Combinatorial Synthesis of New Cationic Lipids and High-Throughput Screening of Their Transfection Properties

Karl Lenssen,[b] Peter Jantscheff,[a] Günter von Kiedrowski,[b] and Ulrich Massing*[a]

Here we describe the first synthesis – screening approach for the identification and optimization of new cationic lipids for gene transfer in various cell lines. Combinatorial solid-phase chemistry was used to synthesize a library of new cationic lipids based on 3-methylamino-1,2-dihydroxypropane as the polar, cationic lipid part. As the nonpolar lipid part, different hydrocarbon chains were bound to the amino group of the scaffold and the amino group was further methylated to afford constantly cationic lipids. Lipids were synthesized in both configurations and as racemates, and the counter ions were also varied. By using a fully automated transfection screening method and COS-7 cells, the cationic lipid N,N-*ditetradecyl*-N-*methyl-amino-2,3-propanediol (KL-1-14) was identified as a candidate lipid for the development of an improved transfection reagent. Screening the transfection properties of KL-1-14 in numerous combinations with the helper lipids dioleoylphosphatidylethanolamine (DOPE) and cholesterol (Chol) revealed that Chol is the most suitable helper lipid and the best KL-1-14/Chol ratio is 0.5 – 0.7. Compared to the standard transfection lipid* N-*[1-(2,3-dioleoyloxy)propyl]*-N,N,N-*trimethylammonium methyl sulfate (DOTAP), transfection efficiency was improved by a factor of about 40. Furthermore, by using* R- *and* S-*configured KL-1-14, it could be shown that the configuration of the lipids had no significant influence on its transfection efficiency. The highest transfection efficiencies were achieved with chloride as the counter ion. The new lipofection reagent was further tested to transfect the cell lines MDA-MB-468, MCF-7, MDCK-C7, and primary dentritic cells (DC), which are important for the development of new anticancer gene therapy strategies. Even in these cells, KL-1-14/Chol (1:0.6) had improved transfection efficiencies, which were about two to four times higher than for DOTAP.*

KEYWORDS:

cationic lipids · combinatorial chemistry · high-throughput screening · lipofection · solid-phase synthesis

Introduction

Gene therapy is a promising strategy for treating acquired or inherited genetic diseases such as cancer or cystic fibrosis[1] and was first suggested in the 1970s.[2] However, despite more than 600 gene therapy studies that have been performed during the last decade,[3] very little or no success (in terms of curing patients) has been reported.

The fundamental hurdle in the transfer of gene therapy from the experimental stage to clinical practice is the development of vectors to deliver genetic material (therapeutic genes) to the appropriate cells in a specific, efficient, and safe manner. This problem of "drug delivery", in which the drug is a gene, is particularly challenging for genes which are large and complex and which require targeting to the nuclei of cells. Most of the vectors currently in use for clinical gene therapy trials are based on attenuated or modified versions of viruses.[4] Although efficient, there are serious safety problems associated with viral vectors including possible activation of the patients immune system, risk of infection with traces of the wild type virus, or insertion mutagenesis.[5] These risks were dramatically underscored by the death of an 18-year-old man in an adenovirus-based gene therapy study in 1999.[6]

A promising alternative to viral gene transfer is gene transfer mediated by cationic lipids (lipofection).[7] Cationic lipids have the advantage of directly interacting with the polyanion DNA, thereby forming lipid – DNA complexes. These mostly positively charged complexes, or lipoplexes, are supposed to bind to and be endocytosed by (negatively charged) cells.[8] The transferred genetic material (DNA, RNA, or oligonucleotides) then has to escape lysosomal degradation and to enter the nucleus to reach its target.[9] Lipofection has some advantages over viral transfection in that there is no restriction on the size of the therapeutic gene and no risk of immunogenicity or infection.[10] Thus, lipofection in vivo can be principally performed several times.[11] Furthermore, cationic lipids can be synthesized in large quantities with relatively little effort. However, in contrast to viral gene transfer, the efficiency of lipofection is still poor,[12] and the mechanism of lipofection is only partly understood. Nonetheless, due to the superior safety profile of cationic lipids and for

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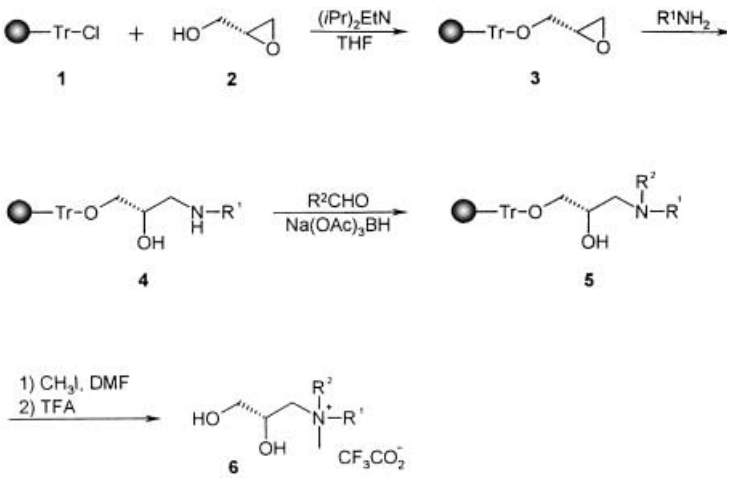
manufacturing reasons, 13% of all gene therapy studies performed so far have been based on lipofection.[3]

