Novel Cationic Lipids Based on Malonic Acid Amides Backbone: Transfection Efficacy and Cell Toxicity Properties

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Gene delivery using nonviral approaches has been extensively studied as a basic tool for intracellular gene transfer. Despite intensive research activity, the aim of creating a vector which meets all necessary demands has still not been reached. One possibility to solve the nonviral vector associated problem of low transfection efficacy is the development of new cationic amphiphiles. Therefore, the non-glycerol-based cationic lipids **1**−**9** have been synthesized and tested for in vitro gene delivery experiments. The backbone structure of the lipids consists of a malonic acid diamide with two long hydrophobic chains. The degree of saturation of the hydrophobic chains and the structure of the polar cationic headgroup were varied. The preparation follows an easy process and facilitates the trouble-free insertion of different alkyl chains. By studying in vitro gene delivery an increase of transfection efficacy was observed when using at least one unsaturated alkyl chain in the hydrophobic part and lysine or bis(2-aminoethyl)aminoethylamid as hydrophilic headgroup. This leads to cationic lipids exhibiting comparable or even higher transfection efficacies compared to the commercially available LipofectAmine and SuperFect.

INTRODUCTION

Without doubt, gene therapy is a very promising method for the treatment of cancer and genetic diseases which are still insufficiently treatable (*1–3*). Although a lot of work has been done creating efficient gene shuttles and progress has been made over the past years, there exists no satisfying gene transfer system. In addition to the application of viral vectors, the design and synthesis of new cationic lipids is a very promising tool for gene therapy. While the transduction with modified viruses is very efficient especially for in vivo gene transfer, viruses have eminent disadvantages due to their immunogenic potential and their possibility to induce cancer (*4–7*). Nonviral gene delivery systems have, on one hand, been demonstrated as standard tools for in vitro transfection with currently more than 60 commercially available kits (*8*). On the other hand, they are regarded as a promising approach for the treatment of genetic diseases (*9, 10*) and cancer (*11–13*). However, nonviral gene delivery suffers from low transfection efficiency. Surely this is the main reason that these gene delivery systems have just a marginal stake in clinical trials (*14*). For this reason, it is of great importance to advance nonviral gene transfer systems. One possibility is the development of new, efficient, and nontoxic cationic lipids.

There are mainly three structures of cationic amphiphiles used for gene delivery: glycerol derived lipids and cholesterol-based and non-glycerol-based compounds. The group of glycerol derived lipids includes in addition to *N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammoniumchloride (DOTMA)[1] , first described by Felgner in his pioneering work (*15*), 1,2-dioleoyloxy-3-(trimethylammonio)propane (DOTAP), 1,2-dimyristyloxypropyl-3-dimethyl(hydroxyethyl)ammoniumbromide (DMRIE), and 2,3-dioleyloxy-*N*-[2-(spermincarboxamido)ethyl]-*N,N*-dimethyl-1-propane-ammoniumtrifluoracetate (DOSPA) (*16–18*). Substances that exhibit no glycerol backbone are also capable of transfering genes into cells, especially by the usage of *N*-methyl-4-(dioleyl)methylpyridiniumchloride (SAINT-2) or 1-[2-(oleoyloxy)ethyl]-2-oeyl-3-(2-hydroxyethyl)imidazoliniumchloride (DOTIM) (*19, 20*).

For a number of these and other cationic amphiphiles, it was shown that hydrophobic chains as well as the polar head and even the connecting backbone have a great influence on the transfection efficiency (*16, 19, 21–23*). Several groups have been working on establishment of a structure−function relationship to define the requirements for safe and efficient gene transfer with cationic lipids (*16, 19–26*).

We have now designed a new class of cationic lipids consisting of the diamide structure of malonic acid as anchor group for the hydrocarbon chains as well as the cationic headgroup inclusive spacer molecules. The idea for the preparation of these new substances is mainly based on three characteristics: First, the new compounds are easy to produce starting from cost-effective materials, and the syntheses facilitate the trouble-free insertion of different alkyl chains. Second, it may be accepted that the compounds should have biocompatibility due to the amide bond as a connection between polar headgroup, linker moiety, and partially the hydrophobic chains. Third, a positive effect may result from the ability of the malonic diamide structure to build an H-bonded network, which could stabilize the complex formation especially under neutral conditions.

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[1]Abbreviations: DOTMA, *N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammoniumchloride; DOTAP, 1,2-dioleoyloxy-3-(trimethylammonio)propane; DMRIE, 1,2-dimyristyloxypropyl-3-dimethyl(hydroxyethyl)ammoniumbromide; DOSPA, 2,3-dioleyloxy-*N*-[2-(spermincarboxamido)ethyl]-*N,N*-dimethyl-1-propane-ammoniumtrifluoracetate; SAINT-2, *N*-methyl-4-(dioleyl)methylpyridiniumchloride; DOTIM, 1-[2-(oleoyloxy)ethyl]-2-oeyl-3-(2-hydroxyethyl)imidazoliniumchloride; EEDQ, 2-ethoxy-1-ethoxycarbonyl-1,2-dihydrochinoline; BOP, (benzotriazol-1-yloxy)-tris-(dimethylamino)-phosphoniumhexafluorophosphate; DCC, *N,N′*-dicyclohexylcarbondiimid; ONPG, O-nitrophenyl-*β*-galactopyranoside; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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The characteristic of the compounds is the specific feature of hydrophobic chain bound to malonic acid amides backbone. One alkyl chain is bound to C2 of the malonic acid, whereas the second hydrophobic chain was linked via an amide bond to one of the carboxylic groups of the diacid. The two alkyl chains that are necessary for satisfying gene delivery vary in the degree of saturation. Starting from two C16 chains, we replaced them by successive substitution with the oleyl residue whereby the chain was elongated. According to the generally accepted finding that spacer molecules enhance the efficacy of transfection (*27*), at first we used only ethylene diamine and tris(aminoethyl)amine as spacer molecules. These compounds were connected with the malonic acid backbone by a second amide bond. Lipids **1**−**3** with the ethylene amine spacer itself were capable of transfering DNA into cells in culture, admittedly to a minor degree. However, this molecule residue was introduced for binding a proper cationic headgroup. The primary amino group was then transformed into a further amide function with boc-lysine resulting in two protonable amine residues after elimination of the protective groups. On the other hand, the tris(aminoethyl)-amine comprises both the spacer and the headgroup resulting from the two aminoethyl residues at the tertiary amine. This branched structure is suitable for the creation of dendrimer-like headgroups (*28*).

The main focus of attention for the new lipids was concentrated on transfection efficacy and toxicity properties compared with LipofectAmine and SuperFect, as they are the frequently used nonviral gene delivery systems. In first tests with these new transfection reagents, we have observed enhanced gene transfection activity with regard to commercially available vectors.

EXPERIMENTAL PROCEDURES

General Procedures and Materials. All materials and reagents were purchased from SigmaAldrich Co. Ltd. unless otherwise stated. All solvents were analytically pure and dried before use. The reactions were performed under argon. TLC was carried out on aluminum sheets precoated with silica gel 60 F254 (Merck) and developed with bromothymol blue dip. Column chromatography was performed using silica gel 60 (0.04−0.063 mm) for middle-pressure liquid chromatography (MPLC, Büchi, Germany) or silica gel 60 (0.063−0.200 mm) for normal-pressure procedure. The ESI−mass spectra were recorded with a Finnigan MAT 710 C (Thermo Separation Products, San Jose, USA) with electron spray ionization energy of 4.5 kV in negative and positive modus. EI−mass spectra were prepared with an AMD 402 (70 eV, AMD Intecta GmbH, Harpstedt, Germany). The ^{1}H NMR and the ^{13}C NMR spectra were recorded on a Varian Gemini 2000. For performing the elemental analysis, a Leco apparatus was used. The final products were fully characterized by ^{1}H NMR, ^{13}C NMR, mass spectrometry, and elemental analysis. p-CMV-SPORT-β-Gal plasmid and LipofectAmine reagent were purchased from Invitrogene Life technologies (Germany). SuperFect and plasmid isolation kit were purchased from QIAGEN (QIAGEN, Hilden, Germany). LLC PK1 cells (ATCC CL−101) were purchased from the American Type Culture Collection (ATCC USA) and A549 cells from German Collection of Microorganisms and Cell Cultures (DSMZ GmbH). Cell culture media, fetal bovine serum, and phosphate buffered saline were supplied from PAA Laboratories (PAA Laboratories GmbH Germany). Antibiotics and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (Sigma Aldrich, Germany). DOPE was purchased from Fluka (Switzerland), and cholesterol was supplied from Roth (Carl Roth GmbH Germany).

Alkyl Malonic Acid Monoethyl Ester IIa,b. The alkyl malonic acid diethyl ester **I** (0.045 mol) was dissolved in ethanol (30 mL). Then, potassium hydroxide (2.92 g, in 30 mL ethanol) was added dropwise during 1 h into the solution with stirring. The mixture was stirred for further 3 h and was then allowed to stand overnight at room temperature. The mixture was heated for a short time to reflux, and the hot solution was filtered to remove bis-potassium salt. From the cooled mixture, the monopotassium salt of hexadecyl malonic acid monoethyl ester was separated by filtration. The salt was dissolved in water and acidified with equimolar amount of 0.1 M hydrochloric acid. The crude monoester was then extracted three times with 30 mL ether. The combined etheral solutions were washed with water and brine, dried over sodium sulfate, and evaporated to dryness. The compound was purified by crystallization from heptane or by column chromatography using silica gel 60.

In the case of **IIb**, no salt precipitates. Therefore, the mixture was concentrated under vacuum to the half of the volume and acidified after cooling. Compound **IIb** was then extracted 3 times with ether, and the combined etheral phase was washed and dried as described above. **IIb** was purified by column chromatography.

Hexadecyl Malonic Acid Monoethyl Ester (IIa). The experimental data were in agreement with the literature (*29*).

