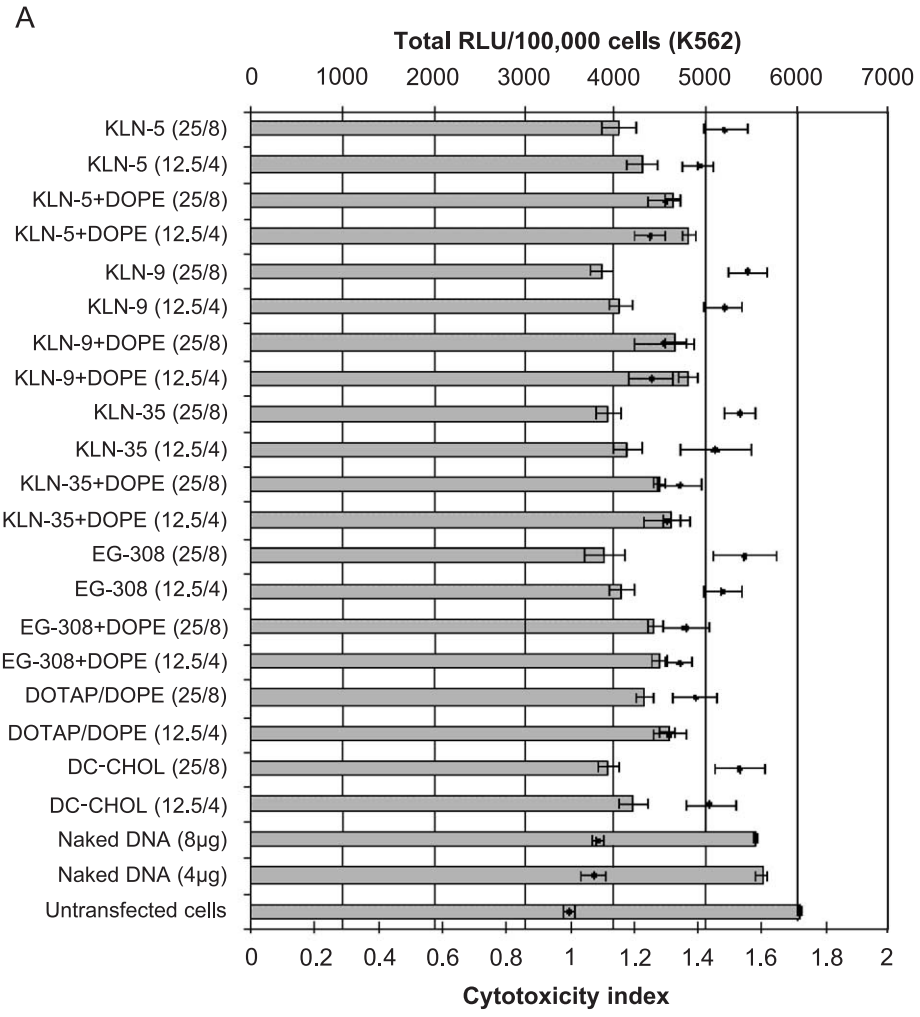

Fig. 4. In vitro cytotoxicity of the compounds on K562 cells depending on the volume delivered (A) and on the charge ratio (B). Luciferase activity results observed 48 h after transfection of K562 cells are summarized in bar form. Each data point indicates the mean value of Total RLU/100,000 cells derived from four transfections and the standard deviation of this mean. The cytotoxicity index is depicted by the solid bracketed lines and was determined as described in Materials and methods. Cells transfected with free DNA (4 and 8μg) were used to evaluate the toxicity of pDNA. Untransfected cells are used as a standard control and the corresponding luminescent results allow to calculate the toxicity index.