Despite several different cationic lipids that have been synthesized and tested for transfection purposes,[13] only a few systematic structure – activity (transfection efficiency) relationship studies have been performed.[14] However, a general structure – transfection-efficiency relationship for cationic lipids could not be drawn from these studies. One reason for the difficulties in linking the chemical structure of cationic lipids with their transfection efficiency is that the structure is not directly responsible for the transfection behavior. Transfection efficiency depends predominantly on the biophysical characteristics of the cationic lipid aggregate (for example, liposomes, lipoplexes), which are only in part dependent on the chemical structure of the lipids. In a previous study with analogues of the transfection lipid *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate (DOTAP)[15] which differ in their nonpolar hydrocarbon chains, it could be shown that the transfection efficiency strongly depended on the biophysical properties of the resulting liposomes and lipoplexes.[16] Minimal alterations of biophysical properties by using lipids with different hydrocarbon chains or by mixing the lipid with different helper lipids could completely allow or prevent transfection. Prediction of transfection properties from the lipid structure was not possible.

Thus, to optimize lipofection, a synthesis – screening approach was performed. The approach described here comprises synthesis of systematically modified cationic lipids by solid-phase combinatorial chemistry, and subsequent testing of the novel lipids for their transfection behavior towards various target cells in a high-throughput screening (HTS).[17] The goal was to identify promising new transfection lipids and, in further steps, to develop optimized lipofection protocols for different cell types.



Scheme 1. *Combinatorial solid-phase synthesis of* N,N-*dialkyl*-N-*methyl-amino-2,3-propanediol with alkyl groups of different length. THF = tetrahydrofuran, DMF =* N,N-*dimethylformamide, TFA = trifluoroacetic acid.*

Results and Discussion

Chemistry

The aim of the synthetic part of the project was the development of a suitable technology for the multiple and combinatorial solid-phase synthesis of new cationic (transfection) lipids. The developed strategy was based on the utilization of 4-methoxytrityl chloride resin. To gain access to a large number of compounds, only commercially available building blocks were used. Protecting groups were omitted if possible. Our synthetic strategy resulted in a new class of cationic lipids **6** (Scheme 1). The structure is based on 3-methylamino-1,2-dihydroxypropane as the polar, cationic lipid part. Different hydrocarbon chains are bound to the amino group of this scaffold to form the nonpolar lipid part. The amino group is further methylated to get a constantly cationic lipid. This synthetic strategy allows us to synthesize the new lipids in different configurations and with different counter ions.

The general synthetic route for the preparation of the cationic lipid **6** is outlined in Scheme 1. The synthesis started with the immobilization of (*R*)-2,3-epoxy-1-propanol (**2**) on the 4-methoxytrityl chloride resin **1**.[18] Reaction of the epoxide **3** with a long-chained amine yielded the polymer-bound secondary amine **4**, with was converted into the tertiary amine **5** by reductive amination.[19] Quaternization of the tertiary amine with methyl iodide,[20] and cleavage from the solid phase gave the cationic lipid **6**, which was further purified by preparative HPLC. Each step of the synthesis could be monitored by means of high-resolution magic angle spinning NMR (HRMAS-NMR) spectroscopy. Lipids with different alkyl chain length, chirality, and counter ions were prepared by this synthetic route (see Table 1). Larger amounts of racemic lipid KL-1-14 containing two tetradecyl hydrocarbon chains were synthesized by alkylation of 3-methylamino-1,2-propanediol with 1-bromotetradecane.

Table 1. *Cationic lipid library of* N,N-*dialkyl*-N-*methyl-amino-2,3-propanediols with alkyl groups of different length.*[a]

Name	R^1	R^2	Name	R^1	R^2
KL-1-1	C_{10}	C_8	KL-1-9	C_{16}	C_{10}
KL-1-2	C_{12}	C_8	KL-1-10	C_{18}	C_{10}
KL-1-3	C_{14}	C_8	KL-1-11	C_{12}	C_{12}
KL-1-4	C_{16}	C_8	KL-1-13	C_{18}	C_{12}
KL-1-5	C_{18}	C_8	KL-1-14	C_{14}	C_{14}
KL-1-6	C_{10}	C_{10}	KL-1-15	C_{14}	C_{16}
KL-1-7	C_{10}	C_{12}	KL-1-16	C_{18}	C_{14}
KL-1-8	C_{10}	C_{14}	KL-1-17	C_{16}	C_{12}

[a] All lipids were synthesized in the *R* or *S* configuration or as a racemate. Chloride, sulfate, methylsulfate, and acetate were used as counter ions. For R^1 and R^2, see Figure 1.