(9*Z*)-Octadec-9-enyl Malonic Acid Monoethyl Ester (IIb). Yield: 68%, colorless oil, $C_{23}H_{42}O_4$, 382.57 g/mol, EI−MS: 382 (M^+).^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.85 (t, 3H, [−C*H*$_3$]), 1.20−1.90 (m, 29H, [$H_3C(CH_2)_6CH_2CH$═$CHCH_2(CH_2)_7$−], [−OCH_2CH_3]), 1.95−2.05 (m, 4H, [−CH_2CH═$CHCH_2$−]), 3.38−3.40 (t, 1H, [−C*H*−]), 4.22 (q, 2H, [−OCH_2CH_3]), 5.30−5.39 (m, 2H, [−C*H*═C*H*−]). Anal. Calcd.: C, 72.20; H, 11.06. Found: C, 71.92; H, 11.17.

2-[(Alkylamino)carbonyl]octadecanoic Acid Ethyl Ester IIIa,b. *Procedure 1.* Compound **IIa** (0.014 mol) and 10 mL thionyl chloride were given into a flask, and the mixture was allowed to stand overnight at room temperature until a clear solution appears. Then, the excess thionyl chloride was removed under vacuo. The residue was dried under vacuum over potassium hydroxide. After that, the crude malonic ester chloride was dissolved in 50 mL dry $CHCl_3$ and cooled down to 0 °C. Hexadecylamine or oleylamine (0.013 mol) and triethyl amine (1.3 g, 0.013 mol), dissolved in 10 mL dry $CHCl_3$, were dropped into the solution with stirring at the same temperature. After 2 h stirring at room temperature, the $CHCl_3$ solution was washed three times with ice water and dried over sodium sulfate. The crude product was crystallized from acetone.

Procedure 2. A suspension of compound **II** (0.014 mol), hexadecylamine or oleylamine (0.014 mol), and 3.5 g (0.014 mol) EEDQ in 100 mL ethanol was heated for 24 h at 50 °C. The solvent was then reduced to half of the volume, diluted with 100 mL $CHCl_3$, and washed with water. After drying the organic layer over sodium sulfate, the solvent was evaporated and the residue recrystallized from acetone.

2-[(Hexadecylamino)carbonyl]octandecanoic Acid Ethyl Ester (IIIa). Yield: 91% (procedure 1), 86% (procedure 2), $C_{37}H_{73}NO_3$, 580.0 g/mol, mp: 72−73 °C. ESI−MS: (M^++Na).^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.85 (t, 6H, 2[−C*H*$_3$]), 1.23−1.86 (m, 61H, [−OCH_2CH_3], [$H_3C(CH_2)_{15}$−], [$H_3C(CH_2)_{14}CH_2NH$−]), 3.14−3.18 (m, 2H, [−CH_2NHCO−]), 3.22−3.28 (m, 1H, [−C*H*−]), 4.16 (q, 2H, [−OCH_2CH_3]), 6.52−6,55 (m, 1H, [−N*H*−]). Anal. Calcd.: C, 76.62; H, 12.69; N, 2.41. Found: C, 76.58; H, 12.70; N, 12.37.

2-{[(9Z)-Octadec-9-enylamino]carbonyl}octadecanoic Acid Ethyl Ester (IIIb). Yield: 83% (procedure 1), 83% (procedure 2), $C_{39}H_{75}NO_3$, 606.03 g/mol, mp: 45−47 °C. ESI−MS: (M^++H). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.85 (t, 6H, 2[−C*H*$_3$]), 1.18−1.86 (m, 57H, [$H_3C(CH_2)_{15}$−], [$H_3C(CH_2)_6CH_2CH$═$CHCH_2(CH_2)_6CH_2NH$−], [−OCH_2CH_3]), 1.90−2.04 (m, 4H, [−CH_2CH═$CHCH_2$−]), 3.14−3.27 (m, 3H, [−CH_2NHCO−], [−C*H*−]), 4.17 (q, 2H, [−OCH_2CH_3]),

5.30−5.35 (m, 2H, [−C*H*═C*H*−]), 6.53−6.56 (m, 1H, [−N*H*−]). Anal. Calcd.: C, 77.29; H, 12.47; N, 2.31. Found: C, 76.96; H, 12.43; N, 2.37.

2-{[(9*Z*)-Octadec-9-enylamino]carbonyl}-(11*Z*)-icos-11-enoic Acid Ethyl Ester (IIIc). Yield: 83% (procedure 2), $C_{41}H_{77}NO_3$, 632.04 g/mol, EI−MS: 632 (M^+, 100), mp: 32−35 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.87 (2t, 6H, 2[−CH_3]), 1.17−1.88 (m, 51 H, [$H_3C(CH_2)_6CH_2CH$═$CHCH_2(CH_2)_7$−], [$H_3C(CH_2)_6CH_2CH$═$CHCH_2(CH_2)_6CH_2NH$−], [−OCH_2CH_3]), 1.90−2.05 (m, 8H, 2[−CH_2CH═$CHCH_2$−]), 3.15−3.19 (m, 2H, [−CH_2NHCO−]), 3.20−3.27 (m, 1H, [−C*H*−]), 4.19 (q, 2H, [−OCH_2CH_3]), 5.30−5.40 (m, 4H, 2[−C*H*═C*H*−]), 6.50−6.54 (m, 1H, [−N*H*−]). Anal. Calcd.: C, 77.91; H, 12.28; N, 2.21. Found: C, 77.83; H, 12.38; N, 2.30.

Saponification of IIIa−c. *General Procedure.* Potassium hydroxide (0.56 g, 0.01 mol) was added to a suspension of compound **III** (2.9 g, 0.005 mol) in ethanol (50 mL), and the mixture was heated under reflux for 5 h. After cooling to room temperature, the precipitated potassium salt was filtered by suction, washed with ether, and dried in vacuum. The salt (3.0 g, 0.0049 mol) was then suspended in 50 mL water containing 1.25 mL conc. HCl under cooling with ice and stirring. After 1 h, the acid **IV** was thrice extracted with $CHCl_3$. The combined $CHCl_3$ layers were dried and evaporated and the residue was crystallized from heptane.

2-[(Hexadecylamino)carbonyl]octadecanoic Acid (IVa). Yield: 85%, white crystals, $C_{35}H_{69}NO_3$, 577.96 g/mol, mp: 74−76 °C. ESI−MS: (M-H).^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD) δ: 0.86 (t, 6H, 2[−CH_3]), 1.23−1.95 (m, 58 H, [$H_3C(CH_2)_{15}$−], [$H_3C(CH_2)_{14}CH_2NH$−]), 3.09−3.18 (m, 2H, [−CH_2NHCO−]), 3.26−3.35 (m, 1H, [−C*H*−]), 5.89−5.94 (m, 1H, [−N*H*−]). Anal. Calcd.: C, 76.16; H, 12.60; N, 2.54. Found: C, 76.16; H, 12.67, N, 2.54.

2-{[(9*Z*)-Octadec-9-enylamino]carbonyl}octadecanoic Acid (IVb). Yield: 82%, white crystals, $C_{37}H_{71}NO_3$, 577.96 g/mol, mp: 74−76 °C. ESI−MS: (M-H).^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD) δ: 0.86 (t, 6H, 2[−CH_3], 1.18−1.89 (m, 54H, [$H_3C(CH_2)_{15}$−], [$H_3C(CH_2)_6CH_2CH$═$CHCH_2(CH_2)_6CH_2NH$−]), 1.92−2.05 (m, 4H, [−CH_2CH═$CHCH_2$−]), 3.14−3.30 (m, 3H, [−CH_2NHCO−], [−C*H*−]), 5.30−5.36 (m, 2H, −C*H*═C*H*−]), 6.45−6.50 (m, 1H, [−N*H*−]). Anal. Calcd.: C, 76.89; H, 12.38; N, 2.42. Found: C, 76.91; H, 12.36; N, 2.42.

2-{[(9*Z*)-Octadec-9-enylamino]carbonyl}-(11*Z*)-icos-11-enoic Acid (IVc). Yield: 85%, white waxy substance, $C_{39}H_{73}NO_3$, 604.00 g/mol, mp: 70−72 °C. ESI−MS: (M-H).^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.80−0.89 (t, 6H, 2 [−CH_3]), 1.20−1.90 (m, 50H, [$H_3C(CH_2)_6CH_2CH$═$CHCH_2(CH_2)_7$−], [$H_3C(CH_2)_6CH_2CH$═$CHCH_2(CH_2)_6CH_2NH$−]), 1.92−2.08 (m, 8H, 2[−CH_2CH═$CHCH_2$−]), 3.08−3.30 (m, 3H, [−CH_2NHCO−], [−C*H*−]), 5.34−5.40 (m, 4H, 2[−C*H*═C*H*−]), 6.05−6.15 (m, 1H, [−N*H*−]). Anal. Calcd.: C, 77.54; H, 12.18; N, 2.32. Found: C, 77.44; H, 12.36; N, 2.30.

Reaction of IVa−c with the Spacer Molecules to Va−f. *Procedure 1.* Compound **IV** (1 mmol) was dissolved in 50 mL CH_2Cl_2 and 140 mg (1.2 mmol) *N*-hydroxysuccinimide were added. Then, 288 mg (1.4 mmol) DCC was added to the slurry, and the mixture was allowed to stir for 12 h at room temperature. After that time, the precipitate of dicyclohexyl urea was filtered off and ethylene diamine or tris(aminoethyl)amine (20 mmol) was added to the solution. The mixture was stirred for 4 h at room temperature. The organic layer was then filtered, washed with water and brine, and dried over sodium sulfate. After evaporation of the solvent, the crude product was purified by column chromatography on silica gel 60 and $CHCl_3$/methanol using gradient technique.

Procedure 2. Triethylamine (0.152 g, 1.5 mmol) was added to a solution of compound **IV** (1 mmol), ethylene diamine or tris(aminoethyl)amine (20 mmol), and BOP (0.442 g, 1 mmol) in CH_2Cl_2 (20 mL). The mixture was stirred at room temperature overnight. The solution was filtered, washed with water, dried over sodium sulfate, and evaporated. The residue was purified by crystallization from heptane.

***N*-(2-Aminoethyl)-*N*′,2-dihexadecyl Propane Diamide (Va).** Yield: 65% (procedure 1), 83% (procedure 2), white substance, 594.01 g/mol, mp: 74−76 °C. ESI−MS: (M^++H), ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD) δ: 0.87 (t, 6H, 2[−CH_3]), 1.00−1.57 (m, 58H, [$H_3C(CH_2)_{15}$−], [$H_3C(CH_2)_{14}CH_2NH$−]), 2.72 (m, 2H, [H_2NCH_2−]), 2.90−3.10 (m, 4H, [−CH_2NHCO−], [$H_2NCH_2CH_2NHCO$−]), 3.25−3.31 (m, 1H, [−C*H*−]), 4.15−4.20 (m, 2H, 2[−N*H*−]). Anal. Calcd.: C, 74.81; H, 12.73; N, 7.07. Found: C, 74.75; H, 12.69; N, 7.05.