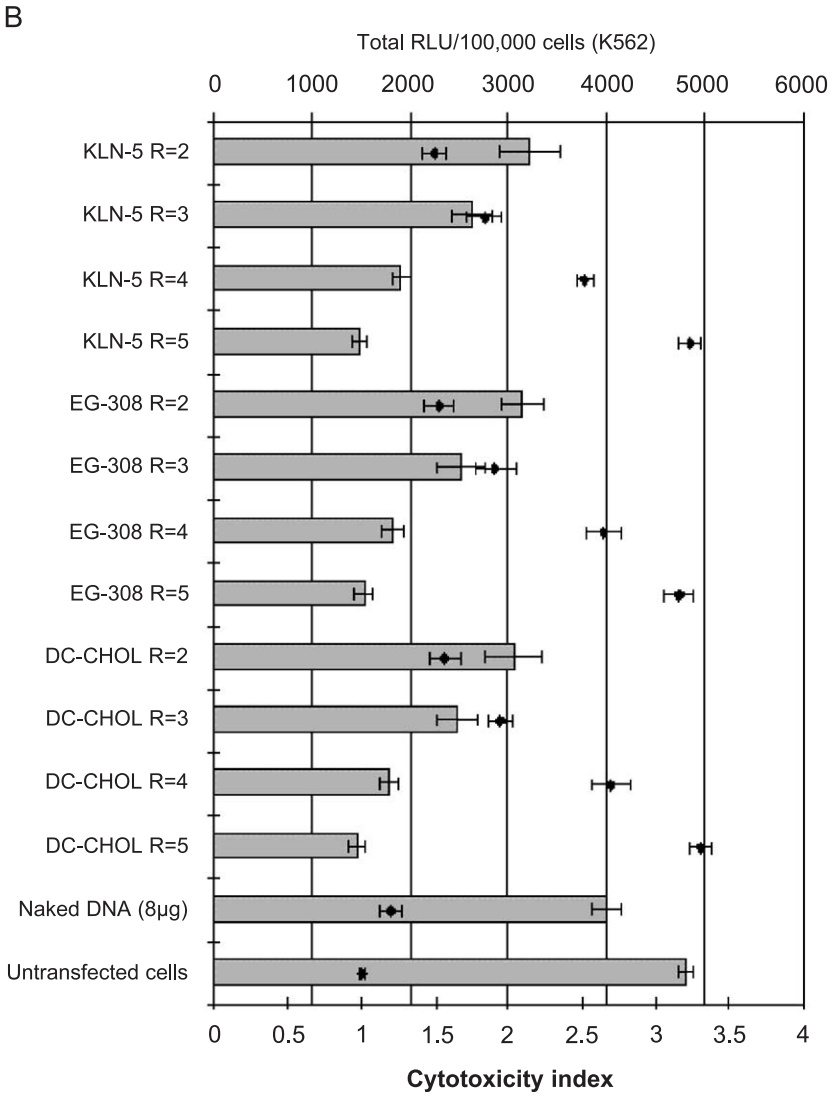

Fig. 4 (*continued*).

(P<0.022) and despite the presence of a phosphoramide link and three carbons in the spacer, similar to KLN-5. We can hypothesize that in this case, the degradation site is fully accessible and hence early division of the lipoplexes can occur, even considering that increasing the distance from the polar head group should itself have a positive effect on transfection efficiency. Finally, the methyl group which is attached to the nitrogen atom of the phosphoramide link in KLN-5 is more basic than the other associated groups and can be more easily protonized in the cell environment. This in turn can lead to the hydrolysis of the phosphorus-to-nitrogen link, the separation of the hydrophobic domain and the release of the polar head group associated with the pDNA from the endosomal vesicles.

The second effect on transfection efficiency concerns the formulation and can be seen with luminescence and FACS-gal assays. Expression of the transgene is shown to be directly influenced by both the formulation of the lipids (volume or charge ratio) and the type of targeted cells. Although the relationship between transgene expression and the charge ratio is direct, the relationship between expression and volume, 12.5 μg of lipid/4 μg of pDNA and 25 μg of lipid/8 μg of pDNA, is more complex. The difference in transgene expression following treatment with each complex is significant but it is not impressive. Recently, Walker et al. [30] showed that increasing the number of DNA copies leads only to a small increase in the number of transfected cells. Our luminescent results with two volumes of lipoplexes also underline that increasing the amount of DNA delivered does not lead to a systematic increase of positive cells and suggest the implication of additional mechanism such as transcription mechanism of exogenous genetic material, which is not yet fully understood.