Screening/lipofection studies

For a broad and reproducible testing of the transfection properties of the new systematically varied cationic lipids, we recently developed a fully automated high-throughput system. This system, which comprises the entire lipofection process from liposome formation to reporter gene assay, allows the identi-

fication of candidate cationic lipids and the development of suitable lipofection reagents and protocols based on those lipids. Automation of this process was necessary, because manually testing the various cationic lipids or lipid mixtures was extremely labor-intensive and high reproducibility could otherwise not be guaranteed.

Screening the combinatorial lipid library for lipofection properties: The goal of this first step was to find the cationic lipid with the best hydrocarbon chain composition for the development of suitable lipofection reagents and protocols. All the *R*-configured lipids with different hydrocarbon chains (lipids KL-1-1 to KL-1-17, Table 1) and with chloride as the counter ion were tested by using a standard protocol: from each lipid, eight different lipoplexes containing equimolar amounts of the helper lipid dioleoylphosphatidylethanolamine (DOPE) were formed by using eight different DNA/lipid charge ratios from 1:1 to 1:15, resulting in a lipofection profile for each lipid (Figure 1). The COS-

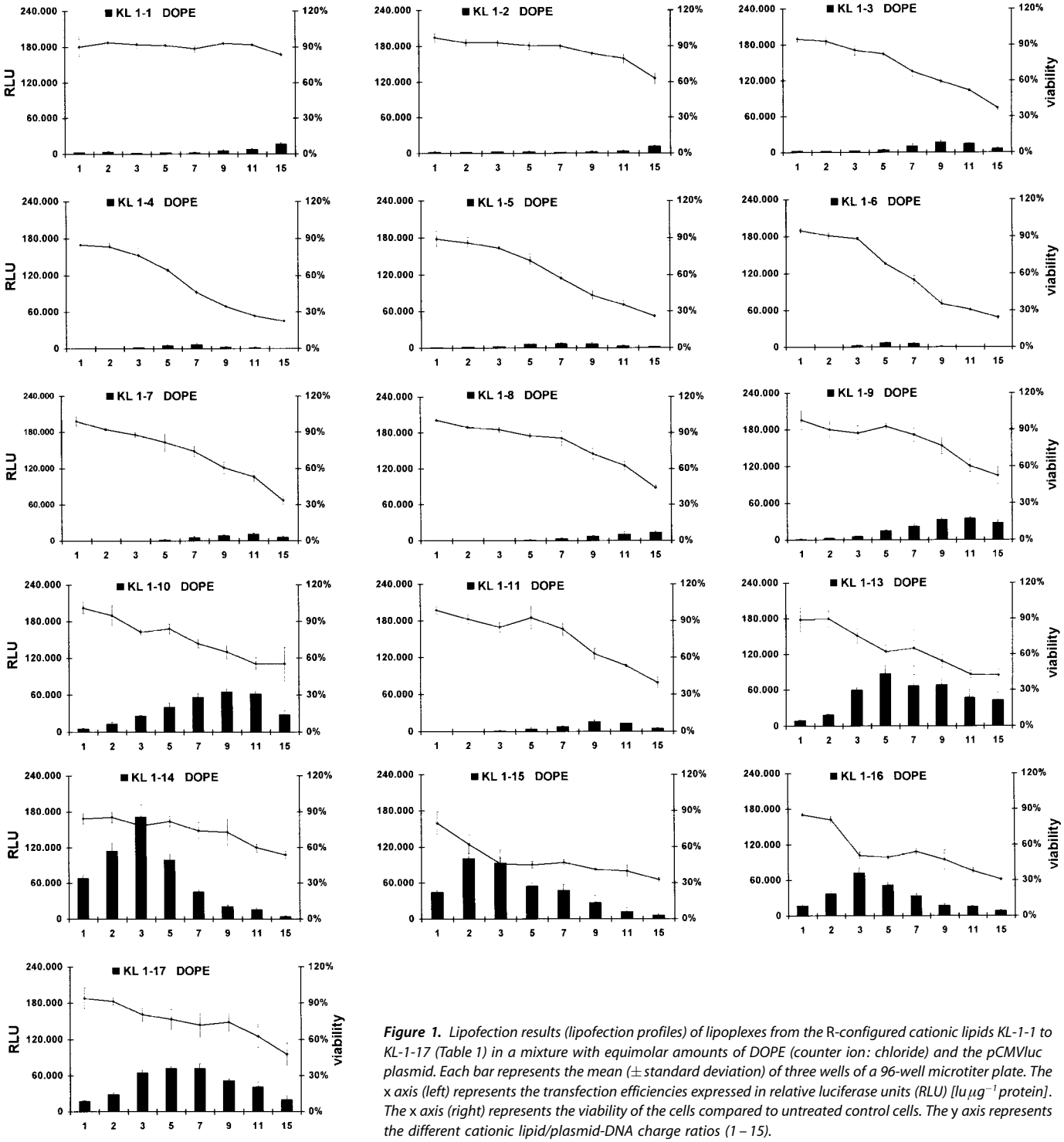


Figure 1. *Lipofection results (lipofection profiles) of lipoplexes from the* R*-configured cationic lipids KL-1-1 to KL-1-17 (Table 1) in a mixture with equimolar amounts of DOPE (counter ion: chloride) and the pCMVluc plasmid. Each bar represents the mean (*$\pm$*standard deviation) of three wells of a 96-well microtiter plate. The* x *axis (left) represents the transfection efficiencies expressed in relative luciferase units (RLU) [lu* μg^{-1} *protein]. The* x *axis (right) represents the viability of the cells compared to untreated control cells. The* y *axis represents the different cationic lipid/plasmid-DNA charge ratios (1 – 15).*

7 cell line was used for this first lipofection study, since COS-7 is easy to transfect and differences between the lipids are easy to identify. For comparison, the transfection efficiency of the well-known cationic lipid DOTAP at its best lipid/DNA charge ratio of 2.5 was also determined in each run.

