Neurons Are Protected from Excitotoxic Death by p53 Antisense Oligonucleotides Delivered in Anionic Liposomes*

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The potential of anionic liposomes for oligonucleotide delivery was explored because the requirement for a net-positive charge on transfection-competent cationic liposome-DNA complexes is ambiguous. Liposomes composed of phosphatidylglycerol and phosphatidylcholine were monodisperse and encapsulated oligonucleotides with 40–60% efficiency. Ionic strength, bilayer charge density, and oligonucleotide chemistry influenced encapsulation. To demonstrate the biological efficacy of this vector, antisense oligonucleotides to p53 delivered in anionic liposomes were tested in an in vitro model of excitotoxicity. Exposure of hippocampal neurons to glutamate increased p53 protein expression 4-fold and decreased neuronal survival to ~35%. Treatment with 1 μM p53 antisense oligonucleotides in anionic liposomes prevented glutamate-induced up-regulation of p53 and increased neuronal survival to ~75%. Encapsulated phosphothioate p53 antisense oligonucleotides were neuroprotective at 5–10-fold lower concentrations than when unencapsulated. Replacing the anionic lipid with phosphatidylserine significantly decreased neuroprotection. p53 antisense oligonucleotides complexed with cationic liposomes were ineffective. Neuroprotection by p53 antisense oligonucleotides in anionic liposomes was comparable with that by glutamate receptor antagonists and a chemical inhibitor of p53. Anionic liposomes were also capable of delivering plasmids and inducing transgene expression in neurons. Anionic liposome-mediated internalization of Cy3-labeled oligonucleotides by neurons and several other cell lines demonstrated the universal applicability of this vector.

Selective inhibition of gene expression with antisense oligonucleotides (AsONs)† is both a popular technique for probing fundamental questions of neuroscience (1) and a potential therapeutic strategy for the treatment of neurodegenerative diseases (2). However, the elegance of the antisense concept belies the considerable challenge of their intracellular delivery (3). Chemical modifications of ONs that enhance nuclease-resistance (e.g. phosphorothioates) have poor cellular uptake (~5–10%) and cause non-sequence-specific effects, raising questions about the efficacy and selectivity of antisense drugs (4). Cationic lipids and polycationic polymers used as ON delivery vectors have met with limited success due to a number of variables that seem to affect vector performance (3, 5). Mechanistic aspects of cationic lipid-mediated delivery are poorly understood because of the physical heterogeneity of cationic lipid-ON complexes (6) that may contribute to their toxicity toward several cell types (7).

Application of antisense technology to the nervous system presents an even greater challenge because of the post-mitotic nature of neurons and their exquisite sensitivity to their microenvironment. Cationic lipids and polymers have been used to deliver nucleic acids to neurons, generally at efficiencies of 0.5–5% (8). Factors that influence transgene expression or target protein inhibition include neuronal maturity at the time of transfection, the type of cationic lipid used (8), and the net charge of the lipid-DNA complex (9). Cationic lipids per se have also been reported to be toxic to neurons (8, 10).

Glutamate, the main excitatory neurotransmitter in the brain, plays a central role in the pathogenesis of stroke, epilepsy, and neurodegenerative diseases such as Alzheimer’s disease. Excitotoxicity results in increased translation (11) and stabilization (12) of the p53 protein, which in turn alters the levels of redox proteins (13), resulting in neuronal loss. p53 has also been shown to accumulate in mitochondria, leading to mitochondrial dysfunction and activation of the caspase cascade (14). As further proof of the involvement of p53 in neurodegeneration, adenovirus-mediated overexpression of p53 causes apoptosis in cultured hippocampal neurons (15), whereas neurons from p53 null mice are resistant to glutamate (16), DNA-damaging agents, and hypoxia (17). Suppression of p53 expression by AsONs protects neurons from apoptosis induced by DNA damage (18).

Although antisense-mediated inhibition of p53 protein expression has therapeutic potential in conditions where neuronal survival is compromised, precise delivery of the oligonucleotides to neurons is imperative for this potential to be fully realized. As the requirement for a net positive charge on transfection-competent cationic lipid-DNA complexes has been ques-
tioned by several recent reports (9, 19–21), we explored the ability of anionic lipids for DNA delivery to neurons. In the present study, zwitierionic and anionic phospholipids were used to design liposomal vectors (anionic liposomes) for oligonucleotide encapsulation. Antisense ONs to p53 delivered by anionic liposomes protected hippocampal neurons from glutamate-induced death by sequence-specific down-regulation of p53 without any discernible toxicity. Uptake of Cy3-labeled ONs delivered by anionic liposomes was studied in primary neurons, immortalized fibroblasts, and cell lines derived from the liver, kidney, ovary, and cervix. Anionic liposomes were successful in delivering Cy3ONs to the entire population of cells within 1 h, regardless of the cell type.