The toxicity studies of the different formulations did not reveal any increase in cytotoxicity related to the phosphoramide structure or additional groups added to the phosphonolipids when formulated to produce optimal conditions (charge ratio=2 and 8 μg of pDNA complexed with 25 μl of

lipid) in comparison with the others reagents. The majority of the cytotoxicity seen with lipophosphoramides appears to be related to the charge ratio in a direct relationship but not to the structure of the transfection reagents. Although the mechanism of release induced by the phosphoramide group is not definitively proved, the cytotoxicity and formulation data are of interest because they clearly highlight the feasibility of generating non-viral carriers, which are more efficient than while retaining their low toxicity. However, several variations of the chemical environment of lipophosphoramides need to be tested in order to obtain a synergy, which leads to a gain of efficiency as seen with KLN-5. Considering the improvements from earlier studies, it seems likely that these new compounds could be further improved, for example by changing the ammonium cation to an arsonium cation in the polar head group.

Difficulties in transfecting nonadherent cells with cationic agents have been previously reported [31,32]. However, we observe a viable transfection efficiency, especially with KLN-5, by using the K562 cell line as well as Jurkat and Daudi cells. Transgene expression levels vary depending on the cells used. Indeed, transfection of Jurkat and Daudi cells, derived from T and B-leukemic cells, respectively, can be achieved but transfection of lymphocytes remains difficult. Some of the difficulties could be overcome by changing the lipoplex formulation [33]. In most reports, the tested lipid formulations are prepared with DOPE [28,34] to enhance the fusion of lipoplexes and cell membranes [35]. The common use of DOPE in previous studies can be explained by the high activity frequently observed when associated with the DOTAP reagent either on adherent cell lines or in vivo. In this study, done on nonadherent cell lines in vitro, the formulation of lipophosphoramides in the presence of DOPE was tested but did not enhance transfection efficiency. Furthermore, no matter the charge ratio or lipoplex volume, optimum results are obtained when the compounds are not formulated with DOPE.

Additionally, FACS-gal assays to measure the percentage of transfected and living cells illustrate the correlation between formulation and transfection efficiency and show the positive development of lipophosphoramides. When the charge ratio increases, the percentage of viable and $LacZ^+$ K562 cells decreases. This effect is not due to a lower