All new lipids had high transfection efficiencies ranging from about 11 000 – 170 000 lu μg^{-1} protein, which corresponds to 250 – 3860 % of the DOTAP values. The transfection efficiencies strongly depended on the hydrocarbon chains of the lipids. In general, increasing the overall length of the hydrocarbon chains resulted in higher transfection efficiencies. An overall length of at least 28 CH units seems to be necessary for transfection efficiencies tenfold higher than those of DOTAP. But, the combination of the hydrocarbon chains also seems important. Of the lipids 10, 14, and 17, which all have a total of 28 CH units, the most effective was lipid 14 which bears two C_{14} hydrocarbon chains.

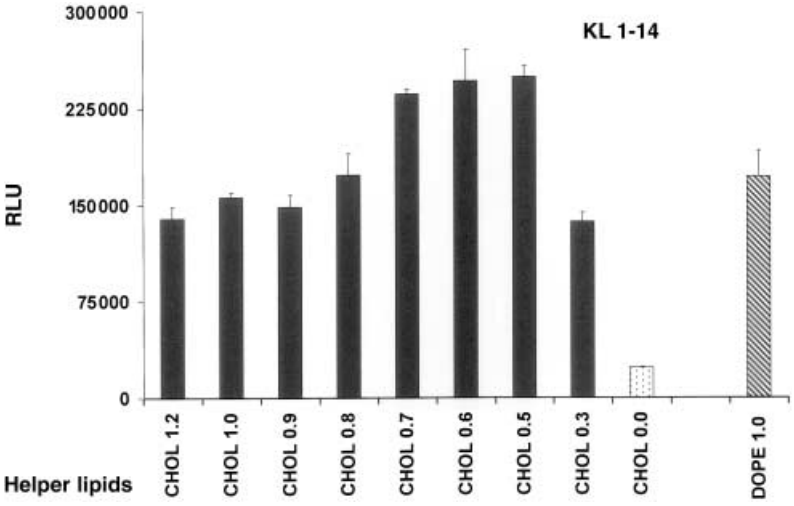


Figure 2. *Maximum lipofection efficiencies in RLU [lu μg^{-1} protein] of the most effective lipoplexes of* R*-configured KL-1-14 in a mixture with Chol in different Chol/KL-1-14 ratios (0.3 – 1.2; counter ion: chloride) and the pCMVluc plasmid (charge ratio: seven or nine). Each bar represents the mean (± standard deviation) of three wells of a 96-well microtiter plate.*

The transfection profiles of the most effective lipids of this group are similar and have a peak (highest transfection efficiency) for lipid/DNA ratios of two to five. For the most effective lipids, the viability of the cells at maximum transfection efficiency usually decreased to roughly 50 %. An exception is the lipid 14, which has the highest transfection efficiency as well as only a minor toxicity of about 70 % viability. We therefore chose lipid 14 for the further development of a versatile transfection reagent.

Influence of helper lipids on transfection efficiencies: The previous screening experiments were performed with lipoplexes containing equimolar amounts of the helper lipid DOPE. Here, the influence of different ratios of the helper lipids DOPE and cholesterol (Chol) on transfection efficiency of KL-1-14 were tested. The transfection behavior of KL-1-14 without any helper lipid was also tested.

Transfection efficiency of KL-1-14 without helper lipids was very low and reached only about twice the transfection efficiency that was found for the standard lipid DOTAP. Independent of the amount of DOPE incorporated into the lipoplexes (ratios of DOPE/KL1-14: 0.3, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, and 1.2), transfection behaviors (maximum transfection efficiencies and transfection profiles) of all mixtures were similar and comparable to the profile of KL-1-14/DOPE (1:1) as shown in Figure 1 (individual data for all mixtures are not shown).