***N*-(2-Aminoethyl)-2-hexadecyl-*N*′-[(9*Z*)-octadec-9-enyl]-propane Diamide (Vb).** Yield: 68% (procedure 1), 80% (procedure 2), white substance, $C_{39}H_{77}N_3O_2$, 620.05 g/mol, mp: 68−71 °C. ESI−MS: (M^++H).^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD) δ: 0.85 (t, 6H, 2[−CH_3]), 1.20−1.88 (m, 54H, [$H_3C(CH_2)_{15}$−], [$H_3C(CH_2)_6CH_2CH$═$CHCH_2(CH_2)_6CH_2NH$−]), 1.92−2.03 (m, 4H, [−CH_2CH═$CHCH_2$−]), 2.79−2.81 (m, 2H, [H_2NCH_2−]), 2.94−3.23 (m, 4H, [−CH_2NHCO−], [$H_2NCH_2CH_2NHCO$−]), 3.26−3.30 (m, 1H, [−C*H*−]), 5.29−5.35 (m, 2H, [−C*H*═C*H*−]), 5.77−5.80 (m, 2H, 2[−CON*H*−]). Anal. Calcd.: C, 75.55; H, 12.52; N, 6.78. Found: C, 75.43; H, 12.37; N, 6.69.

***N*-(2-Aminoethyl)-*N*′,2-di[(9*Z*)-octadec-9-enyl]propane Diamide (Vc).** Yield: 82% (procedure 2), white substance, $C_{41}H_{79}N_3O_2$, 646.06 g/mol, ESI−MS: (M^++H). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.86 (t, 6H, 2[−CH_3]), 1.20−1.90 (m, 48H, [$H_3C(CH_2)_6CH_2CH$═$CHCH_2(CH_2)_7$−], [$H_3C(CH_2)_6CH_2CH$═$CHCH_2(CH_2)_6CH_2NH$−]), 1.92−2.05 (m, 8H, 2[−CH_2CH═$CHCH_2$−]), 2.78−2.82 (m, 2H, [$H_2NCH_2CH_2NH$−]), 3.18−3.22 (m, 4H, −CH_2NHCO−], [$H_2NCH_2CH_2NHCO$−], 3.25−3.32 (m, 1H, [−C*H*−]), 5.25−5.40 (m, 4H, 2[−C*H*═C*H*−]), 5.80−5.89 (m, 2H, 2[−CON*H*−]). Anal. Calcd.: C, 76.22; H, 12.32; N, 6.50. Found: C, 76.22; H, 12.38; N, 6.42.

***N*-[Bis(2-aminoethyl)amino]ethyl-*N*′,2-dihexadecylpropane Diamide (Vd).** Yield: 49% (procedure 1), 76% (procedure 2), white substance, $C_{41}H_{85}N_5O_2$, 680.15 g/mol, mp: 84−86 °C. ESI−MS: (M^++H). ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD) δ: 0.84 (t, 6H, 2[−CH_3]), 1.22−1.79 (m, 58H, [$H_3C(CH_2)_{15}$−], [$H_3C(CH_2)_{14}CH_2NH$−]), 2.51−2.56 (m, 4H, 2[$H_2NCH_2CH_2$−]), 2.73−2.76 (m, 4H, 2[$H_2NCH_2CH_2$−]), 2.96 (t, 2H, [−NCH_2CH_2NH−]), 3.13−3.20 (m, 2H, [−CH_2NHCO−]), 3.21−3.35 (m, 2H, [−NCH_2CH_2NHCO−]), 3.60−3.63 (m, 1H, [−C*H*−]), 5,77−5.89 (m, 2H, [−CON*H*−]). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.56, 23.22, 27.48, 28.13, 29.83, 29.88, 30.00, 30.15, 30.17, 30.20, 30.24, 32.47, 33.33, 38.01, 39.59, 40.05, 40.18, 54.02, 54.87, 56.98, 172.00, 172.16. Anal. Calcd. for $C_{41}H_{85}N_5O_2 \times H_2O$: C, 70.53; H, 12.56; N, 10.03. Found: C, 70.37; H, 12.64; N, 9.95.

***N*-[Bis(2-aminoethyl)amino]ethyl-2-hexadecyl-*N*′-[(9*Z*)-octadec-9-enyl]propane Diamide (Ve).** Yield: 49% (procedure 1), 71% (procedure 2), white waxy substance, $C_{43}H_{87}N_5O_2$, 706.18 g/mol, mp: 35 °C. ESI−MS: (M^++H). ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD) δ: 0.85 (t, 6H, 2[−CH_3], 1.20−1.89 (m, 54H, [$H_3C(CH_2)_{15}$−], [$H_3C(CH_2)_6CH_2CH$═$CHCH_2(CH_2)_6CH_2NH$−]), 1.95−2.05 (m, 4H, [−CH_2CH═$CHCH_2$−]), 2.50−2.56 (m, 4H, 2[$H_2NCH_2CH_2$−]), 2.79−2.81 (m, 4H, 2[$H_2NCH_2CH_2$−]), 2.94−3.23 (m, 4H, [−CH_2NHCO−], [−NCH_2CH_2NHCO−]), 3.26−3.30 (m, 1H, [−C*H*−]), 5.29−5.35 (m, 2H, [−C*H*═C*H*−]), 5.77−5.86 (m, 2H, 2[−CON*H*−]). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.31, 23.14, 25.84, 27.48, 27.67, 28.00, 29.69, 29.77, 29.79, 29.84, 29.91, 30.01, 30.10, 30.14, 30.19, 30.24, 30.28, 31.04, 31.40, 32.42, 33.05, 37.72, 37.97, 40.20, 52.35, 54.19, 54.39, 54.56, 130.14, 130.34, 172.12,

172.24. Anal. Calcd. for $C_{43}H_{87}N_5O_2 \times H_2O$: C, 71.31; H, 12.38; N, 9.67. Found: C, 71.03; H, 12.45; N, 9.52.

***N*-[Bis(2-aminoethyl)amino]ethyl-*N′*,2-di[(9*Z*)-octadec-9-enyl]propane Diamide (Vf).** Yield: 74% (procedure 2), white waxy substance, $C_{45}H_{89}N_5O_2$, 732.22 g/mol. ESI−MS: $(M^+ + H)$. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.87 (t, 6H, 2[−C*H*$_3$]), 1.20−1.90 (m, 50H, [$H_3C(CH_2)_6CH_2CH$═$CHCH_2(CH_2)_6CH_2NH$−], [$H_3C(CH_2)_6CH_2CH$═$CHCH_2(CH_2)_7$−), 1.90−2.05 (m, 8H, 2[−C*H*$_2$CH═CHC*H*$_2$−]), 2.49−2.6 (m, 4H, 2[$H_2NCH_2CH_2N$−]), 2.80−2.92 (m, 6H, 2[$H_2NCH_2CH_2N$−], [−N*CH*$_2$CH$_2$NH−), 3.16−3.30 (m, 4H, [−C*H*$_2$NHCO−], [−NCH$_2$C*H*$_2$NHCO−]), 3.50−3.59 (m, 1H, [−C*H*−]), 5.30−5.38 (m, 2H, [−C*H*═C*H*−]), 5.80−5.89 (m, 2H, [−CON*H*−]). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 13.93, 22.50, 26.81, 27.05, 27.50, 29.00, 29.13, 29.23, 29.27, 29.31, 29.34, 29.36, 29.43, 29.48, 29.52, 29.59, 31.72, 32.41, 32.66, 37.70, 39.32, 39.41, 53.40, 54.49, 56.46, 129.36, 129.38, 129.53, 129.55, 170.78, 171.11. Anal. Calcd. for $C_{45}H_{89}N_5O_2 \times H_2O$: C, 72.04; H, 12.22; N, 9.34. Found: C, 72.38; H, 12.17; N, 9.25.

Reaction Va−c to Compounds VIa−c. *General Procedure.* 0.84 mmol of compound **V** was dissolved in 30 mL CH_2Cl_2. 0.9 mmol (0.4 g) diboc-lysin-*N*-hydroxysuccinimid ester was given to this solution. The mixture was stirred for 1 h at room temperature and then for another 10 h under reflux. After that time, the solvent was evaporated, the residue was chromatographed using a short column and dry silicagel with $CHCl_3$/methanol (98:2, v/v) as eluent. The so-purified product was dissolved in 15 mL CH_2Cl_2, and 1 mL trifluoro acetic acid was added. The mixture was allowed to stir for 4 h, then mixture was diluted with 60 mL $CHCl_3$. 100 mL diluted ammonia was poured into the mixture, which was shaken until two clear phases evolved. The organic layer was separated, dried over sodium sulfate, and evaporated. The crude products **VI** were then purified by column chromatography using silicagel 60 and $CHCl_3$/methanol/NH_3 with gradient technique.

***N′*-2[(2,6-Diamino-1-oxohexyl)amino]ethyl-2,*N*-(dihexadecyl)propane Diamide (VIa).** Yield: 54%, white waxy substance, $C_{43}H_{87}N_5O_3$, 722.18 g/mol, mp: 111−112 °C. ESI−MS: $(M^+ + H)$.^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.86 (t, 6H, [−C*H*$_3$]), 1.18−1.88 (m, 64H, [$H_3C(CH_2)_{15}$−], [$H_3C(CH_2)_{14}CH_2NH$−], [−$(CH_2)_3CH_2NH_2$]), 2.82−2.87 (m, 2H, [−C*H*$_2$NH$_2$]), 3.05−3.09 (m, 1H, [−C*H*−]), 3.20−3.38 (m, 5H, [−CONHC*H*$_2$CH$_2$NHCO−], [−CONHCH$_2$C*H*$_2$NHCO−], [−COC*H*(NH$_2$)CH$_2$−]), 6.58−6.70 (m, 2H, 2[−CON*H*−]). ^{13}C NMR (100 MHz, $CDCl_3$/CD_3OD) δ: 13.11, 22.03, 22.08, 22.11, 26.32, 26.85, 28.61, 28.73, 28.85, 28.96, 29.00, 29.03, 29.07, 30.18, 31.16, 31.24, 31.32, 34.03, 38.26, 38.42, 38.95, 40.00, 53.62, 54.26, 170.51, 171.35. Anal. Calcd. for $C_{43}H_{87}N_5O_3 \times H_2O$: C, 69.77; H, 12.12; N, 9.46. Found: C, 70.05; H, 12.16; N, 9.39.