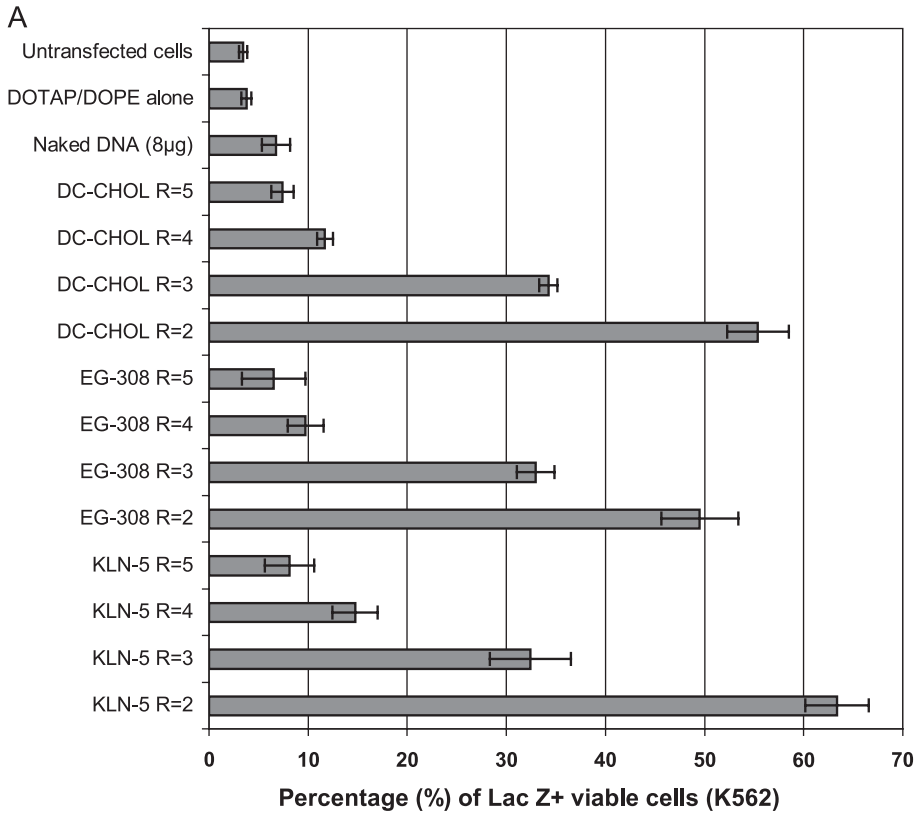

Fig. 5. Flow cytometry analysis to determine the percentage of positive cells among the K562 cells transfected by pCMV-βGal plasmid DNA mixed with the most efficient transfection reagents (DC-CHOL, EG 308 and KLN-5). Cells transfected by free DNA were used to evaluate the transfection capacity of uncomplexed DNA. The autofluorescent background was estimated from untransfected cells and cells in contact with DOTAP/DOPE alone. The cells were assayed 2 days after transfection. (A) summarizes the percentage of $LacZ^+$ viable cells for the most efficient reagents (KLN-5, EG-308 and DC-CHOL) and their different charge ratios. (B) represents the most efficient transfection results for the three reagents with R=2. On the left graph, the purple curves represent the dead cells. The red curves represent the living cells and the cursor (M1) indicates the percentage of positive cells (LAC Z^+) among them. On the right graph, the large zone defines the percentage of the living cells in the whole cell population and the small one defines the dead cells and cellular fragments. The final percentage of $LacZ^+$ viable cells is determined by the proportion of $LacZ^+$ cells not included in the nonviable cell peak.