**EXPERIMENTAL PROCEDURES**

**Design and Synthesis of p53 Oligonucleotides**—The 18-mer p53 antisense ON used in this study targets the translation initiation site of the rat p53 mRNA and is complementary to nucleotides 21–38 (5’-CTGGTAATCTCCATTGCCA-3’). GenBank™ accession number X13058 (22) with 50% GC content for optimal hybridization. Scrambled (5’-TGCATCTGACGATGCTC-3’) and mismatched (5’-GAGTAGATGCATCATGGG-3’) sequences were also designed for use as negative controls. The sequences had no similarity to other mammalian genes (BLAST search (23)) and exhibited minimal self-complementarity (Vector NTI, Informax, Inc.). All ONs, synthesized as lyophilized powders by Midland Certified Reagent Co. (Midlands, TX), were reconstituted in sterile, nuclease-free Tris-EDTA buffer (10 mM Tris HCl, 1 mM EDTA, pH 7.4) and stored at −20 °C. The concentrations of ONs in solution were routinely determined by absorbance measurements at 260 nm. Cy3-labeled oligonucleotides were synthesized byIntegrated DNA Technologies, Coralville, IA.

**Liposome Preparation**—DOPC, DOPG, DOPS, DOPE, DC-Chol, and DOTAP were purchased from Avanti Polar Lipids, Alabaster, AL and stored at −20 °C as stock solutions of 2 mg/ml in chloroform. Anionic liposomes were prepared by a modification of the classic film hydration-extrusion procedure. Briefly, the lipid mixture was dried to a thin film under a stream of high purity nitrogen and hydrated with a solution of ONs in 10 mM HEPES buffer (pH 7.4) with 5 mM NaCl (except when indicated otherwise) with intermittent heating and vortexing. After complete hydration, the suspension was transferred to a Lipofast™ miniextruder system (Avestin, Inc., Ottawa, Canada) and extruded through a series of polycarbonate membranes down to a pore size of 0.1 μm. Unencapsulated ONs were removed by polyethyleneimine (Aldrich) and encapsulated ONs on a Sephadex G-50 column (7 × 100 cm) with 10 mM HEPES buffer and extruded to 200 nm. The liposomes were dialyzed against 10 mM HEPES buffer and 140 mM NaCl, and stored at −20 °C. The size of the liposomes was measured by light scattering and the zeta potential was determined by laser Doppler electrophoresis.

**Transfection of Neurons**—Aliquots (~20 μl) of the liposome suspensions were diluted to 500 μl with distilled water, and 500 μl of chloroform/methanol (1:1 v/v) was added to dissolve the liposomes. Aqueous and organic phases (containing the ONs and lipids, respectively) were separated by centrifugation at 1400 × g for 10 min. This extraction procedure was repeated twice, and organic solvents dissolved in the aqueous phase were removed by heating in a 95 °C water bath for 15 min. Known volumes of the extracted ONs were diluted to 100 μl with Tris-EDTA buffer (10 mM Tris HCl, 1 mM EDTA) and added to a 90-μl well of a 48-well plate pre-coated with 200 ng of OligoGreen™ (Molecular Probes, Eugene, OR) in 10 μl of a 0.5× dilution of 10 μl of a 0.5× dilution of OligoGreen™ (Molecular Probes, Eugene, OR) in 10 μl of a 0.5× dilution of OligoGreen™ (Molecular Probes, Eugene, OR) in 10 μl of a 0.5× dilution of OligoGreen™ (Molecular Probes, Eugene, OR). The fluorescence increase upon binding of the dye to ON was measured using a FLUOSStar microplate fluorometer (BMG Labtechnologies GmbH, Offenburg, Germany) with excitation and emission wavelengths of 480 and 535 nm. Because OligoGreen™ exhibits significant base selectivity, the amount of ON in the liposomes was calculated from standard curves generated with a known concentration of that particular ON in solution. For Cy3-labeled ONs, Cy3 fluorescence in the aqueous phase after extraction was measured directly at excitation and emission wavelengths of 544 and 590 nm. The amount of ONs present in the extracted aqueous phase relative to the amount initially added to the liposomes was determined by adding chloroform and ammonium ferrioxycyanate (AFT) to the dried extracted organic phases. The mixture was vortexed to induce formation of the colored AFT-phospholipid complex that partitions into the chloroform phase (25), and absorbance of the complex was measured at 562 nm (Beckman Instruments Inc., Fullerton, CA). 

