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Hydrophobic Oxime Ethers: A Versatile Class of pDNA and siRNA Transfection Lipids

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Abstract

The manipulation of cationic lipid structure to increase polynucleotide binding and delivery properties while also minimizing associated cytotoxicity has been a principal strategy for developing next-generation transfection agents. The polar (DNA-binding) and hydrophobic domains of transfection lipids have been extensively studied; however, the linking domain comprising the substructure used to tether the polar and hydrophobic domains has attracted considerably less attention as an optimization variable. Here, we examine the use of an oxime ether as the linking domain. Hydrophobic oxime ethers were readily assembled via click chemistry by oximation of hydrophobic aldehydes using an aminooxy salt. The facile ligation reaction delivered the title compounds with hydrophobic domain asymmetry. Using the MCF-7 breast cancer, H1792 lung cancer and PAR C10 salivary epithelial cell lines, our findings show that lipoplexes derived from the oxime ether lipids transfect in the presence of serum at higher levels than commonly used liposome formulations, based on both luciferase and GFP assays. Given the biological compatibility of oxime ethers and their ease of formation, this functional group should find much application as a linking domain in future designs of transfection vectors.

Keywords

gene transfer; non-viral; transfection; oximation; aminooxy

Introduction

Cationic lipids and their derived liposomes have become the most well-studied and widely used synthetic, non-viral gene delivery vehicles since Felgner *et al.*^[1] first disclosed DOTMA-mediated gene transfer in 1987.^[2,3] Due to the major limitations of viral vectors, such as associated immune responses, limited polynucleotide carrying capacity and high cost,^[4,5] cationic lipids remain an attractive alternative. The advantages of low immunogenicity, the ability to transfect RNA or DNA of nearly unlimited size,^[6,7] and the relative ease of lipoplex (cationic lipid-plasmid DNA/RNA complex) formulation^[8] continue to attract interest aimed at developing safer and more efficient cationic lipids.^[9]

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Cationic lipid molecules, such as the prototypical dual chain lipids DOTMA^[1] and DOTAP^[10] (Figure 1), contain a polar, positively charged (DNA-binding) headgroup connected to a hydrophobic domain via linking functionality. These three principal structural components of cationic glycerol-type lipids have been extensively studied in efforts to improve lipid-mediated intracellular delivery of polynucleotides to mammalian cells.^[11] Many structure-activity relationships have been determined,^[12,13] particularly for the hydrophobic domain. Indeed, the hydrophobic domain structural variables of chain length, degree of unsaturation, and domain asymmetry are among the strongest contributors to transfection efficacy.^[14] Fewer direct structural comparisons of changes in the cationic lipid backbone, or linking domain, have been reported, with the most well known comparison being that of the di-ether DOTMA vs. di-ester DOTAP. The linking functionality, and to a lesser extent the cationic headgroup, seem to be the principal determinants of toxicity.^[15–17] The linker determines conformational flexibility, degree of stability, and biodegradability. Among the most studied chemical functionalities comprising the linking domain of transfection lipids are the ether, ester, ortho ester,^[18,19] carbamate,^[20] amide,^[21,22] and phosphono^[23] moieties.

The use of oxime ether linkages for applications in chemical biology and medicinal chemistry research has increased dramatically in the past several years as the benefits of chemoselectivity have become better understood. Chemoselective click ligation^[24] of an aldehyde or ketone carbonyl group with an aminooxy counterpart to form an oxime ether linkage (i.e., oximation, Figure 2) is the key step in the synthesis of numerous bioconjugates.^[25–27] Oxime ether linkages also are used as a mechanism for pro-drug generation,^[28] and are present in FDA-approved pharmaceuticals, such as fluv-oxamine.^[29] Although oximation has been used to attach ligands to transfection lipids, such as the ligation of carbohydrates,^[30] and although an oxime ether-based assembly for siRNA delivery has been reported,^[31] no studies have examined the use of the robust yet biodegradable oxime ether linkage as the backbone feature of cationic lipids for DNA delivery.

Our program in developing lipid based gene delivery vectors^[32] and interest in click chemistry^[33,34] inspired us to use oximation as a facile approach to prepare low toxicity transfection-active oxime ethers (Figure 2). We report herein the physico-chemical characterization of this new class of lipids and *in vitro* evaluation of a hydrophobic oxime ether panel in pDNA and siRNA delivery experiments using epithelial cell lines, including two human cancer cell lines.