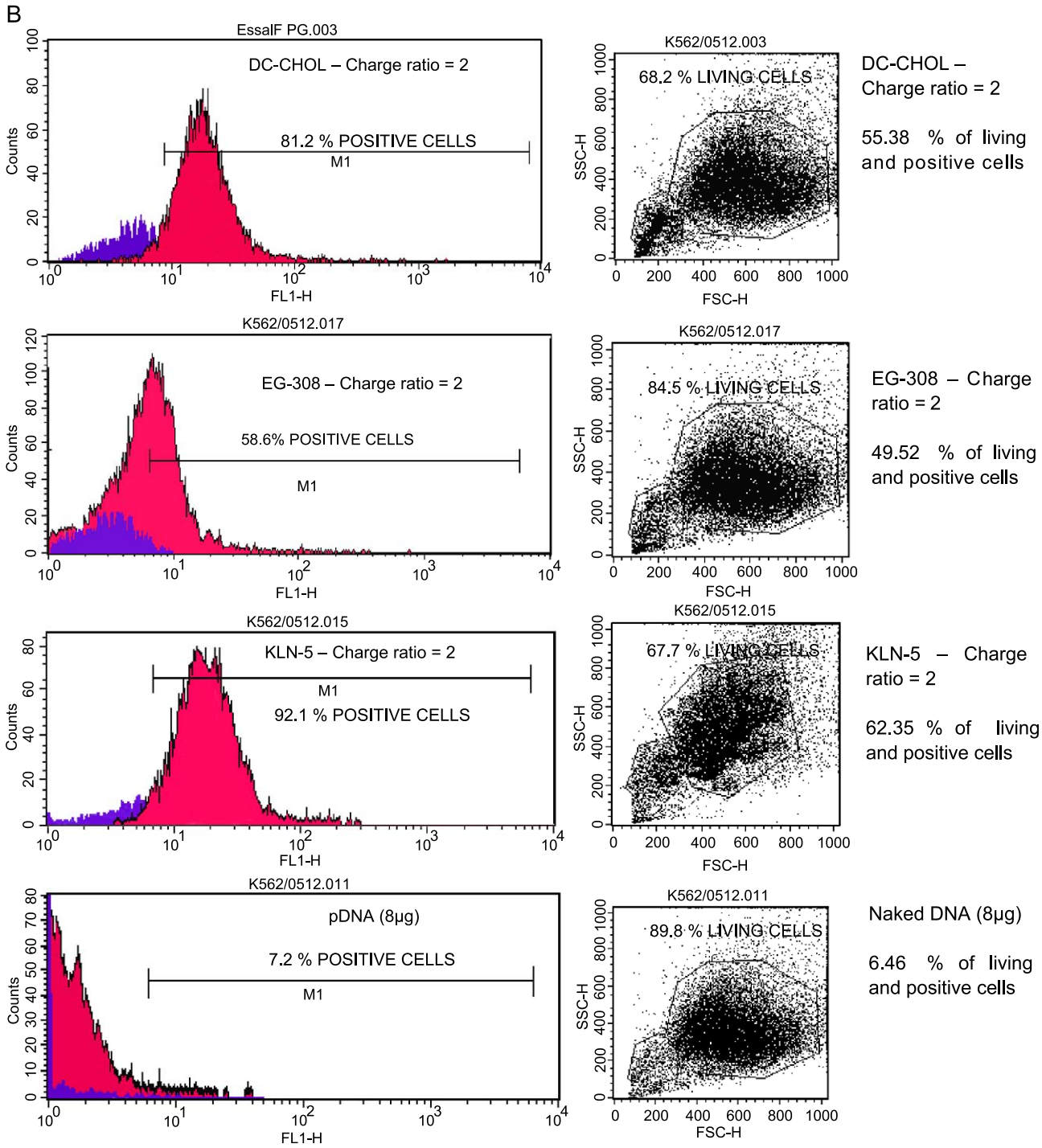

Fig. 5 (*continued*).

transfection capacity but is more likely caused by an increase in cytotoxicity. These results can be explained mostly on the basis of a higher transfection capacity and a lower toxicity level with KLN-5 rather than on the use of the other synthetic delivery systems, except with a charge ratio of five. Indeed, cytotoxicity assays did not show any significant difference between these three transfection reagents, whereas luminescence and FACS-gal experiments highlight the superior transfection capacity of KLN-5. Finally, these results raise the question of gene transfer approach since increasing the charge ratio leads to a decrease in the percentage of living cells and to an increase in the absolute expression level per cell.

Our results also show that the appropriate choice of transfection strategy depends on both maximal gene expression and minimal cytotoxicity. The balance obviously varies depending on whether the transfection is attempted as part of in vitro experiments or as part of ex vivo