**Hippocampal Cell Culture**—Primary cultures of hippocampal neurons were prepared from neonatal rat pups (P1 or P2) as previously described (26). Neurons were plated at a density of 60,000 cells/cm² onto polylysine-coated plastic 12-well plates for the toxicity experiments or 100-mm dishes (Becton Dickinson, Franklin Lakes, NJ) for the immunoprecipitation studies in neurobasal medium with B27 supplements (Life Technologies, Inc.) and 0.5 mM glutamine. Fluoroceuroxazine (15 μg/ml) was added to the cultures 24 h after plating to inhibit glial growth. Under these culture conditions, and growth of non-neuronal cells was minimized. Cells were maintained at 37 °C in 95% air, 5% CO₂ and were used between 6–8 days in vitro.

**Neuroprotection Experiments**—ONs (unencapsulated or in liposome preparations) were added to the medium at 3 h after final concentrations of 0.1 to 5 μM, depending upon the experimental paradigm, and the neurons were then exposed to 50 μM glutamate. MK-801 and CNQX (final concentrations 20 μM each) were added 1–2 min before glutamate addition, and pifithrin-α (final concentration 10 μM) was added 3 h before glutamate addition. Neuronal survival was assessed by an observer blinded to the treatments 48 h after glutamate exposure by counting viable cells in preselected fields based on trypan blue exclusion (27). The ratio of viable cells to the total number of neurons in the preselected fields was calculated for quantifying survival.

**p53 Immunoprecipitation**—Neurons (~5 million cells/100-mm dish) were treated with 1 μM p53 antisense or scrambled ONs in anionic liposomes for 3 h and exposed to glutamate for 15 h. Cells were detached by scraping and sonicated in lysis buffer containing 0.1% SDS, 0.1% glycerol in 85 mM Tris HCl (pH 6.8) and protease inhibitor set III (Calbiochem). After preclearing with Protein G-agarose (ImmunoPure®, Pierce), lysates were immunoprecipitated with the G5-12 monoclonal p53 antibody (2 μg/million cells, Pharmingen, San Diego, CA) and protein G-agarose. Immunoprecipitates and p53 positive control (Oncogene Research Products, Cambridge, MA) were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (15% SDS-polyacrylamide gel electrophoresis, and proteins were transferred to an Immobilon-P membrane (Millipore, Bedford, MA). Blots were incubated with the CM1 rabbit polyclonal p53 antibody (1:1000, Novocasta Laboratories, Newcastle upon Tyne, UK) and then probed with horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:5000, Chemicon International, Inc., Temecula, CA). Detection was performed by enhanced chemiluminescence (ECL kit, Amersham Pharmacia Biotech, Inc.), and p53 bands were quantified using a Personal Densitometer SI and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

**Transfection of Neurons**—Neurons were cultured on 8-chambered glass slides (Nalgene Nunc, Naperville, IL) in minimum essential medium with 10% NuSerum (Collaborative Research). A plasmid coding for the enhanced green fluorescent protein driven by a cytomegalovirus promoter (pEGFP-N1, CLONTECH, Palo Alto, CA) was condensed with LipofectAMINE (Aldrich) and encapsulated in anionic DOPC/DOPG liposomes as described above. Neurons were incubated with pEGFP-N1 either alone or in anionic liposomes for 48 h in serum-containing medium, after which they were fixed in 4% paraformaldehyde and imaged for EGFP expression.

**Liposome Uptake**—The Chinese hamster ovary cell line CHO-K1, human hepatoma cell line HuH-7, cervical carcinoma cells HeLa, canine kidney cell line MDCK, and mouse embryonic fibroblast cell line, MEF-1 (ATCC, Manassas, VA), were cultured on eight-chambered glass slides in Dulbecco’s modified Eagle’s medium containing penicillin and streptomycin with 10% cosmic calf serum. Cy3-labeled oligonucleotides were added to the culture medium, either without any delivery vector, encapsulated in anionic DOPC/DOPG liposomes, or complexed with cationic DC-Chol/DOPC liposomes at a final concentration of 1 μM. After 1 h, the cells were rinsed with phosphate-buffered saline and fixed.