Results and Discussion

Synthesis of hydrophobic oxime ethers

We prepared a panel of oxime ethers by reacting bis(aminooxy) ammonium salt **1**^[35] with hydrophobic aldehydes dodecanal, tetradecanal, or (*Z*)-octadec-9-enal (oleyl aldehyde) in methanol at room temperature to afford oxime ethers **2**, **3** and **5**, respectively (Figure 2). In each case, the lipid was obtained as a mixture containing two stereoisomers (*ca.* ranging between 2.3:1 to 5.3:1). The major diastereomer exhibited (*E*)-stereochemistry about each oxime C=N bond (as depicted). The minor diastereomer featured an (*E*)-oxime ether as well as a (*Z*)-oxime ether linkage. The (*E*, *E*):(*E*, *Z*) ratio was readily measured by integration of the well-separated oximyl proton shifts in ¹H NMR,^[36] and the ratio was found to be susceptible to a variety of factors including exposure to mild acid and heat. The stereochemical integrity of the oxime ether lipids is inconsequential in that isomerization can be expected *in vivo*. Of greater interest, however, is that the oximation approach used to prepare these compounds delivers the lipids with intrinsic asymmetry in the hydrophobic domain. We have recently noted the beneficial influence of unsymmetrical hydrophobic

domains in cationic lipid-mediated DNA transfection.^[32] Consequently, we used the oxime ether lipids as obtained directly in transfection experiments. To probe further the influence of an unsymmetrical hydrophobic domain, we also prepared unsymmetrical analog **4** (Figure 2) by successively condensing **1** with dodecanal and tetradecanal. Hydrophobic oxime ethers **2–5** were formulated as cationic liposomes using equimolar quantities of co-lipid dioleoylphosphatidylethanolamine (DOPE) for subsequent studies.

Size and charge characterization

Particle size and zeta potential measurements on the oxime ether liposome suspensions revealed that the particles were net positively charged with electrostatic potentials in the range 45–66 mV (Figure 3). Liposomes derived from bis- C_{18} lipid **5** were the largest, with particle diameters exceeding 200 nm, and the most highly charged. Not unexpectedly, the liposomes derived from the shortest oxime ether lipids **2** and **4**, lipids prepared using dodecanal, had the smallest mean diameters, near 150 nm. The trend in particle size vs. particle charge correlated well for this series of lipids.

Of significance as a predictor of transfection activity is the net charge of the lipid-pDNA complex (lipoplex) formed on mixing a liposome suspension with pDNA.^[13,37] Complexation of the oxime ether:DOPE liposomes with pDNA (pCMV-Luc) at different ammonium nitrogen:DNA phosphate ratios (N:P ratio) gave the corresponding lipoplexes. Zeta potential measurements of the oxime ether-derived lipoplexes at N:P ratios of 3, 5 and 7 showed high positive values in the range of 31–53 mV (Figure 4). As can be expected at these electrostatic potentials, minimal lipoplex aggregation ensued — the oxime ether:DNA lipoplex suspensions were stable at concentrations up to 0.33 mg oxime ether lipid/mL beyond one week. Particle size measurements of the lipoplexes (data not shown) indicated that the mean effective diameter for all the N/P formulations remained under 250 nm. Lipoplex size mirrored closely the precursor liposome, with the lipoplex generally having an increase in diameter by 20–50 nm.

Transfection activity

We evaluated the transfection activity of the dual-chain oxime ether lipid panel in human breast cancer cells (MCF-7) and in human lung cancer cells (H1792) using a CMV-luciferase reporter plasmid in the presence of serum (Figure 5). Whereas the shortest-chain lipid **2** was essentially ineffective at all N:P formulations examined in MCF-7 cells (data not shown), we were gratified to find that the transfection efficiency of lipid **3** in this cell line at N:P=7 was notably superior (>2.5 order of magnitude higher activity) to the commercial transfection standard Lipofectamine 2000 (RLU 3500 ± 990) as well as to the popular^[38] transfection formulation jetPrime (RLU 2500 ± 209) (Figure 5A). The unsaturated lipid **5** also was found to be efficacious in MCF-7 cells, although we observed greater deviation in the measurement of transgene expression at the higher N:P ratio using this lipid (Figure 5A). We did not observe an additional boost in transfection activity on further amplifying the hydrophobic domain asymmetry by using dissymmetric lipid **4**, relative to the activities of lipids **2** and **5** in this cell line.