***N′*-2-[(2,6-Diamino-1-oxo-hexyl)amino]ethyl-2-hexadecyl-*N*-[(9*Z*)-octadec-9-enyl]propane Diamide (VIb).** Yield: 56%, white waxy substance, $C_{45}H_{89}N_5O_3$, 748.22 g/mol, mp: 104−105 °C, ESI−MS: $(M^+ + H)$. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.85 (2t, 6H, [−C*H*$_3$]), 1.20−1.80 (m, 60H, [$H_3C(CH_2)_{15}$−], [$H_3C(CH_2)_6CH_2CH$═$CHCH_2(CH_2)_6CH_2NH$−], [−$(CH_2)_3CH_2NH_2$]), 1.92−2.04, (m, 4H, [−C*H*$_2$CH═CHC*H*$_2$−]), 2.80−2.85 (m, 2H, [−C*H*$_2$NH$_2$]), 3.05−3.10 (m, 1H, [−C*H*−]), 3.22−3.43 (m, 5H, [−CONHC*H*$_2$CH$_2$NHCO−], [−CONHCH$_2$C*H*$_2$NHCO−], [−COC*H*(NH$_2$)CH$_2$−]), 5.33−5.41 (m, 2H, [−C*H*═C*H*−]), 6.55−6.65 (2m, 2H, 2[−CON*H*−]). ^{13}C NMR (100 MHz, $CDCl_3$/CD_3OD) δ: 13.99, 22.38, 26.66, 26.91, 27.20, 28.94, 29.01, 29.06, 29.16, 29.22, 29.31, 29.36, 29.40, 29.47, 31.63, 31.91, 32.28, 34.32, 38.58, 39.32, 40.30, 53.97, 54.45, 129.29, 129.51, 170.68, 171.70. Anal. Calcd. for $C_{45}H_{89}N_5O_3 \times H_2O$: C, 70.54; H, 11.97; N, 9.14. Found: C, 70.71; H, 11.63; N, 9.17.

***N′*-2-[(2,6-Diamino-1-oxo-hexyl)amino]ethyl-2,*N*-di[(9*Z*)-octadec-9-enyl]propane Diamide (VIc).** Yield: 53%, white pasty substance, $C_{47}H_{91}N_5O_3$, 774.26 g/mol, mp: 109−111 °C. ESI−MS: $(M^+ + H)$. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.86 (t, 6H, 2[−C*H*$_3$]), 1.15−1.88 (m, 56H, [$H_3C(CH_2)_6CH_2CH$═$CHCH_2(CH_2)_7$−], [$H_3C(CH_2)_6CH_2CH$═$CHCH_2(CH_2)_6CH_2NH$−], [−$(CH_2)_3CH_2NH_2$]), 1.90−2.05 (m, 8H, 2[−C*H*$_2$CH═CHC*H*$_2$−]), 2.80−2.85 (m, 2H, [H_2NCH_2−]), 3.05−3.10 (m, 1H, [−C*H*−]), 3.15−3.25 (m, 2H, [−C*H*$_2$NHCO−]), 3.25−3.40 (m, 5H, [−CONHC*H*$_2$CH$_2$NHCO−], [−CONHCH$_2$C*H*$_2$NHCO−], [−COC*H*(NH$_2$)CH$_2$−]), 5.30−5.40 (m, 4H, 2[−C*H*═C*H*−]), 6.60−6.65 (2 m, 2H, 2[−CON*H*−]). ^{13}C NMR (100 MHz, $CDCl_3$/CD_3OD) δ: 13.66, 22.38, 26.67, 26.92, 27.21, 28.94, 29.01, 29.14, 29.22, 29.41, 29.47, 30.62, 31.62, 32.29, 34.32, 38.58, 39.32, 40.35, 53.99, 54.47, 129.31, 129.50, 170.67, 171.70. Anal. Calcd. for $C_{47}H_{91}N_5O_3 \times H_2O$: C, 71.25; H, 11.83; N, 8.84. Found: C, 71.37; H, 11.57; N, 8.86.

Preparation of Liposomes. A $CHCl_3$/methanol solution (9/1, v/v) of the pure cationic lipid was combined with a $CHCl_3$ solution of DOPE or cholesterol. The mixtures were dried under reduced pressure to remove the organic solvent, and the dried film was vacuum-desiccated for 3 h. The lipid film was dissolved in distilled water at room temperature to a final concentration of 2 mg lipid/mL. The film was allowed to swell in a water bath at 40 °C for at least 15 min. Subsequently, the lipid formulations were vortexed and sonicated to clarity in a bath sonicator. The samples were stored in the refrigerator at 4 °C before experiments.

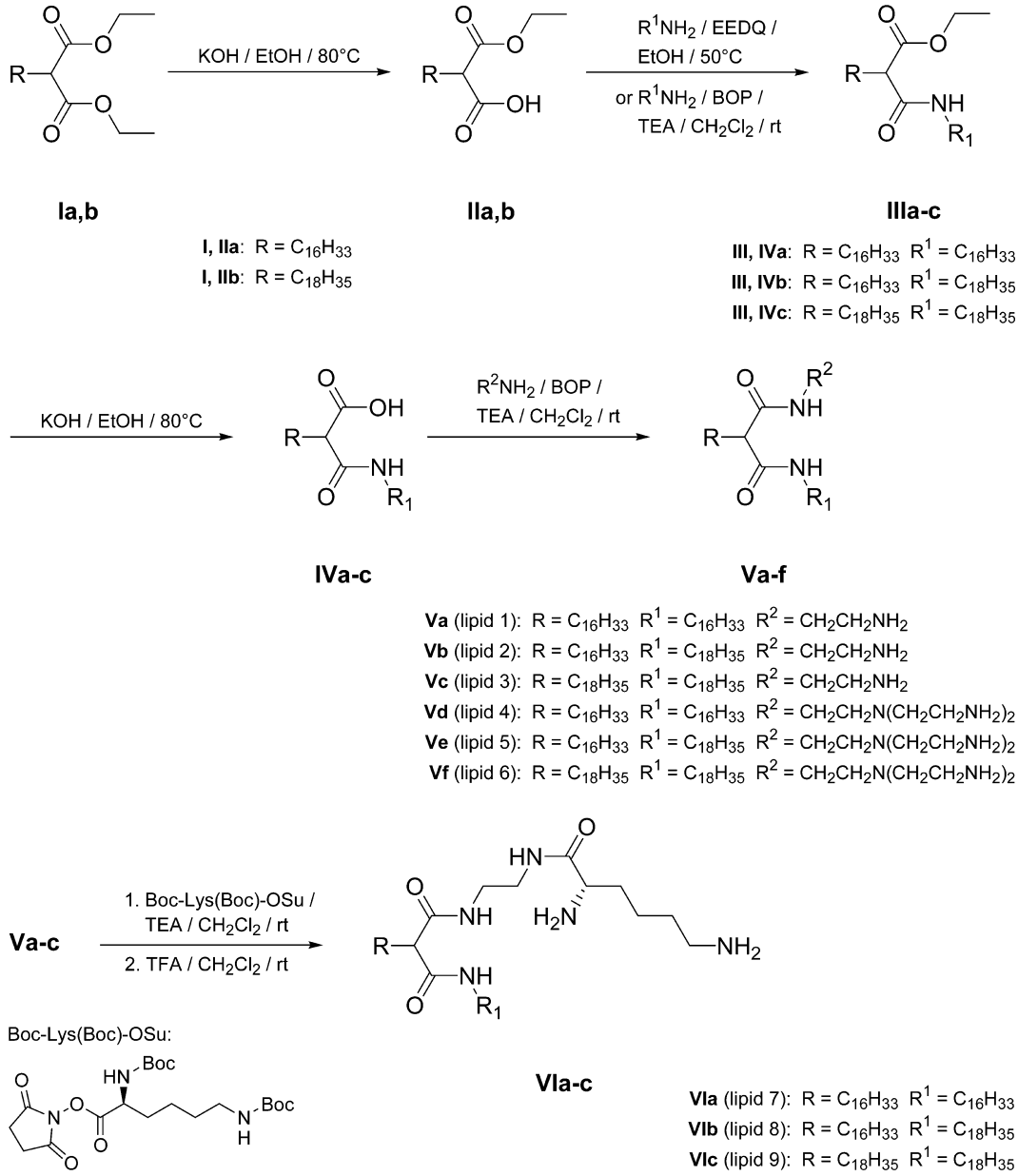
Preparation of Plasmid DNA. pCMV Sport β-Gal was isolated from *Escherichia coli* DH5α (Invitrogene life technologies) using a Quiagen plasmid mega kit (Quiagen) following the manufacturer's instructions (*30*). DNA with an $OD_{260}/OD_{280} = 1.94$ was used for experiments.

Cell Culture. LLC PK1 cells (porcine kidney epithelial cells) were cultured in 75 cm^2 tissue culture flasks in Medium 199 adjusted to contain 2.2 g/L sodium bicarbonate, 10% fetal bovine serum (FBS), and 0.05 mg/mL gentamycin at 37 °C and 5% CO_2. The cells were grown confluently and were regularly split twice a week. Only cells that had undergone fewer than 30 passages were used for transfection and MTT experiments.

A549 cells (human lung carcinoma cells) were cultured in 75 cm^2 tissue culture flasks in Dulbecos's modified eagle medium (DMEM) adjusted to contain 4.5 g/L glucose, 10% FBS, and 0.05 mg/mL gentamycin at 37 °C and 5% CO_2. The cells were grown confluently and were regularly split twice a week. For experiments, only cells in the range of passages 20−60 were used.