**Confocal Microscopy and Image Analysis**—Imaging was performed on a Leica TCS 4D confocal microscope (Deerfield, IL) equipped with an argon/krypton laser. The entire volume of the cells was scanned in 0.5-μm increments, and optimal images were obtained by averaging 16
Characterization of Anionic Liposomes Encapsulating ONs—Liposomes composed of DOPC and 12 mol % anionic lipids DOPG, DOPS, or DOPA were monodisperse suspensions with narrow Gaussian size distributions (Fig. 1A) and encapsulated 40–60% of the initial ON amount (Table I) depending on the liposome composition. The amount of ON encapsulated in the liposomes was measured using the OliGreen™ dye, which is highly specific for single-stranded nucleic acids, with a 1000-fold increase in dye fluorescence upon binding to a 20-mer ON (28). We also measured encapsulation of Cy3-labeled ONs in anionic liposomes by directly measuring Cy3 fluorescence and obtained identical results. Phospholipid content in the final preparations was 60–70% of the initial amount, reflecting losses during extrusion and purification by minicolumn centrifugation (Table I).

** Ionic Strength, Anionic Charge Density, and Oligonucleotide Chemistry Influence Encapsulation—Anionic liposomes composed of DOPC with 12 mol % DOPG (DOPC/DOPG liposomes) were prepared in 10 mM HEPES buffer (pH 7.4) with 5, 50, or 150 mM NaCl, and ON encapsulation was measured. Increasing the ionic strength of the hydration buffer dramatically decreased ON encapsulation (Fig. 1B). The buffer with 5 mM NaCl was used for all subsequent studies because this allowed for maximum encapsulation. To investigate the role of anionic charge density on encapsulation, we varied the mol % of anionic lipid in liposomes. Again, increasing the anionic charge of the lipid bilayer decreased encapsulation (Fig. 1C). We also compared the encapsulation of phosphodiester ONs in anionic liposomes with that of phosphorothioate ONs, as a function of mol % DOPG. Phosphorothioate ONs were encapsulated to a lesser extent than phosphodiester ONs, and this decreased further with increasing anionic lipid content (Fig. 1C).

**p53 Antisense ONs Delivered by Anionic Liposomes Elicit a Sequence-specific Neuroprotective Effect—**The ability of anionic liposomes to effectively deliver ONs to hippocampal neurons was evaluated in an *in vitro* model of glutamate toxicity. Neurons exposed to glutamate alone for 48 h exhibited apoptotic features such as condensed, granular soma, neurite blebbing, and fragmentation (Fig. 2A, *Veh* + glu). Neurons treated with 1 µM p53 AsONs delivered by anionic DOPC/DOPG liposomes retained intact processes and smooth soma after glutamate treatment, irrespective of the chemical nature of the ONs used (Fig. 2A, *AL-dAs* and *AL-sAs*, anionic liposomes with phosphodiester and phosphorothioate p53 antisense ONs, respectively). Treatment with 0.5 and 1 µM p53 phosphodiester AsONs in DOPC/DOPG liposomes significantly increased the survival of neurons exposed to glutamate (Fig. 2B, *AL-dAs*). This neuroprotection was sequence-specific as anionic liposomes with buffer alone or with 1 µM p53 scrambled ONs (Figs. 2A and B, *AL-buf* and *AL-dScr*, respectively) were ineffective. p53 protein levels in neurons treated with glutamate and ONs in DOPC/DOPG liposomes were determined by immunoprecipitation (Figs. 2C and D). Exposure of hippocampal neurons to 50 µM glutamate for 15 h increased p53 expression ~4-fold relative to untreated neurons. Pretreatment of neurons with 1 µM p53 AsONs in DOPC/DOPG liposomes prevented the glutamate-induced increase in p53 protein levels by antisense-mediated down-regulation of p53 expression. In contrast, pretreatment with 1 µM scrambled oligonucleotides in anionic

| Lipid   | Mean diameter (nm) ± S.D. | % ON encapsulated | % Phospholipid recovered |
|---------|---------------------------|-------------------|--------------------------|
| DOPC/DOPG | 216.0 ± 75               | 56.8 ± 3.0        | 72.0 ± 6.1               |
| DOPC/DOPS | 242.3 ± 90               | 46.3 ± 10.7       | 69.3 ± 7.3               |
| DOPC/DOPA | 229.0 ± 92               | 44.4 ± 6.2        | 60.2 ± 8.7               |

*a* Mean ± S.D. of >5 independent experiments.
Liposomes did not significantly alter the glutamate-induced increase in p53 expression, proving the specificity of p53 antisense sequence used in this study.