On a scale of 1 μ g DNA/well, transfection of MCF-7 cells in a 6-well format using lipoplexes derived from lipid **3** at a 7:1 N:P ratio gave ~70-fold higher luciferase expression (as assessed by RLU/ μ g protein) than did Lipofectamine 2000-derived lipoplexes (see Supporting Information). This result shows that the oxime ether lipid **3** is effective over a wide range of DNA dose.

Only lipids **4** and **5** led to high transgene expression in H1792 cells (Figure 5B). In agreement with previous studies on lung cell transfections,^[14,39] the presence of

hydrophobic domain un-saturation, such as present in lipid **5**, proved to be beneficial for activity. Lipoplexes derived from lipid **5** effectively transfected H1792 cells relative to the commercial controls (*ca.* >60 fold higher activity at N:P=7). Interestingly, the enhanced asymmetry of lipid **4** resulted in improved transfection of this cell line relative to the other saturated lipids examined.

To further examine the gene transfer capability of lipids with an oxime ether linker domain, we used a green fluorescent protein (GFP) reporter vector to estimate the transfection efficiency in MCF-7 cells (Figure 6). Examination of cells by fluorescence microscopy using an EVOS fluorescent microscope determined that the results were in agreement with luciferase assay.

MCF-7 and H1792 cells are both cancer cell lines. To test the lipids in a non-cancer epithelial cell line, GFP was expressed in the salivary gland cell line PAR C10.^[40] Lipoplexes were formulated at an N:P ratio of 1.5, the optimal ratio determined for lipids **2–5** in this cell line. GFP expression was evaluated 48h post-transfection. The fraction of transfected cells was quantitated by flow cytometry. Lipid **3** transfected approximately 40% of the cells, lipid **2** transfected about 20% while lipids **4** and **5** exhibited less than 10% transfection (Figure 7A). Lipofectamine was slightly more effective in this cell line when used in excess (>3X recommended dose, Figure 7A) and comparable to lipid **3** when used at the vendor's recommended dose (Figure 7B). These results suggest that lipids with an oxime ether linker domain are suitable for pDNA transfection of epithelial cell lines and, depending on hydrophobic domain composition, competitive with the transfection standard Lipofectamine.

Transfection with siRNA

In addition to pDNA transfection we tested the ability of lipid **3** to transfect epithelial cells with siRNA. The cellular “house-keeping” gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as a target and transfection efficiency was determined as percent reduction of GAPDH activity. Lipid **3** reduced GAPDH activity $55\% \pm 9\%$ (mean $\pm$ SEM) while the control lipid Lipofectamine RNAiMAX reduced GAPDH activity by $71\% \pm 6\%$ (N=9). This difference was not statistically significant ($P=0.23$). These results suggest that lipid **3** effectively introduces siRNA into epithelial cells.

Cytotoxicity measurements

To measure the cytotoxicity of the lipids in MCF-7 and in H1792 cells, we performed cell viability studies using the vital dye trypan blue to stain dead cells. The percentage of live cells remaining after treatment was calculated (Figure 8). Our lipids were found to be less toxic than Lipofectamine and jetPrime in both cell lines, with a live cell percentage comparable to untreated cells at all N:P formulations examined.

For PAR C10 cells, the viability was determined by reduction of alamar blue (Figure 9). None of the lipids reduced cell viability significantly compared to untreated cells, confirming the low toxicity of lipids with an oxime ether linker domain.

Conclusion

We have demonstrated that the facile aminooxy-carbonyl click reaction can be used to rapidly assemble transfection lipids furnished with asymmetric hydrophobic domains. The data show that lipids containing an oxime ether linking domain are well tolerated by both cancerous and non-cancerous epithelial cells. Furthermore, with appropriate tuning of hydrophobic domain composition and N:P formulation, lipoplexes derived from the oxime

ether lipids readily transfected representative epithelial cell lines with excellent activity relative to established transfection reagents. The present study shows that oxime ethers are a suitable choice for linker domain. Given the biological compatibility of oxime ethers and their ease of formation, this functional group should find much application in future designs of transfection vectors.