Transfection Biology. 16−24 h before transfection, cells were seeded into a 96 well plate at a density of 8000−10 000 cells/well by usage of LLC PK1 and 18 000−20 000 in the case of A549 cells. Lipoplex mixtures were prepared by combining plasmid DNA (0.1 μg per well = 2.5 μg/mL) with varying amounts of cationic liposome suspension in the absence or presence of 10% serum in Medium 199 or DMEM. The samples were incubated for 15 min at room temperature (total volume 40 μL/well). The N/P ratios were varied from 1:1 to 4:1 or even higher in cases when the results of experiments foreshadow increasing transfection efficacies at higher charge ratios. During this time, cells were washed once with phosphate buffered saline (PBS). Then the complexes were added to the cells. After 4 h of incubation, 160 μL of Medium 199 or DMEM was added to the cells in a way that the final concentration of FBS reached 10%. The medium was refreshed after 24 h, and the reporter gene activity was estimated after 48 h. Therefore, the cells were washed with PBS lysed for 15 min in lysis buffer (5 mM Chaps in 50 mM Hepes buffer). The solution was taken out of the wells, reunited in safe-lock tubes (Eppendorf), centrifuged, and stored on ice. It was pipeted again in a 96 well plate and substrate solution [1.33 mg/mL of *O*-nitrophenyl-β-galactopyranoside (ONPG), $MgCl_2$ 2 mM, β-mercaptoethanol 100 mM in 0.2 M sodium phosphate pH 7.3] was added and incubated for 30 min at 37 °C. The reaction was stopped with 1 M sodium carbonate. Absorption at 405 nm was converted to β-galactosi-

Scheme 1. Synthesis of Lipids 1−9 Starting from Alkyl Malonic Acid Diethyl Ester



dase units using a calibration curve constructed with pure commercial β-galactosidase enzyme. Protein concentration was detected with bichinonic acid reaction (*31*). Absorption at 570 nm was converted by using a calibration curve made of bovine serum albumin. All experiments were carried out sixfold. Every experiment was repeated three to five times on different days.

Toxicity Assay. Cytotoxicity of the lipoplexes was assessed by the MTT reduction assay (*32*). The cytotoxicity assays were performed in 96 well plates by maintaining the same ratio and number of cells and lipoplexes as used in the transfection experiments. MTT was added after refreshing the medium (24 h after addition of lipoplexes to the cells) and incubated for 3 h at 37 °C. Afterward, the cells were lysed with a mixture of 10% sodium dodecyl sulfate in acetic acid/dimethyl sulfoxide. Absorption was measured at 570 nm. The results were expressed as percent viability = $[A_{570}$ (treated cells) − background$]/[A_{570}$ (untreated cells) − background$] \times 100$.

Size Measurements. The sizes of liposomes and lipoplexes were measured in distilled water by photon correlation spectroscopy on a HPPS−ET (Malvern, U.K.) with sample refractive index of 1.25 and a viscosity of 0.8872 at 25 °C. Lipoplexes were prepared by following the instructions used for transfection experiments and measured with 2.5 μg DNA/mL sample. The *z*-average and PDI were calculated by using the automatic mode. Every sample was measured by three spectroscopic runs consisting of ten consecutive scans, each of 10 s in duration. Results shown are the average of these three values.

X-Gal Staining. To estimate a successful transfection, cells were histochemically stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). β-Galactosidase expressing cells can be indicated by blue color. The experiments were carried out in 24 well plates with a density of 40 000−50 000 cells per well and DNA concentration of 2.5 μg/mL (1.25 μg/well in 24 well plate). 48 h after transfection, cells were washed two times with PBS (2×500 μL) and fixed with 0.2% glutaraldehyde and 2.0% formaldehyde in PBS for 10 min at room temperature. Afterward, the cells were washed with PBS again (2×500 μL) and were stained subsequently with 1 mg/mL X-gal in PBS containing 2 mM $MgSO_4$ and 4 mM $K_3[Fe(CN)_6]$ and 4 mM $K_4[Fe(CN)_6]$ for 3 h at 37 °C.

Statistical Analysis. All measurements were collected (n = 3−9) and expressed as means ± standard deviation. One-way

ANOVA was used in conjunction with Bonferoni's multiple comparison test to assess the statistical significance.

RESULTS

Synthesis of Malonic Acid Amides Cationic Lipids. The synthesis of the compounds is shown in Scheme 1. Starting from long-chain monoalkylated malonic acid esters, which are easy to prepare by alkylation reaction of malonic acid diesters (*33*) with the corresponding alkyl halides or methansulfonates, one of the ester groups was saponified according to published methods (*34*). We have also studied the preparation of the monoester starting from malonic esters with different alcoholic residues followed by a selective saponification. Because of the simplicity of the first reaction, this procedure was used. Compounds **IIa**,**b** were then transformed into the 2-alkylmalonic acid ester amides **IIIa**−**c** by reaction with hexadecyl or oleyl amine using condensing reagents. The most effective reagents were 2-ethoxy-1-ethoxycarbonyl-1,2-dihydrochinoline (EEDQ) and the benzotriazole derived compounds known from peptide synthesis, like (benzotriazol-1-yloxy)-tris-(dimethylamino)-phosphoniumhexaflourophospahte (BOP), leading to the products in high yield.

For the synthesis of **IIIa**,**b**, the activation via the corresponding acid chloride was also a high-yield procedure, but in the case of the unsaturated chain of **IIb**, we used only the modern condensation agents. The saponification of the ester with potassium hydroxide yields the acids. For the second coupling reaction with ethylene diamine or tris(aminoethyl)amine to **Va**−**f**, *N*-hydroxysuccinimide/*N,N*′-dicyclohexylcarbondiimid (DCC) or better BOP was used as condensing reagent. We found that a 20-fold excess of the di- or triamine is necessary. This ensures yields of more than 70%. On the other hand, the reaction with only a 5- to 10-fold excess leads only to a yield of 20%, also in the case of BOP as the most effective condensing reagent. The introduction of a lysine residue into compounds **Va**−**c** was realized in good yields using the commercially available diboc-lysine-*N*-hydroxysuccinimide ester. The crude products were purified to some extent by filtration using silica gel. For the removal of the protective groups, trifluoroacetic acid in methylene chloride was used. The yields after the two steps were more than 50%.

In Vitro Transfection and Cell Toxicity of Malonic Acid Amides Cationic Liposomes. To form liposomes for gene transfer experiments, cationic amphiphiles are usually mixed with colipids. Most frequently, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and cholesterol are used (*26, 35–37*). Their influence on transfection is ascribed to their tendency to adopt the inverted hexagonal H_{II} phase that seems to be necessary for the DNA release from the endosomal compartment (*36, 38–42*). For that reason, all of the lipids **1**−**9** were mixed in different molar ratios with DOPE or cholesterol to determine the most auspicious combination. To identify the kind of structure/activity relationship for the malonic acid amides, nearly 60 samples were prepared and tested for their ability to transfect cells in culture.

Figure 1 summarizes the relative in vitro gene delivery efficiencies of lipid **8** mixed with colipids. Lipoplex (cationic liposome/DNA complex) mixtures were prepared by combining plasmid DNA (0.1 μg per well = 2.5 μg/mL) with varying amounts of cationic liposome suspension in plain Medium 199. The N/P ratios were varied from 1:1 to 4:1. Lipid **8** has been proven to be successful for gene transfer into cells in culture. The majority of the analyzed samples exhibit high transfection efficacies. All tested combinations of this lipid with cholesterol show equal or higher transfection efficacy than LipofectAmine and SuperFect. Merely the maximum values were found at different N/P ratios. The use of DOPE as colipid decreases the transfection efficiencies. Only lipid **8**/DOPE 1:1 (*n*/*n*) is comparable to commercially available gene transfer kits.

Table 1 summarizes the results of all lipids **1**−**9** in combination with the used helper lipids. It shows the most auspicious combination of any lipid with any colipid in gene transfer experiments. Furthermore, the N/P ratio, the MTT based percent cell viability, and the results of size measurements are listed. Apart from four samples, for all new lipids transfection was detected, but only lipid **6**, lipid **8**, and lipid **9** exhibit transfection efficacies that make them comparable to commercially available vectors. Liposomes that consist of lipid **6** or lipid **8** and cholesterol as colipid lead to increasing transfection efficacies. As shown in Figure 2, this effect is statistically significant compared to LipofectAmine (*) or SuperFect (^), whereas the effects on cell survival are similar.

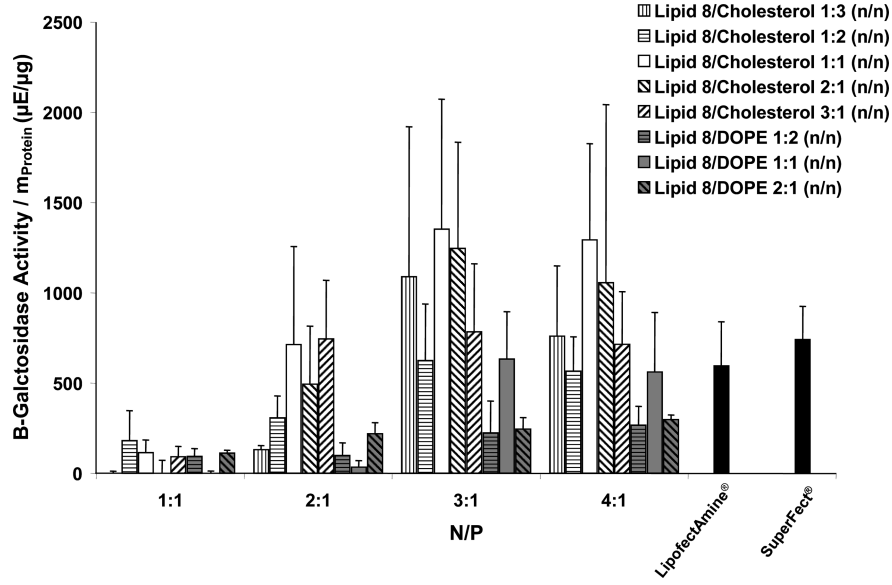


Figure 1. In vitro transfection efficacies of lipid **8** in LLC PK1 cells using cholesterol and DOPE as colipid (at different lipid/cholesterol or lipid/DOPE molar ratios). Micro units of β-galactosidase related to cell protein concentration were plotted against the varying N/P ratios. The transfection efficiencies of the liposome samples were compared to those of the commercially available LipofectAmine and SuperFect. The experiments were performed as described in the text. The transfection values shown are the average of at least three experiments carried out on three different days.