Using Chol as the helper lipid for KL-1-14, the transfection efficiencies were no longer similar for the different Chol/KL-1-14 ratios (Figure 2). The highest transfection efficiencies were found for Chol/KL-1-14 ratios of 0.5 – 0.7. Higher or lower ratios led to lower transfection efficiencies, which were similar to those of the KL-1-14/DOPE mixtures. The toxicity of all mixtures was similar and moderate (about 70 % survival at maximum transfection efficiency, data not shown).

Influence of the configuration, methylation, and the counter ion of KL-1-14 on transfection efficiency: We compared the transfection efficiency and toxicity of KL-1-14 synthesized in the *R* and *S* configurations (with 0.6 mol % Chol as the helper lipid (see above)). The transfection efficiencies for both lipids were statistically similar. Thus, for further experiments, KL-1-14 was synthesized as the racemate.

Methylation of KL-1-14 was an important prerequisite for its transfection properties. A KL-1-14 analogue that was not methylated did not transfect at all. It could be assumed that the nonmethylated KL-1-14 was not sufficiently protonated at physiological pH values so that the formation of a bilayer structure from these lipids was not possible. As previously shown for DOTAP analogues, formation of a lipid bilayer is an important prerequisite for a cationic lipid to be a transfection lipid.[16, 21]

We further investigated the influence of four different counter ions on the transfection behavior of KL-1-14 (methylsulfate, sulfate, chloride, and acetate). As shown in Figure 3, the use of chloride as counter ion resulted in the highest transfection efficiency. With methylsulfate or sulfate as the counter ion, transfection efficiency was reduced to 70 – 73 % of the values found for chloride. Use of acetate as the counter ion led to the lowest transfection efficiency, which was only 57 % of the chloride values.

Transfection properties toward different cell lines: For testing the transfection properties of KL-1-14 toward the mamma carcinoma cell lines MDA-MB-468 and MCF-7, the polarized cell line MDCK-C7, and the primary dentritic cells, KL-1-14 was used in its racemic form with chloride as the counter ion and as a mixture with 60 mol % Chol. The transfection efficiencies were generally lower relative to those found for COS-7 cells (see above) as shown in Table 2. For the mamma carcinoma cell lines MDA-MB-468 and MCF-7, transfection efficiencies were reduced by a factor of about 10, for the MDCK-C7-cells by a factor of about 80, and for the dentritic cells by a factor of about 500.

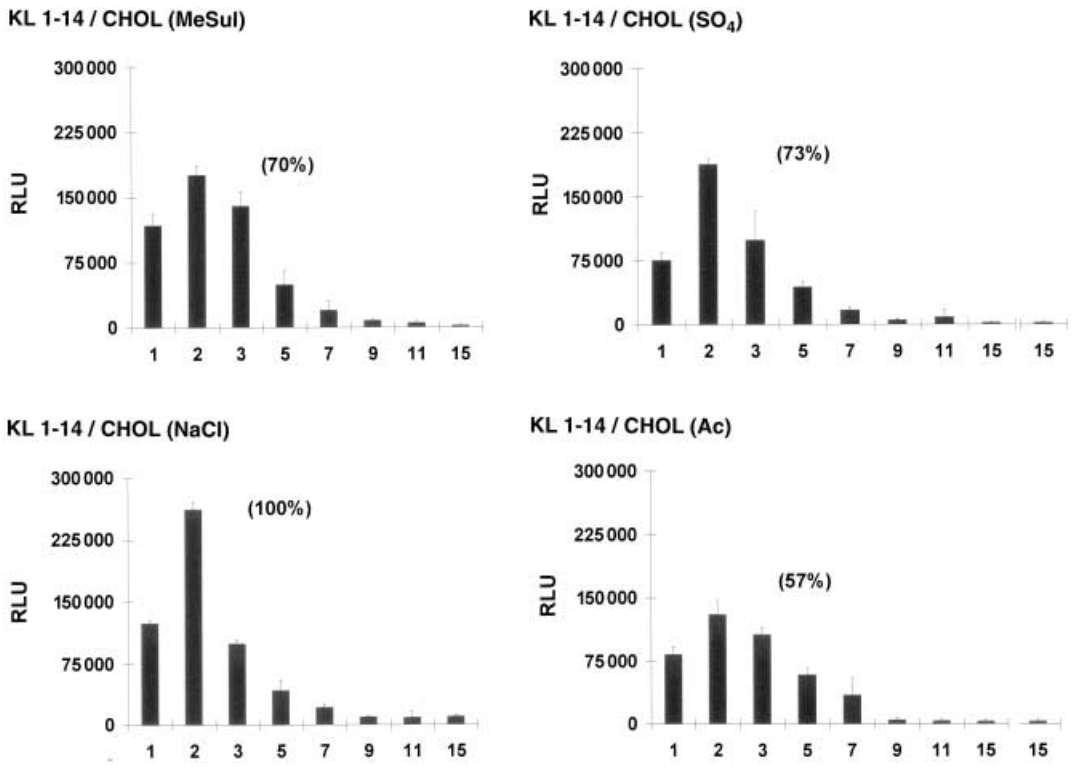


Figure 3. *Lipofection results (lipofection profiles) of lipoplexes from the racemic KL-1-14 in a mixture with Chol (ratio 0.7) with different counter ions (MeSul = methylsulfate, SO_4 = sulfate, Cl = chloride, Ac = acetate) and the pCMVluc plasmid. Each bar represents the mean (± standard deviation) of three wells of a 96-well microtiter plate.*