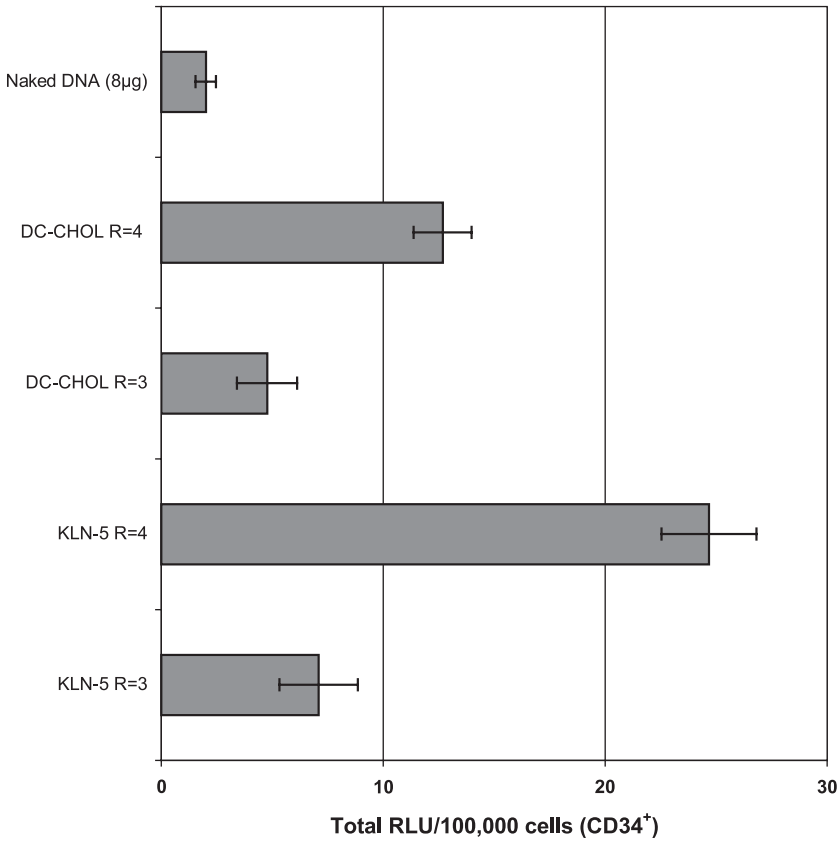

Fig. 6. Ex vivo transfection efficiency of KLN-5 and DC-CHOL on human haematopoietic progenitor $CD34^+$ cells (purity equals 97%) depending on the charge ratio of the complexes. The nonadherent cells were transfected in 24-well plates as described in Materials and methods. Cells transfected with free DNA were used as controls (8 μg) and Luciferase activity was measured using a luminescent assay 2 days after transfection. The values of Total RLU/100,000 cells were obtained by summing the RLU values of eight wells of microtiter plates and were plotted for each reagent and charge ratio. Each data point indicates the mean value of Total RLU/100,000 cells derived from three transfections and the standard deviation of this mean.

observations. Depending on the difficulty in transfecting or isolating the cells, if they are abundant or not, the strategy and the formulation should be adapted in order to optimize the transfection capacity of the reagents. Therefore, chemical innovations guided by mechanistic studies are essential to make progress in the transfection efficiency of all non-viral carriers and these studies should be accompanied by formulation assays and an approach adapted in order to produce optimal conditions.

Regarding cells of clinical interest, such as $CD34^+$, our previous assays using GLB compounds failed [29]. With KLN-5, significant transgene expression was induced in these cells, albeit lower than that observed with the in vitro cell line studies. In addition, the optimal charge ratio is inverted in comparison with the in vitro assays and is observed here at R=4. This phenomenon could be explained by the fact that $CD34^+$ cells do not proliferate much and have low phagocytosis activity, with phagocytosis being the main transfection mechanism in cell lines [14,36]. Consequently, the charge ratio itself induces an electrostatic interaction between the lipoplex and the cell membrane and allows the penetration of the complex into the cell. Under these conditions, the increase of the charge ratio is essential, but the same toxic phenomenon as in in vitro experiments could be problematic. In other words, cytotoxicity occurs when the charge ratio of the lipoplex continually increases. However, we did not verify this point in this work, but previous in vivo studies using phosphonolipids showed that the best results were obtained when the charge ratio was four, whereas higher charge ratios led to hepatic necrosis [11]. These observations support that the ex vivo approach could be closer to in vivo conditions than in vitro models and this approach represents a valuable alternative provided that the reagents can successfully transfect primary cells. This is an additional example of the necessity of adapting the synthetic delivery reagent formulation to the targeted cells, in addition to improving chemical structures of the reagents for higher transfection efficiency.

In conclusion, efficient gene delivery into primary cells and hard-to-transfect cell lines has been a challenge for non-viral systems. Although the initial transfection results reported here are still low in comparison with those obtained with viruses, these new molecules demonstrated their relevant effectiveness directed towards haematopoietic cell lines and $CD34^+$ human progenitor cells. This increase represents a strong improvement over what was previously seen with non-viral systems, and while the transfection efficiency of $CD34^+$ cells is still below clinically beneficial

levels, the results generated with monocationic lipophosphoramides support the use of these reagents and provide the first step to their successful therapeutic applications. Efficient and safe gene transfer into haematopoietic cells could become a highly valuable therapeutic tool for the treatment of genetic haematological disorders with severe defects, such as adenosine deaminase deficiency, or for the development of anti-tumors strategies such as the induction of a specific immune response [37].

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