The ability to transfect epithelial cells could have clinical applications, as the epithelial surfaces are readily accessible and are attractive targets for gene therapy protocols that aim to express therapeutic proteins in vivo.^[41] The ability to simultaneously transfect pDNA and siRNA with a single reagent now opens potential applications to express therapeutic proteins in cells that are simultaneously modified by gene knock-down.

Experimental Section

Chemistry

All ¹H NMR (400 MHz) and ¹³C NMR (100 MHz) spectra were recorded in CD₃OD. HRMS was performed by the CREAM facility at the University of Louisville.

***N,N*-dimethyl-*N*-2-((dodecylideneamino)oxy)-(2-((dodecylideneamino)oxy)ethyl)ethanaminium iodide (2)**—Bis(amino-oxy)ammonium iodide **1** (26.5 mg, 0.091 mmol) and dodecanal (35.4 mg, 0.19 mmol) were dissolved in methanol (3 mL) and stirred at room temperature. After 3.5 h, the solvent was removed by rotary evaporation and the residue was purified by column chromatography (SiO₂), eluting with a 3% mixture of methanol:CH₂Cl₂ to afford oxime ether lipid **2** as a white solid (34.6 mg, 61%) (5.3:1 mixture of (*E, E*):(*E, Z*) oxime ether diastereomers); *R*_f = 0.52 (CH₂Cl₂/MeOH, 9:1); mp 155 °C (dec); ¹H NMR (400 MHz, CD₃OD): (major diastereomer) δ=0.86 (t, *J* = 6.8 Hz, 6H), 1.25 (m, 28H), 1.43–1.48 (m, 4H), 2.17 (dd, *J* = 6.8, 7.2 Hz, 4H), 3.20 (s, 6H), 3.73–3.75 (m, 4H), 4.40–4.41 (m, 4H), 7.46 ppm (t, *J* = 6.0 Hz, 2H); ¹³C NMR (100 MHz, CD₃OD): δ=14.6, 23.9, 27.1, 27.3, 27.7, 30.4, 30.6, 30.9, 33.2, 53.4, 64.9, 68.0, 68.3, 155.0, 155.5 ppm; IR (neat): $\tilde{\nu}$ =1735, 1468, 966, 920 cm⁻¹; HRMS *m/z* [*M*⁺] calcd for C₃₀H₆₂N₃O₂⁺: 496.4837, found: 496.4839.

***N,N*-dimethyl-*N*-2-((tetradecylideneamino)oxy)-(2-((tetradecylideneamino)oxy)ethyl)ethan-aminium iodide (3)** was prepared as previously described.^[35]

***N,N*-dimethyl-*N*-2-((dodecylideneamino)oxy)-(2-((tetradecylideneamino)oxy)ethyl)ethan-aminium iodide (4)**—Bis(amino-oxy)ammonium iodide **1** (22.6 mg, 0.078 mmol) and dodecanal (7.2 mg, 0.034 mmol) were dissolved in methanol (1 mL) and stirred at room temperature. After 14 h, the solvent was removed by rotary evaporation and the residue was purified by column chromatography (SiO₂), eluting with a 5% mixture of methanol:CH₂Cl₂ to afford the inter-mediate mono-oxime ether as a yellow oil (14.0 mg, 39%) (1.7:1 mixture of (*E, E*):(*E, Z*) oxime ether diastereomers); *R*_f = 0.25 (CH₂Cl₂/MeOH, 9:1); ¹H NMR (400 MHz, CD₃OD): (major diastereomer) δ=0.85 (t, *J* = 6.8 Hz, 3H), 1.24 (m, 16H), 1.45 (dd, *J* = 6.4, 6.8 Hz, 2H), 2.16 (dd, *J* = 6.4, 7.4 Hz, 2H), 3.17–3.20 (m, 6H), 3.65–3.74 (m, 4H), 4.04 (m, 2H), 4.40 (m, 2H), 7.45 ppm (t, *J* = 6.0 Hz, 1H); ¹³C NMR (100 MHz, CD₃OD): δ=14.6, 23.9, 27.1, 27.3, 27.6, 30.4, 30.6, 30.8, 30.9, 33.2, 53.3, 64.7, 68.0, 68.4, 70.0, 134.0, 155.0, 155.5 ppm; HRMS *m/z* [*M*⁺] calcd for C₁₈H₄₀N₃O₂⁺: 330.3115, found: 330.3118.