Table 1. In Vitro Transfection Efficacies, Size Measurements, and MTT Assays of Lipids 1−9 in LLC PK1 Cells Using DOPE and Cholesterol as Colipid (at Different Lipid/Colipid Molar Ratios)[a]

sample	N/P ratio	*z*-average ± *s* [nm] liposomes	*z*-average ± *s* [nm] lipoplexes	activity$_{\beta\text{-gal}}$/m_{protein} ± *s* [μE/μg]	viability ± *s* [%]
Lipid 1/DOPE 2:1 (*n*/*n*)	4:1	50 ± 1	185 ± 1	no transfection	86 ± 11
Lipid 1/Cholesterol 1:1 (*n*/*n*)	3:1	147 ± 5	330 ± 11	no transfection	69 ± 9
Lipid 2/DOPE 1:1 (*n*/*n*)	4:1	99 ± 10	205 ± 2	no transfection	91 ± 7
Lipid 2/Cholesterol 1:1 (*n*/*n*)	1:1	108 ± 3	136 ± 1	352 ± 191	93 ± 17
Lipid 3/DOPE 2:1 (*n*/*n*)	4:1	120 ± 1	171 ± 2	276 ± 157	85 ± 13
Lipid 3/Cholesterol 2:1 (*n*/*n*)	1:1	151 ± 2	681 ± 33	29 ± 13	94 ± 13
Lipid 4/DOPE 1:2 (*n*/*n*)	3:1	104 ± 7	423 ± 6	190 ± 69	84 ± 14
Lipid 4/Cholesterol 1:2 (*n*/*n*)	6:1	120 ± 7	189 ± 6	16 ± 7	94 ± 11
Lipid 5/DOPE 3:1 (*n*/*n*)	6:1	112 ± 50	113 ± 1	156 ± 38	92 ± 6
Lipid 5/Cholesterol 1:1 (*n*/*n*)	2:1	100 ± 3	116 ± 1	31 ± 25	102 ± 13
Lipid 6/DOPE 2:1 (*n*/*n*)	6:1	87 ± 16	189 ± 1	335 ± 104	77 ± 15
Lipid 6/Cholesterol 2:1 (*n*/*n*)	7:1	88 ± 17	134 ± 1	1018 ± 206	82 ± 8
Lipid 7/DOPE 1:1 (*n*/*n*)	2:1	108 ± 1	1132 ± 114	no transfection	84 ± 9
Lipid 7/Cholesterol 1:1 (*n*/*n*)	2.5:1	139 ± 1	642 ± 12	117 ± 37	83 ± 7
Lipid 8/DOPE 1:1 (*n*/*n*)	3:1	131 ± 46	268 ± 7	633 ± 263	89 ± 11
Lipid 8/Cholesterol 1:1 (*n*/*n*)	4:1	90 ± 4	137 ± 1	1294 ± 511	85 ± 14
Lipid 9/DOPE 2:1 (*n*/*n*)	2:1	121 ± 2	583 ± 2	309 ± 211	86 ± 10
Lipid 9/Cholesterol 1:1 (*n*/*n*)	2:1	94 ± 1	258 ± 1	692 ± 190	90 ± 8

[a] The transfection and viability values listed are the average of at least three experiments carried out on different days.

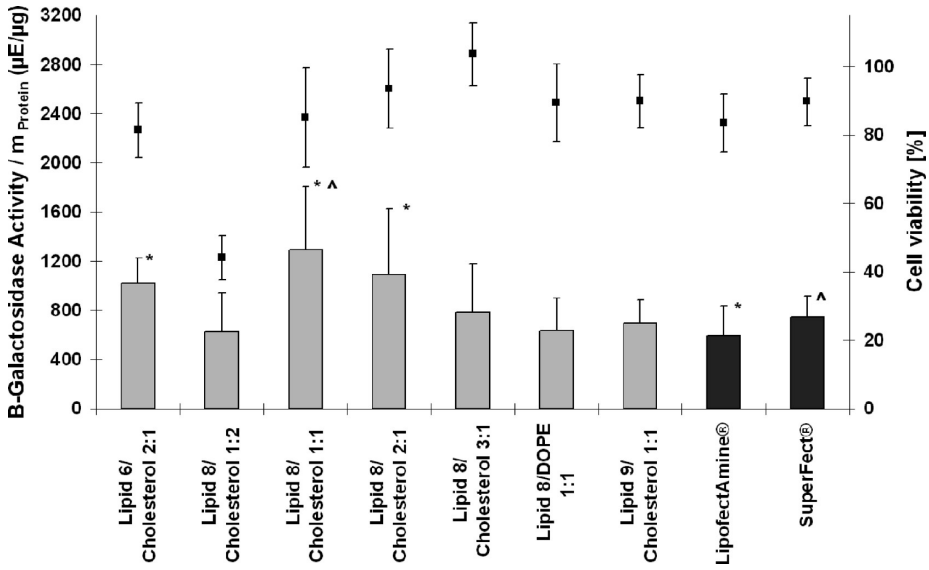


Figure 2. In vitro transfection efficacies and cell viability values of lipids **6**, **8**, and **9** in LLC PK1 cells using cholesterol or DOPE as colipid (at different lipid/cholesterol or lipid/DOPE molar ratios). Micro units of β-galactosidase related to cell protein concentration and cell viability in % were plotted against different samples. The transfection efficiencies of the liposomes were compared to that of commercially available LipofectAmine and SuperFect. Percent cell viability is plotted on secondary *y*-axis via scatter diagram. The transfection and cytotoxicity values shown are the average of at least three experiments carried out on three different days. Statistical analyses were carried out with $p < 0.05$, one-way ANOVA, and Bonferoni's multiple comparison tests (*^).

Figure 3 summarizes the results of gene transfer experiments in LLC PK1 cells with LipofectAmine (A), lipid **1** (B), and lipid **6** (C) visualized by histochemical staining (X-Gal assay). Whereas LipofectAmine and lipid **6**/cholesterol 2:1 (*n*/*n*) lead to considerable staining of wells, no colored cells were found when using lipoplexes consisting of lipid **1** as gene transfer reagent.

In the majority of cases, the cell viability fluctuates between 80% and 90% (cf. Table 1). These results suggest that the applied liposomes have an influence on the cell survival but that the efficacy of the gene transfer is not directly coupled with the toxicity. For instance, lipid **1** and lipid **7** with no or low transfection efficacy have similar or even stronger influence on the cell viability than lipid **6**, lipid **8**, or lipid **9**, although those are proven to be successful for the transfection process.

Figure 4 shows the results of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) measurements of lipid **1**, lipid **7**, and lipid **8** and lipid **9** lipoplexes. The addition of lipoplexes decreases the cell viability. This effect was observed for all lipids and all N/P ratios. Furthermore, there is no statistically significant difference between the charge ratios of one sample, which suggests that the cell viability is already decreased at low lipid concentrations. An increase of cationic lipid concentration shows little effect on the toxicity at least in applied N/P ratios. Interestingly, the influence on cell viability of lipid **1** in combination with cholesterol is more distinct than that of lipid **8** or lipid **9**. Liposomes, which are not qualified for in vitro gene delivery, influence cell survival to the same degree or even more than efficiently transfecting samples. By comparing lipoplexes that consist of lipid **9** with those of lipid **1** and lipid **7**, the differences in cell viability are statistically significant just like the comparison of lipid **1** and lipid **8**. The results suggest that the chemical structure of the cationic lipid and the composition of liposomes play a major role in cell damaging characteristics, as well as in the ability to transfer nucleic acids. It was observed that the addition of lipoplexes leads to derogation of LLC PK1 cell survival independent of successful or unsuccessful gene transfer. Transfection of cells in culture is to a certain level connected with toxicity, but the reverse is not compulsory. To look for other parameters, which

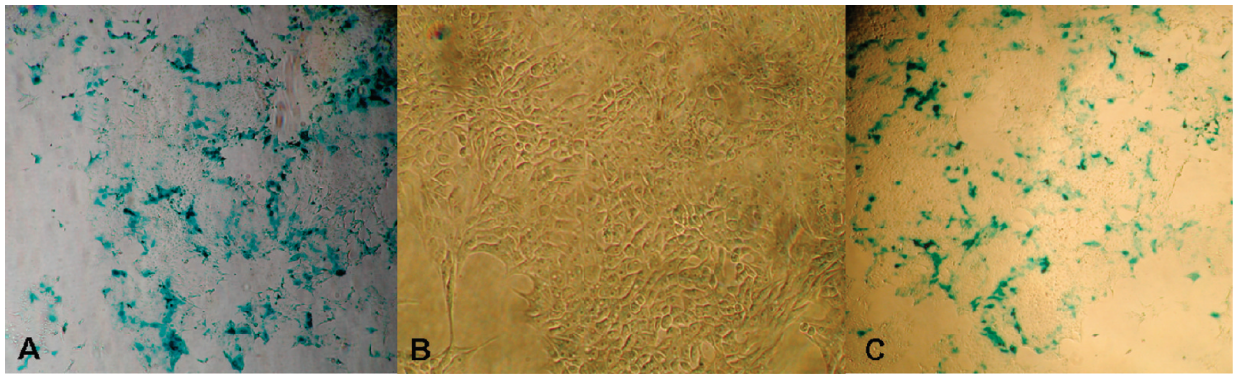


Figure 3. Pictures of X-Gal assay in LLC PK1 cells using lipid **1**, lipid **6**, and LipofectAmine. Cells were incubated with 2.5 μg/mL of DNA and stained 48 h after starting transfection. (A) LipofectAmine, magnification 1:10. (B) Lipid 1/DOPE 1:1 (*n*/*n*), magnification 1:200. (C) Lipid 6/Cholesterol 2:1 (*n*/*n*), magnification 1:10.