Table 2. *Transfection efficiencies of KL-1-14 in a mixture with Chol (ratio: 0.7) or DOPE (ratio: 1.0) toward the mamma carcinoma cell lines MDA-MB-468 and MCF-7, the polarized cell line MDCK-C7, and the primary dentritic cells.[a]*

Lipofection reagent	Mamma carcinoma cells		Polarized cells	Primary cells
	MDA-MB-468	MCF-7	MDCK-C7	DC (5 days)
KL-1 – 14/Chol	19640 ± 8725 (n = 6), 424 %[b]	19985 ± 19295 (n = 5), 393 %[b]	2490 ± 175 (n = 3), 187 %[b]	395 ± 135 (n = 3), 134 %[b]
KL-1-14/DOPE	17515 ± 5760 (n = 5), 378 %[b]	9250 ± 1865 (n = 5), 182 %[b]	–	410 ± 115 (n = 3), 139 %[b]
DOTAP	4630 ± 910 (n = 9)	5085 ± 3005 (n = 7)	1330 ± 165 (n = 3)	295 ± 160 (n = 3)

[a] Transfection efficiencies of the KL-1-14 lipoplexes were compared to the transfection efficiency achieved with the standard transfection lipid DOTAP. Results were given in RLU [lu μg^{-1} protein] and, for easier comparison, standardized on the lipofection efficiency of DOTAP-lipoplexes which was set to 100 %. [b] Compared to the respective DOTAP value.

Nevertheless, the transfection efficiencies found for KL-1-14/Chol (1:0.6) were generally higher than with DOTAP. For the mamma carcinoma cell lines, transfection efficiencies with KL-1-14/Chol (1:0.6) were four times higher than with DOTAP. For MDCK-C7 and dentritic cells, the increase was 1.3 – 2.5-fold. We also tested KL-1-14 as an equimolar mixture with DOPE for its transfection efficiencies toward the mamma carcinoma cell lines and the dentritic cells. Again, transfection efficiencies were greatly reduced even for the KL-1-14/DOPE mixture and were similar to the values found for KL-1-14/Chol (1:0.6).

Conclusion

Improving and easing the process of transfection is the key to establishing gene therapy in clinical practice. Today, compared to viral transfection techniques, lipofection is less effective, but has strong advantages concerning safety aspects, versatility, and manufacturing aspects. To become a routinely used method, lipofection has to be improved.

This study describes the first synthesis – screening approach for the identification and optimization of new cationic lipids for gene transfer in various cell lines. For the first time, combinatorial solid-phase chemistry was used to synthesize a library of new cationic lipids, differing in their nonpolar lipid part, configuration, and counter ions.

From the combinatorial cationic lipid library, the lipid *N*,*N*-ditetradecyl-*N*-methyl-amino-2,3-propanediol (KL-1-14) was identified by systematic screening as a suitable lipid for the development of a new transfection reagent. Continuing systematic investigations of the transfection conditions revealed that Chol is a suitable helper lipid, and that the best KL-1-14/Chol ratio is 0.5 – 0.7. Furthermore, it could be shown that the

configuration of the lipids has no influence on the transfection efficiency and that use of chloride as the counter ion led to the highest transfection efficiencies.

By using this new transfection reagent, we compared its transfection behavior toward various cells, which are important for the development of new gene therapy strategies. It could be demonstrated that even in these cell types KL-1-14/Chol (1:0.6) had improved transfection efficiencies, which were 1.3 – 8 times higher than those with the well-known cationic lipid DOTAP.

The synthesis – screening approach presented in this study is a powerful tool to identify promising cationic transfection lipids and to optimize the transfection protocol for individual cell lines. Thus, this method might also be useful for the identification of optimal transfection reagents for therapeutic gene transfer. Further studies with additional cell systems and new lipid libraries might contribute to transfer gene therapy from an experimental stage to a widely used clinical standard.

Experimental Section

General: THF was distilled over sodium prior to use. CH_2Cl_2 and DMF were distilled over CaH_2. 4-Methoxytrityl chloride resin was purchased from Advanced Chemtech. All other chemicals were of the highest commercial purity and purchased from Sigma, Fluka, or Acros. ^{1}H HRMAS-NMR spectra (600 MHz) were recorded on a Bruker DRX-600 instrument in $CDCl_3$. The identities of the products were verified by MALDI-TOF mass spectrometry (2,5-dihydroxybenzoic acid (DHB) as matrix) and ^{1}H NMR spectroscopy (400 MHz). Lipid libraries were synthesized by a SYRO II parallel synthesizer. Preparative HPLC purification was performed on a Shimadzu LC-8A chromatograph, equipped with an evaporative light scattering detector (column material: Kromasil 5 NH_2; $CHCl_3$/MeOH (95:5)).