The C₁₂ mono-oxime ether intermediate (13.1 mg, 0.029 mmol) and tetradecanal (9.1 mg, 0.043 mmol) were dissolved in methanol (1 mL) and stirred at room temperature. After 14 h, the solvent was removed by rotary evaporation and the residue was purified by column chromatography (SiO₂), eluting with a 3% mixture of methanol:CH₂Cl₂ to afford

unsymmetrical lipid **5** as a yellow oil (13 mg, 69%) (2.3:1 mixture of (*E, E*):(*E, Z*) oxime ether diastereomers); $R_f = 0.48$ ($\text{CH}_2\text{Cl}_2/\text{MeOH}$, 9:1); ^1H NMR (400 MHz, CD_3OD): (major diastereomer) $\delta = 0.84$ (t, $J = 6.8$ Hz, 6H), 1.23–1.26 (m, 36H), 1.44 (dd, $J = 6.8, 7.2$ Hz, 4H), 2.15 (dd, $J = 6.4, 7.2$ Hz, 4H), 3.18 (s, 6H), 3.69–3.72 (m, 4H), 4.38–4.40 (m, 4H), 7.44 ppm (t, $J = 6.2$ Hz, 2H); ^{13}C NMR (100 MHz, CD_3OD): $\delta = 14.6, 23.9, 27.1, 27.3, 27.6, 30.4, 30.6, 30.7, 30.8, 30.9, 33.2, 53.3, 64.9, 68.0, 68.4, 155.0, 155.5$ ppm; IR (neat): $\tilde{\nu} = 1728, 1467, 964, 942\text{ cm}^{-1}$; HRMS m/z [M^+] calcd for $\text{C}_{32}\text{H}_{66}\text{N}_3\text{O}_2^+$: 524.5150, found: 524.5172.

***N,N*-dimethyl-*N*-2-((((*Z*)-octadec-9-en-1-ylidene)amino)oxy)-(2-((((*Z*)-octadec-9-en-1-ylidene)amino)oxy)ethyl)ethanaminium iodide (**5**)—**

Bis(aminoxy)ammonium iodide **1** (19.7 mg, 0.068 mmol) and *cis*-9-octadecenal (37.8 mg, 0.142 mmol) were dissolved in methanol (3 mL) and stirred at room temperature. After 3.5 h, the solvent was removed by rotary evaporation and the residue was purified by column chromatography (SiO_2), eluting with a 3% mixture of methanol: CH_2Cl_2 to afford oxime ether lipid **4** as a yellow oil (45.8 mg, 90.0%) (3.0:1 mixture of (*E, E*):(*E, Z*) oxime ether diastereomers); $R_f = 0.51$ ($\text{CH}_2\text{Cl}_2/\text{MeOH}$, 9:1); ^1H NMR (400 MHz, CD_3OD): (major diastereomer) $\delta = 0.84$ (t, $J = 6.8$ Hz, 6H), 1.23–1.27 (m, 40H), 1.42–1.47 (m, 4H), 1.96–1.97 (m, 8H), 2.15 (dd, $J = 6.4, 7.2$ Hz, 4H), 3.16 (s, 6H), 3.68–3.71 (m, 4H), 4.38–4.39 (m, 4H), 5.27–5.29 (m, 4H), 7.43 ppm (t, $J = 6.0$ Hz, 2H); ^{13}C NMR (100 MHz, CD_3OD): $\delta = 14.6, 23.9, 27.6, 28.3, 30.4, 30.5, 30.6, 30.8, 31.0, 33.2, 53.3, 64.9, 68.0, 130.9, 131.1, 155.0$ ppm; IR (neat): $\tilde{\nu} = 1730, 1464, 964, 942\text{ cm}^{-1}$; HRMS m/z [M^+] calcd for $\text{C}_{42}\text{H}_{82}\text{N}_3\text{O}_2^+$: 660.6401, found: 660.6395.

Zeta potential and particle size—Measurements of liposome and lipoplex zeta potential and particle size were performed using a ZetaPALS dynamic light scattering detector (Brookhaven Instruments Corporation; Model 90 Plus). All measurements were taken in water.