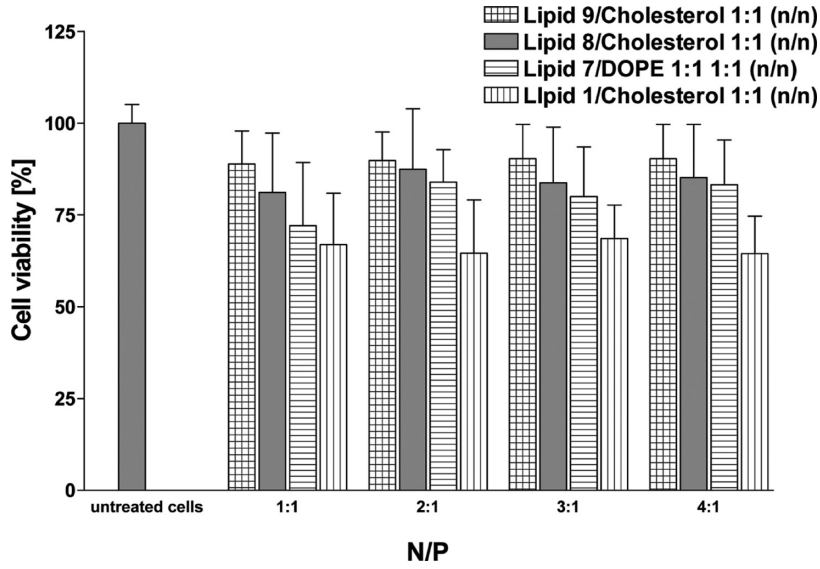


Figure 4. MTT-based cellular cytotoxicities of lipids **1**, **7**, **8**, and **9** in LLC PK1 cells using DOPE or cholesterol as colipid (at a lipid/colipid molar ratio of 1:1). The percent cell viability was plotted against the varying N/P ratios. The experiments were performed as described in the text. The cytotoxicity values shown are the average of at least three experiments carried out on different days.

are important for cell damaging characteristics of cationic liposomes, MTT measurements of lipoplexes consisting of lipid **8** were specified.

Figure 5 summarizes these results in LLC PK1 cells. As shown, the N/P ratios are varied from 1:1 to 4:1 for all applied lipoplexes. The concentration of lipid **8** is the same in all samples and increases merely with increasing N/P ratios (in the range 4.05−16.2 μmol/L). It is shown that lipoplexes consisting of a larger percentage of colipids exhibit more cytotoxic potential as lipoplexes consisting of larger percentage of lipid **8**. With increasing concentration of the helper-lipid, cell survival decreases. This effect is highly pronounced using cholesterol, but also visible using DOPE as colipid. The concentration of the colipid also seems to play a decisive role in cell damaging characteristics of cationic liposomes. As the mixed liposomes influence the shape and structure of lipoplexes, variances in the ratio between the cationic and the helper lipids has an impact not only on transfection efficacy, but also on compatibility and consequently the cell viability.

Figure 6 summarizes the results of PCS measurements for selected malonic acid amides. Since particle size seems to be crucial for transfection efficiencies compared with lipoplex charge or zeta potential (*41*), the size measurements of all lipoplexes were used to find a correlation of transfection efficacy and particle size. For DOTAP lipoplexes, it was shown that particles with 500 nm sizes were more effective in cell culture experiments than 100 nm lipoplexes (*41, 43*). For successful in vivo gene transfer, particles should have a size less than 150 nm, because larger ones are quickly cleared from blood (*44, 45*).

The results of size measurements of six liposomes and lipoplexes consisting of lipid **1**, lipid **2**, lipid **7**, lipid **8**, and lipid **9** are plotted. They were chosen to distinguish between well-transfecting and nontransfecting lipoplexes. All of them disclose some similarities. The increasing particle size in connection with increasing cationic lipid concentrations has a maximum value at charge equalization (*17*). The displaced maximum values are presumably due to varied capability to condense DNA. By comparing lipoplexes consisting of lipid **7**, lipid **8**, and lipid **9**, it is conspicuous that not only the number of amine residues and the cationic lipid concentration but also the fluidity of liposome membranes is important for charge equalization. Unfortunately, the differences in particle size between transfecting and nontransfecting liposomes are marginal, so that differentiations between the samples are hardly assessable. Certainly, it should be noted that lipoplexes, which exhibit appreciable transfection features, have *z*-average values which are only slightly larger than those of the pure liposomes. The particle size of the majority of lipoplexes is less than 500 nm (cf. Table 1).

As a matter of course, lipid **6**, lipid **8**, and lipid **9** were analyzed for their ability to transfer genes in another cell strain. Figure 7 summarizes the results of in vitro gene delivery and

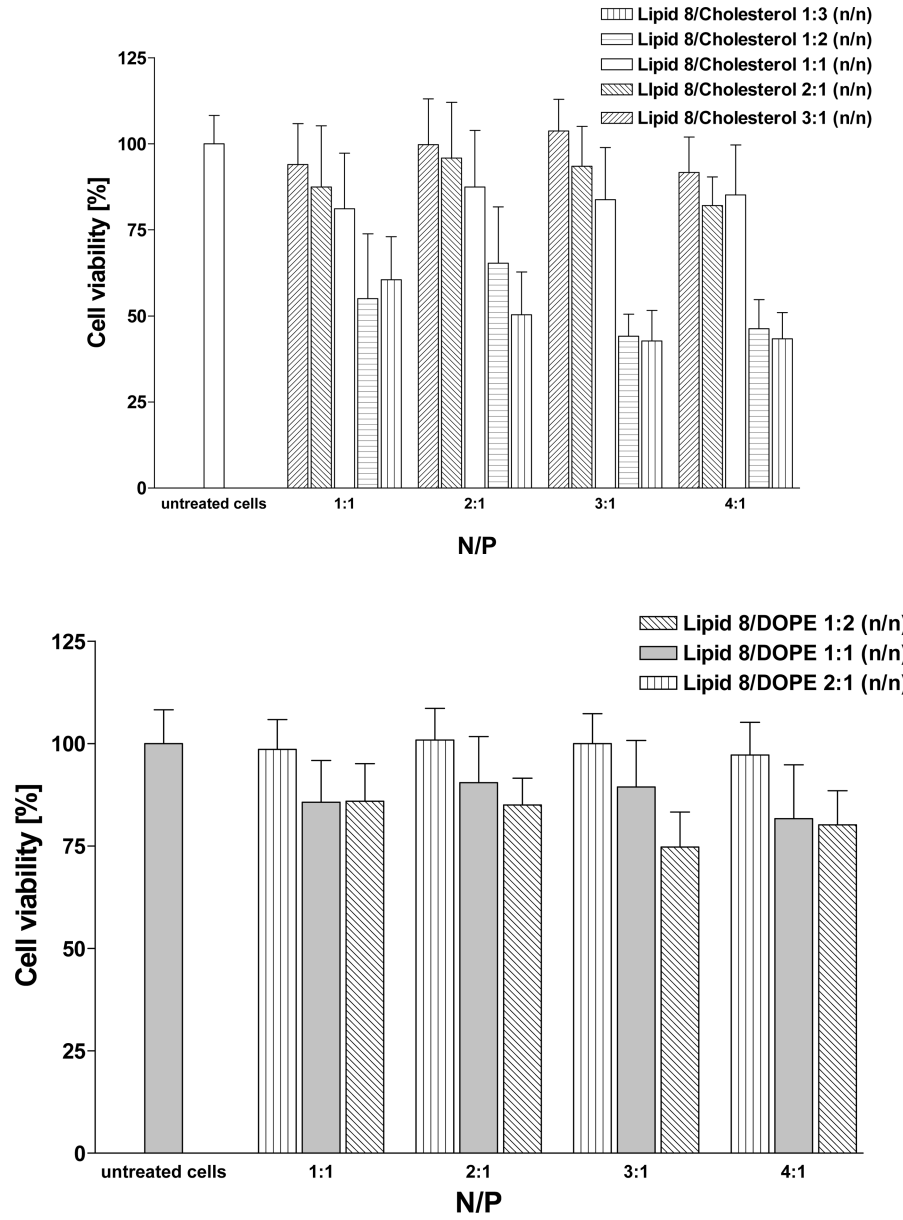


Figure 5. MTT-based cellular cytotoxicities of lipid **8** in LLC PK1 cells using cholesterol and DOPE as colipid (at different lipid/colipid molar ratios). The percent cell viability was plotted against the varying N/P ratios.

MTT based cell viability assays in A549 cells. Even if transfection efficacy of lipid **8** is not as appreciable as in LLC PK1 cells, it is still comparable to LipofectAmine and SuperFect. In human lung carcinoma cells, the multivalent cationic lipids **6** and **9** with two unsaturated alkyl chains were found to transfer DNA statistically significantly better than commercially available vectors at similar cell viability values (*).

Figure 8 summarizes the experiments of gene transfer in the presence of FBS in LLC PK1 cells. Lipoplexes were incubated with the cells as described in the Experimental Procedures section, certainly the concentration of serum was adjusted to 10% from the outset of experiments and kept for 48 h. Admittedly, total protein concentration of human serum that is between 66 and 83 g/L (*46*) is just reached with a level of 60%, but information about serum compatibility of applied lipoplexes is still possible. A slight influence of serum was observed for SuperFect and lipid **8**/cholesterol 2:1 (*n*/*n*), whereas lipid **8**/cholesterol 2:1 (*n*/*n*) offers nearly twice the amount of transfection efficacy. Notably, for LipofectAmine, which contains DOPE, a decrease of gene transfer was observed. Lipoplexes consisting of lipid **6** and lipid **9** deteriorate in transfection efficacy in regard to gene transfer without serum over a period of 4 h. Nevertheless, these samples exhibit gene transfer rates in excess of LipofectAmine that are partially statistically significant (*).

DISCUSSION

Since their introduction as gene carriers in 1987 (*15*), cationic liposomes have become well-studied nonviral vectors (*47*). Lipoplex mediated gene delivery systems are currently the most favorable means to achieve transgene expression in cells in culture. In the meantime, about 1300 clinical trials in gene therapy research have been carried out (*14*). Although hundreds of cationic lipids have been studied (*48*), there is still a need to develop new gene transfer systems.