General synthetic route for the preparation of *N,N*-dialkyl-*N*-methyl-amino-2,3-propanediols:

As an example, the synthesis is described for the lipid KL-1-17 containing a C_{16} and a C_{12} hydrocarbon chain (Table 1).

Immobilization reaction: A solution of (*R*)-2,3-epoxy-1-propanol (**2**; 65 mg) and *N,N*-diisopropylethylamine (135 mg) in anhydrous THF (4 mL) was added to 4-methoxytrityl chloride resin **1** (50 mg, 0.085 mmol). After stirring the suspension for 48 h, the resin was filtered, washed several times with CH_2Cl_2/methanol/*N,N*-diisopropylethylamine (17:2:1), THF, and CH_2Cl_2, and dried in vacuo. ^{1}H HRMAS-NMR (600 MHz, $CDCl_3$): $\delta = 2.5$ (s; CHOC*H*H), 2.7 (s; HOC*H*H), 3.0 (s; CH_2C*H*O), 3.3 (s; CHOCH*H*) 3.4 (s; HOCH*H*), 3.7 (s; OCH_3) ppm.

Synthesis of secondary amine 4: The resin-bound epoxide **3** was suspended in absolute ethanol (4 mL). Hexadecyl amine (210 mg; dodecyl, tetradecyl, and octadecyl amine were also used) was added, and the reaction mixture was heated to 65 °C for 12 h. The resin was filtered, washed several times with ethanol, THF, and CH_2Cl_2, and dried in vacuo. ^{1}H HRMAS-NMR (600 MHz, $CDCl_3$): $\delta = 0.9$ (s; CH_3), 1.3 (s; CH_2), 3.6 (s; OCH_2, CHO, CH_2N), 3.7 (s; OCH_3) ppm.

Synthesis of tertiary amine 5: The intermediate resin **4** and sodium triacetoxyborohydride (185 mg) were suspended in anhydrous CH_2Cl_2. Dodecanal (160 mg; octanal, decanal, and tetradecanal were also used) was added and the reaction mixture was stirred for 3 h. The resin was filtered, washed with methanol and CH_2Cl_2, and dried in vacuo. ^{1}H HRMAS-NMR (600 MHz, $CDCl_3$): $\delta = 0.9$ (s; CH_3), 1.3 (s; CH_2), 3.6 (s; OCH_2, CHO, CH_2N), 3.7 (s; OCH_3) ppm.

Quaternization of tertiary amine 5: The resin-bound tertiary amine **5** was suspended in anhydrous DMF. Methyl iodide (120 mg) was added and the mixture was stirred for 12 h. The resin was then filtered, washed with DMF and dichloromethane, and dried in vacuo. ^{1}H HRMAS-NMR (600 MHz, $CDCl_3$): $\delta = 0.9$ (s; CH_3), 1.3 (s; CH_2), 3.6 (s; OCH_2, CHO, CH_2N), 3.7 (s; OCH_3) ppm.

Cleavage of lipid 6 from the resin: Resin-bound lipid **6** was stirred in a 5% solution of trifluoroacetic acid in dichloromethane. The resin was filtered and washed several times with $CHCl_3$/CH_3OH (4:1). The solutions were combined and evaporated under reduced pressure, and the product was purified by preparative HPLC. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 0.8$ (t, 6H, $J = 6.5$ Hz; CH_3), 1.2 (br s, 52H; CH_2), 3.2 (s, 3H; N^+–CH_3), 3.3 (m, 2H; NCH_2), 3.4 (m, 2H; CH_2O), 3.6 (m, 1H; CHO) ppm; MS (MALDI-TOF, DAB): *m/z*: 498.9 $[M]^+$. Generally, yields were in the region of 75 – 85%.

Exchange of counter ions (for example, acetate, chloride, sulfate, or methylsulfate) was achieved by subsequent extraction of a solution of the respective lipid in $CHCl_3$/MeOH (4:1) with a saturated aqueous solution of sodium acetate, sodium chloride, sodium sulfate, or sodium methylsulfate.

Transfection studies:

Cell culture: Mamma CA cell lines MDA-MB468 (American Type Culture Collection (ATCC) HTB-132) and MCF7 (ATCC MTB-22), canine normal kidney epithelial cell line MDCK (NBL-2; ATCC CCL-34), and SV40-transformed African-green-monkey kidney cell line COS-7 (ATCC CRL-1651) were routinely passaged in Dulbecco's modified Eagle's medium (DMEM) and minimum essential medium with Earle's salts (EMEM; both from GIBCO BRL, Karlsruhe, Germany) supplemented with 1% L-glutamin solution (100 ×), 1% penicillin/streptomycin solution (100 ×) (GIBCO BRL, Karlsruhe, Germany), and 10% fetal calf serum (FCS; BioWhittaker, Verviers, Belgium) at 37 °C with 5% CO_2 in a humidified atmosphere. For the screening, cells were maintained in 96-well culture plates (Greiner, Frickenhausen, Germany) in EMEM or DMEM/10% FCS/1% penicillin/streptomycin (200 μL) in a humidified atmosphere at 37 °C with 5% CO_2. The cells were seeded 24 h prior to transfection into the microtiter plate at 5000 – 10000 cells per well to reach a confluence of 50% at the time of transfection.