Liposome Composition Influences the Extent of Neuroprotection by p53 AsONs—The influence of liposomal lipids on the biological performance of the vector was studied by comparing the extent of neuroprotection by p53 AsONs delivered in DOPC/DOPG liposomes with that achieved by AsONs delivered (a) in liposomes where the anionic lipid DOPG was replaced with DOPS or (b) as complexes with cationic liposomes composed of DC-Chol/DOPE. DC-Chol was the model cationic lipid in our studies as it was best tolerated by neurons based on initial toxicity screens of DC-Chol, DOTAP, and commercial transfection reagents TransFast™ and Tfx-20™ (Table II).

p53 antisense ONs delivered by both anionic vectors caused a dose-dependent increase in neuronal survival after glutamate exposure, whereas AsONs complexed with DC-Chol/DOPE were largely ineffective (Fig. 3A). However, greater neuroprotection was observed with p53 AsONs delivered by DOPC/DOPS liposomes compared with DOPC/DOPS liposomes at AsON doses of 0.5, 0.7, and 1 μM. To test whether the lipids themselves could exacerbate glutamate toxicity, we treated neurons with liposomes made solely of DOPG, DOPS, or DC-Chol/DOPE (without AsONs), followed by exposure to a submaximal dose of glutamate (10 μM). The addition of increasing amounts of DOPG did not appreciably change neuronal survival from the 71% seen after a 48-h exposure to 10 μM glutamate (Fig. 3B). However, treatment with 40 μg of DOPS (equivalent to the amount present in liposomes for a final ON concentration of 1 μM) decreased neuronal survival to 48%. Neurons were treated with amounts of cationic lipid required to complex 1 μM ONs in 1/100/100/100 charge ratios (μmol of lipid/μmol of ON) of 1/2, 1.6/1, 3.2/1, and 8/1 (6.25, 20, 40, and 100 μg of DC-Chol, respectively). Only those neurons treated with 6.25 μg of DC-Chol, i.e. where the complex has a net negative with ONs in anionic liposomes before glutamate exposure and, 48 h later, visualized by differential interference contrast microscopy (A) or survival was quantified (B). Veh, control neurons treated with vehicle alone; glu, 50 μM glutamate; AL-dAs, 1 μM phosphodiester p53 AsONs in anionic liposomes; AL-sAs, 1 μM phosphorothioate p53 AsONs in anionic liposomes; AL-buf, anionic liposomes containing buffer alone; AL-dScr, 1 μM phosphodiester p53 scrambled ONs in anionic liposomes. Scale bar, 20 μm. •, neuronal survival significantly greater than neurons treated with glutamate alone or AL-Scr and glutamate, p < 0.05. Mean ± S.E., n > 9. C, neurons treated with p53 antisense ONs or scrambled ONs in anionic DOPC/DOPG liposomes for 3 h followed by a 15-h exposure to 50 μM glutamate were harvested for measurement of p53 protein levels by immunoprecipitation. The Western blot shown is from a typical experiment. Veh, control neurons treated with vehicle; AL-As, 1 μM p53 antisense ONs in anionic liposomes; AL-Scr, 1 μM p53 scrambled ONs in anionic liposomes; glu, 50 μM glutamate. •, p53 expression significantly lower than that in neurons treated with glutamate alone or AL-Scr and glutamate, p < 0.05.