By engineering new lipids for nonviral gene delivery, it is important to investigate structure/activity relationships to expedite the reasonable development of these substances. In the case of malonic acid amides, several effects of structure−function relationships were demonstrated. Independent of the chemical structure of the hydrophilic molecule part or the colipid, it was observed that the insertion of at

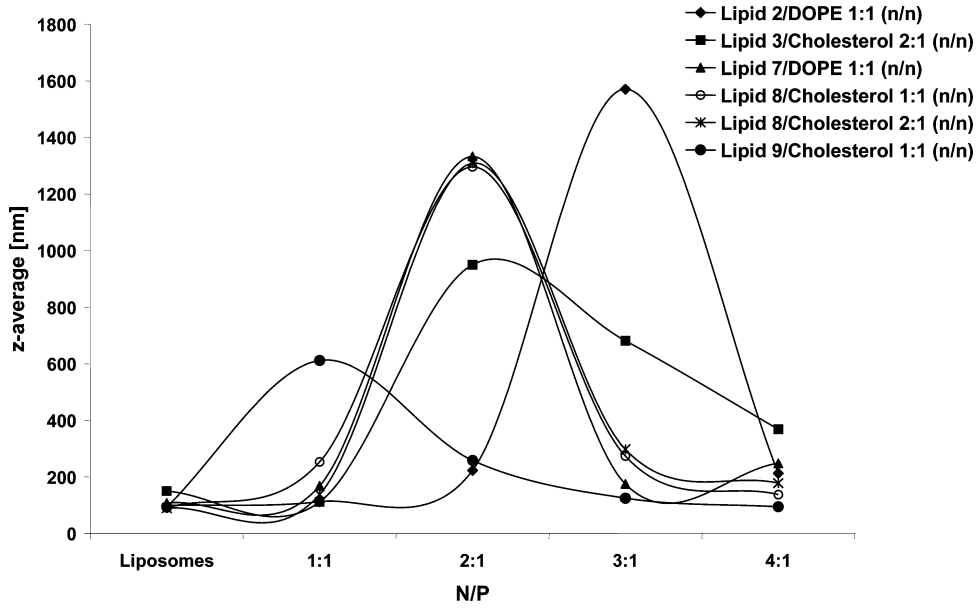


Figure 6. Photon correlation spectroscopy analyses of liposomes and lipoplex mixtures: Changes of particle size as a function of increasing lipid concentration (concentration of DNA = 2.5 μg/mL). The average diameters of lipoplex particles in each set of lipoplex mixtures were determined by three spectroscopic runs comprising ten consecutive scans, each of 10 s in duration.

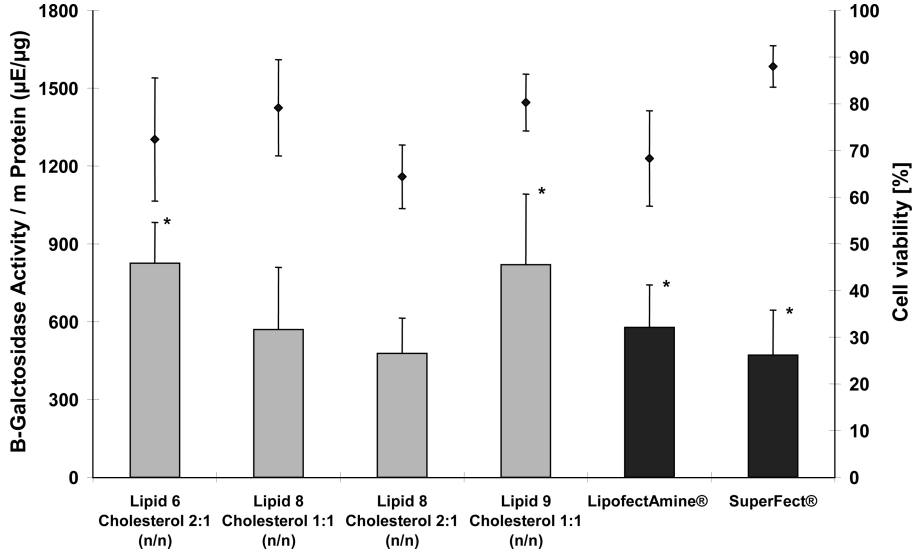


Figure 7. In vitro transfection efficacies and MTT based cell viability values of lipids **6**, **8**, and **9** in A549 cells using cholesterol as colipid. The transfection efficacies and percent cell viability values were compared with LipofectAmine and SuperFect. Percent cell viability is plotted on a secondary *y*-axis via scatter diagram. The transfection and cell viability values shown are the averages of at least three experiments carried out on different days. Statistical analyses were carried out with $p < 0.05$, one-way ANOVA, and Bonferoni's multiple comparison tests (*).

least one unsaturated alkyl chain leads to higher transfection efficacies. Already, Felgner et al. could demonstrate that increasing membrane fluidity often leads to increasing transfection efficacies (*16*). This effect has been attributed to an enhanced capability of fluid amphiphiles to condense DNA (*16, 49*). Besides the electrostatic interaction of cationic lipids and DNA, packing properties of the lipids seem to be important for the condensation of DNA (*40*). It was shown that the insertion of tris(aminoethyl)amine and lysine as hydrophilic headgroup increase transfection efficacy. Although lipids **1**−**3** are able to adsorb DNA in monolayer experiments and protect partially nucleic acids in ethidium bromide exclusion assay (data not shown), multivalent headgroups seem to be more suitable for lipoplex-mediated gene transfer. Except for monovalent substances, all lipids exhibit higher transfection efficacies in combination with cholesterol as colipid. Helper lipids mainly affect the tertiary structure of the plasmid DNA in lipoplexes (*8*). Although this does not affect the transfection effiency (*39, 50*), the influence of colipids for gene transfection is indisputable. As mentioned above, colipids influence the transfection because of their tendency to adopt the inverted hexagonal H_{II} phase that seems to be necessary for the DNA release from the endosomal compartment. Furthermore, DOPE promotes the lipid exchange between liposome and the endosome membrane, which is also crucial for the endosome releasing process (*51, 52*).

The fact that cholesterol was found to be a more efficacious colipid than DOPE is an encouraging result in regard to in vivo gene transfer experiments. Although the application of cationic liposomes as gene delivery systems in cells in culture is popular, the in vivo application still has serious drawbacks (*53*). Due to the chemical structure of the lipids used, liposomes interact with different components of the human

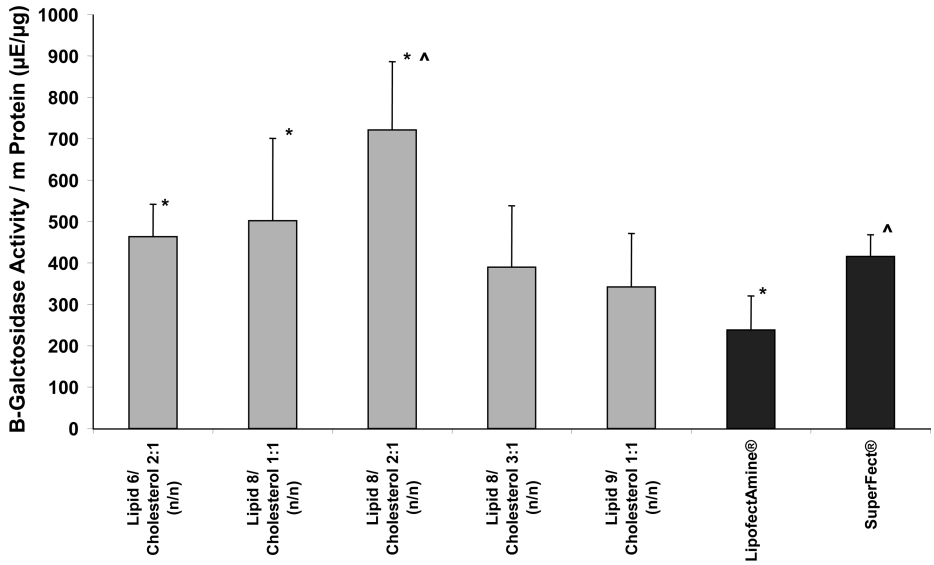


Figure 8. In vitro transfection efficacies of lipids **6**, **8**, and **9** in LLC PK1 cells in the presence of serum compared with LipofectAmine and SuperFect. Concentration of FBS was adjusted to 10% from the outset of experiment and kept up for 48 h. N/P ratios: lipid **6**, 6:1; lipid **8**, 4:1 and 4:1; lipid **9**, 3:1. The transfection values shown are the average of leastwise three experiments carried out on three different days. Statistical analyses were carried out with $p < 0.05$, one-way ANOVA, and Bonferoni's multiple comparison tests (*^).

organism, especially negatively charged amphipathic proteins and polysaccharides that are present in blood, mucus, or tissue matrix (*47*). Plasma proteins like albumin adsorb on the liposome surface and make them recognizable for cells of the reticuloendothelial system (*54*). For liposomes consisting of phospholipids, it is well-known that lipoproteins lead to membrane disintegrations as a consequence of lipid exchange. Mainly liposomes containing lipids with unsaturated fatty acids interact notably with lipoproteins (*55*). For that reason, DOPE is unsuitable for in vivo gene transfer (*56*). On the contrary, cholesterol was found to stabilize the lipoplex structure in blood (*57, 58*). To minimize disadvantageous effects of nonviral vectors in systemic gene delivery, PEG−lipid conjugates were successfully incorporated in lipoplexes (*59*). On the other hand, lipoplexes exhibiting certain serum stability without modification could advance in vivo gene delivery. As shown, cationic liposomes that consist of malonic acid amides have the ability to transfer nucleic acids even in the presence of FBS.

Malonic acid amides describe an interesting novel group of cationic amphiphiles. Nine substances were synthesized and mixed with DOPE or cholesterol, and liposomes were tested for their ability to transfect cells in culture. It was shown that three of them transfect cells at comparable or even higher levels than commercially available LipofectAmine and SuperFect. Thereby, it was observed that lipids containing at least one unsaturated alkyl chain in the hydrophobic molecule part and multivalent headgroups are very successful in gene transfer experiments. Along with this, the cell damaging characteristics of lipoplexes were analyzed. Although the addition of lipoplexes is connected with decreasing cell survival, in the majority of cases the applied lipoplexes show remarkably high cell viability (>80%).

Furthermore, some lipids proved to be successful in gene transfer experiments notwithstanding the presence of serum. Certainly, despite these promising results, further research is clearly needed to clarify the complex mechanism of cationic lipid mediated gene transfer. The presented malonic acid amides are qualified for these requirements. The preparation facilitates the trouble-free variation of alkyl chain length. In addition, multivalent hydrophilic molecule parts are suited to link other cationic residues or molecules for functionalization. Thereby, we expect further developments from this kind of cationic lipids toward successful in vivo gene transfer.

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