Dentritic cells: Dendritic cells (DC) were derived from highly purified circulating CD14(+) monocytes. In a first step, peripheral blood mononuclear leukocytes (PBL) were isolated by Ficoll – Paque (Pharmacia Biotech AB, Uppsala, Sweden) density centrifugation from the blood of healthy control persons. The following immunomagnetic purification of the monocytes from PBL was performed by using CD14 Microbeads and MiniMACS high-gradient magnetic separation columns (Miltenyi Biotech, Bergisch Gladbach, Germany) according to manufacturer's instructions. Isolated monocytes (5×10^5 mL^{-1}) were cultured in RPMI-1460 (Gibco BRL, Karlsruhe, Germany) medium supplemented with 10% human recombinant GM-CSF (0.05 μg mL^{-1}) and IL-4 (0.025 μg mL^{-1}; PromoCell, Heidelberg, Germany) in six-well plates. Aliquots of the cells were reanalyzed by fluorescence activated cell sorter (FACS) with anti-CD14 monoclonal antibodies (mAbs; Pharmingen, San Diego, CA). After 5 – 6 days, nonadherent cells were collected and the purity of DCs was determined by FACS analysis with a FACSort flow cytometer (Becton Dickinson, Mountain View, CA) and the CellQuest software for data processing as recently described elsewhere.[22] For this FACS analysis, HLA class I (clone W6/32), HLA-DR (clone HB55; Dr. G. DeLibero, Basel, Switzerland), CD1a, CD3, CD11c, CD14, CD54, CD80, CD83, and CD86 specific mAbs (Pharmingen, San Diego, CA), and

R-PE-goat anti-mouse immunoglobulin (Ig; Southern Biotechnology Associates, Inc., Birmingham, AL) were used. Binding specificity was determined by using IgG1 (MOPC-21), IgG2a (UPC-10), or IgG2b (MOPC-141) isotype control mAbs (Sigma-Aldrich Chemie, Buchs, Switzerland).

Transfection vector: Vector pEGFPLuc (BD Biosciences Clontech, Palo Alto, CA) is a commercially available reporter vector encoding a fusion protein of green fluorescent protein (GFP) and luciferase (Luc). All information is available at the Clontech homepage (http://www.clontech.com/techinfo/vectors/vectorsE/pEGFPLuc.shtml).

Determination of the transfection properties: Determination of the transfection behavior of the new lipids was performed by using an automated assay system as described in detail by Regelin et al.[17] In brief, the method consists of two parts: 1) the preparation of liposomes and formation of lipoplexes from the different lipids, and 2) cell transfection and determination of transfection properties.

1. Pretransfection assays: Cationic lipids in organic solvents were transferred into glass test tubes. The organic solvents were removed under a stream of nitrogen to create a thin lipid film on the surface of the glass tube. 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffer (20 mM plus 130 mM NaCl, pH 7.4) was added and the tubes were transported into a water bath sonicator in which a dispersion of small cationic liposomes is formed. The liposomal dispersions are distributed to the 96 wells of a deep-well plate, and reporter plasmid DNA is added to allow formation (1 h) of the lipid/DNA complexes (lipoplexes). For the subsequent quantification of transfection efficiency (part 2), a reporter plasmid carrying the firefly luciferase gene under the control of the CMV promoter is used to form lipoplexes.

2. Transfection assays: After lipoplex formation, the robot took the cell culture microtiter plates from the incubator and removed the lid from the plates. Immediately prior to transfection, culture medium (100 μL) was carefully removed from each well by the robot and lipoplex dispersions (90 μL) were dispensed into the wells (triplicates). The FCS concentration during transfection was therefore 5.3%. The lids were replaced and the microtiter plates (MTP) were returned to the incubator. After 4 h, the cells were automatically retrieved, the cell monolayers were carefully washed by using the special drop mode of the plate washer, fresh medium was added, and the cells were incubated for a further 42 h before harvesting. The MTP containing the cells were transported to the robot, the medium was removed, and the cells were washed by using the drip mode of the microplate washer and then lysed. Cell lysates were diluted and aliquots were transferred to white microplates for the luciferase activity assay and transparent plates for the bicinchoninic acid (BCA) protein assay (Interchim, Montlucon, France). Assay-specific standards and controls were added to the microplates, and luciferase activity and protein content of the lysates were measured. Transfection efficiencies were calculated by dividing luciferase activity by protein content. Dividing protein content of transfected cells by protein content of nontransfected control cells resulted in a relative measure of cytotoxicity.

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