Lipoplexes were formulated at a final concentration of 0.1 mg oxime ether lipid/mL water. To formulate lipoplexes at different N:P ratios, 100 μL of the liposome formulation was added to the required quantity of pDNA (taken from a stock solution of 0.1 mg pDNA/mL) in an Eppendorf tube. After incubating each lipoplex 15 min, the solutions were diluted to 1 mL by adding ultra pure water. Measurements were taken within minutes of formulation.

Biology

Tissue culture—Human breast cancer cells (MCF-7) and lung tumor cells (H1792) were purchased from American Type Culture Collection VA, USA. Cells were grown to 50–60% confluency prior to transfection. MCF-7 cells were cultured in DMEM (Invitrogen, Carlsbad CA), 1% Pennstrep (Mediatech Inc, VA), 10% Fetal Bovine Serum (FBS) (Valley Biomedical, Winchester, VA). H1792 cells were cultured in RPMI (Invitrogen), 1% PennStrep, 10% FBS.

The PAR C10 cell line was immortalized from rat parotid acinar cells.^[40] Experiments were performed on cells of passage number 40–60. The cultures were grown to confluence in DMEM-Ham's F12 (1:1) containing 2.5% (vol/vol) fetal bovine serum (GIBCO BRL, Gaithersburg, MD) and the following supplements: 0.1 μM retinoic acid, 80 ng/mL epidermal growth factor, 2 nM triiodothyronine, 5 mM glutamine, 0.4 $\mu\text{g/mL}$ hydrocortisone, 5 $\mu\text{g/mL}$ insulin, 5 $\mu\text{g/mL}$ transferrin, 5 ng/mL sodium selenite and 50 $\mu\text{g/mL}$ gentamicin (all from Sigma, St. Louis, MO). Cells were cultured at 37 °C in a humidified atmosphere of 95% air-5% CO_2 .

Liposome formulation—To the oxime ether lipid (1 mg) in CHCl_3 (0.20 mL) was added an equimolar amount of DOPE from a stock solution (10 mg DOPE/mL chloroform). The solvent was evaporated and the resultant thin lipid film was dried under vacuum 4h. To the dry lipid film was added ultra pure water (3 mL) and the suspension was sonicated (bath sonicator) 1 min at room temperature to furnish the liposome formulation (0.33 mg oxime ether lipid/mL).

Luciferase expression—Luciferase transfections were performed in triplicate using 0.025 μg of pDNA (pCMV-Luc)/well in MCF-7 and H1792 cells. Cells were seeded up to 1×10^5 cells/well in a 24-well plate to give 50–60% confluence, and 400 μL of media containing 10% FBS was added to each cell well. Lipoplexes (lipid pDNA complex) were prepared at N:P charge ratios of 3, 5 and 7 by adding the required volume of liposome solution to a pDNA solution (3 μL , 0.025 μg DNA/ μL) and incubated for 5 min. 100 μL of serum free DMEM was then added to each lipoplex solution followed by incubation for 20 min at room temperature with occasional gentle vortex mixing. The lipoplex solutions were diluted to 600 μL with serum free DMEM, and then 200 μL of the final lipoplex formulation was added directly to each cell well. No additional media containing FBS was added to the cells during transfection. After 18h incubation at 37.5°C , the cells were lysed and luciferase expression was quantified using a commercial kit (Promega) and luminometer according to the vendor's protocol. Lipofectamine 2000 (Invitrogen) and jetPrime (Polyplus-transfection SA, France) lipoplexes were prepared at the vendor's recommended stoichiometry (2 μL stock/ μg DNA).

GFP expression—Transfection studies were performed using a GFP reporter gene in MCF-7 cells using 0.1 μg of pEGFP (Clontech, WI) DNA/well in 24-well plate with a similar transfection protocol as for the luciferase assays. Cells were examined 40h post-transfection by fluorescence microscopy (EVOS, Advanced Microscopy Group, Bothell, WA) and photographed.

GFP expression also was assessed in PAR C10 cells using the plasmid pZsGreen1-N1(Clontech). Transfection of PAR C10 cells proceeded by plating the cells at a density of 5×10^4 cells/ cm^2 in 24-well plates in complete medium (described above) and 200 ng of pDNA was used/well. Lipoplexes were formed at the N:P charge ratio 1.5 in 200 μL of complete medium containing 2.5% FBS. Media was taken off and cells were transfected with lipoplexes. After 3h, each well was replenished with 250 μL of complete medium. Lipofectamine was used as a positive control. After 48h, GFP expressing cells were analyzed using a FACS Calibur flow cytometer (BectonDickinson, San Jose, CA, U.S.A.) and CELL QUEST software.

Cytotoxicity assay—Lipid cytotoxicity was evaluated using the vital dye trypan blue to distinguish between live and dead cells. MCF-7 and H1792 cells were seeded at 1×10^4 cells/well and then grown to 50–60% confluency in 96-well plates. Transfections were performed as described above. The media was removed 18 h post transfection. Cells were then trypsinized by adding 100 μL trypsin to each well and incubated for 5 min at 37.5°C . Trypsin was neutralized by adding 1X PBS and then 10 μL of extract from each well was mixed with 10 μL of trypan blue. Dead cells and live cells were counted under an inverted microscope to determine the percentage cell viability using a hemocytometer.

Cell viability in PAR C10 cells was assessed using the alamarBlueTM cell viability reagent (Invitrogen). The PAR C10 cells were transfected as described above using lipoplexes formulated from lipids 2–5 at the optimal N:P charge ratio 1.5, and Lipofectamine at 1.6 μL . At 48h post-transfection, the viability assay was performed using the manufacturer's protocol. Absorbance was measured at 600 nm and 570 nm using a Synergy HT Multi-Mode

Microplate Reader (Bio-Tek). The reduction of alamar blue is proportional to proliferation of the cells and is expressed as a percentage (% reduced).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Structural domains of common transfection lipids.

Figure 2.
Oximation route to hydrophobic oxime ethers.

Figure 3.
Zeta potential and particle size measurements for oxime ether:DOPE liposomes (0.1 mg cationic lipid/mL H₂O).

Figure 4. Lipoplex zeta potential measurements. Lipoplexes were formulated at 0.1 mg cationic lipid/mL H₂O with pDNA (pCMV-Luc) at different N:P charge ratios. Results are expressed as the mean values of n=3 measurements.

Figure 5. Transfection of (A) MCF-7 cells and (B) H1792 cells using lipoplexes formulated at different N:P charge ratios. Results are expressed as total relative light units (RLU). Transfections were performed in 24-well tissue culture plates using 0.025 μ g luciferase reporter construct (pCMVLuc) per well with 18h transfection time. Each data point reflects the mean value of three separate transfections. Error bars show the standard deviation from the mean. LFT = Lipofectamine 2000, jetP = jetPRIME.

Figure 6.

MCF-7 cells were transfected with a GFP expression plasmid (0.1 $\mu\text{g}/\text{well}$ in a 24-well plate). Figures are presented as an overlay of green fluorescence (visualized by fluorescent microscopy after 20 hours of transfection) over the phase contrast image: **(A)** cells treated with pDNA (negative control); **(B)** cells transfected with lipoplexes derived from Lipofectamine 2000 (positive control, vendor's recommended dose, 0.25 μL); **(C)** cells transfected with lipoplexes derived from lipid **3** at N:P=7.

Figure 7. GFP expression in PAR C10 cells

(A) Cells were transfected (0.2 μ g GFP reporter construct) with lipoplex formulations derived from lipids 2–5 (N:P 1.5) and excess LFT (positive control, Lipofectamine 2000, 1.6 μ L); (B) Comparison of lipid 3 formulations at the indicated N:P ratio with LFT at the vendor's suggested dose (0.5 μ L). The transfection percentage of GFP expressing cells was analyzed by flow cytometry 48h post-transfection. Each data point reflects the mean $\pm$ SD from three independent experiments.

Figure 8. Relative cytotoxicity

MCF-7 (dark bars) and H1792 cells (light bars) were transfected using either lipofectamine 2000 (LFT), jetPrime (jP) or oxime ether lipoplexes at the specified N:P ratio (value in parenthesis). Transfections were performed using a 96-well format. Dead and live cells were counted 18 h post transfection using a trypan blue stain (n = 3).

Figure 9. Cell viability assay

PAR C10 cells were transfected (N:P 1.5) with lipoplex formulations derived from lipids **2–5** and Lipofectamine 2000 (1.6 μ L). After 48h, the cell viability was measured by calculating the % of alamar blue dye reduction. The data are shown as the mean $\pm$ SD of three independent experiments.