![Fig. 2](http://www.jbc.org/)

**Fig. 2.** p53 antisense ONs delivered by anionic DOPC/DOPG liposomes protect glutamate-treated hippocampal neurons by down-regulating p53 expression. Neurons were incubated for 3 h with ONs in anionic liposomes before glutamate exposure and, 48 h later, visualized by differential interference contrast microscopy (A) or survival was quantified (B). Veh, control neurons treated with vehicle alone; glu, 50 μM glutamate; AL-dAs, 1 μM phosphodiester p53 AsONs in anionic liposomes; AL-sAs, 1 μM phosphorothioate p53 AsONs in anionic liposomes; AL-buf, anionic liposomes containing buffer alone; AL-dScr, 1 μM phosphodiester p53 scrambled ONs in anionic liposomes. Scale bar, 20 μm. •, neuronal survival significantly greater than neurons treated with glutamate alone or AL-Scr and glutamate, p < 0.05. Mean ± S.E., n > 9. C, neurons treated with p53 antisense ONs or scrambled ONs in anionic DOPC/DOPG liposomes for 3 h followed by a 15-h exposure to 50 μM glutamate were harvested for measurement of p53 protein levels by immunoprecipitation. The Western blot shown is from a typical experiment. D, quantified results are the mean ± S.E. of three independent experiments. Veh, control neurons treated with vehicle; AL-As, 1 μM p53 antisense ONs in anionic liposomes; AL-Scr, 1 μM p53 scrambled ONs in anionic liposomes; glu, 50 μM glutamate. •, p53 expression significantly lower than that in neurons treated with glutamate alone or AL-Scr and glutamate, p < 0.05.
Anionic Liposomal Delivery of p53 Phosphorothioate AsONs

**Fig. 3.** Lipid composition and charge influence the efficacy and toxicity of the delivery system. A, comparison of the neuroprotective dose-response curves of p53 antisense ONs encapsulated in DOPC/DOPG (circles) or DOPC/DOPS liposomes (squares) or complexed to cationic DC-Chol/DOPE liposomes in a +/− charge ratio of 1/2 (triangles). Neurons were treated with AsONs for 3 h before glutamate exposure (50 μM, 48 h). Survival significantly greater than the corresponding AsON dose delivered in DOPC/DOPG liposomes, *p < 0.001; §, p < 0.05. B, DOPC (squares) and DC-Chol/DOPE (triangles), but not DOPG (circles), dose-dependently exacerbate toxicity associated with a sub-maximal concentration (10 μM) of glutamate. Arrowhead, amount of anionic lipid present in liposomes corresponding to a 1 μM final concentration of ON; arrow, amount of cationic lipid present in complexes corresponding to a +/− charge ratio of 1/2 and 1 μM final concentration of ON. 20, 40, and 100 μg of DC-Chol/DOPE correspond to amounts present in complexes of +/− charge ratio 1.6/1, 3.2/1, and 8/1 (μmol of lipid/μmol of ON), respectively, for a 1 μM final concentration. For both A and B, data are expressed as the mean ± S.E.; n > 9.

charges, survived 48 h post-glutamate. Amounts of DC-Chol where the complex would be near neutral or have a net positive charge caused extensive neuronal loss.

Anionic Liposomal Delivery of p53 Phosphorothioate AsONs Potentiates Antisense-mediated Neuroprotection—Although p53 phosphodiester AsONs were not neuroprotective when delivered “free”, i.e., without encapsulation in anionic liposomes, free p53 phosphorothioate AsONs, at a dose of 5 μM, significantly increased neuronal survival (Fig. 4A, sAs) compared with neurons treated with glutamate alone. Phosphorothioate AsONs, when delivered via DOPC/DOPG liposomes (Fig. 4A, AL−sAs), provided significantly more neuroprotection at concentrations of 0.5 and 1 μM than 5 μM free sAs. Neither phosphorothioate p53-scrambled ONs nor a sequence with 6 mismatches to p53 AsON were neuroprotective (Fig. 4A, sScr and sMm, respectively, 5 μM each). Neuronal survival was also not increased by 1 μM phosphorothioate-scrambled ON in anionic liposomes (data not shown). Phosphorothioate ONs at concentrations greater than 5 μM caused neurons to detach from the culture substrate within 12 h of exposure and were not tested.

Neuroprotection by p53 AsONs Delivered by Anionic Lipo­somes Is Comparable with That by the p53 Inhibitor, Pifithrin-α (PFT-α), and Glutamate Receptor Antagonists—PFT-α is a chemical inhibitor of p53 that was shown to protect cells from p53-induced apoptosis caused by genotoxic stress (29). Antagonists to the N-methyl-D-aspartate and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid glutamate receptors, MK-801 and CNQX, 20 μM each, used individually or together, and 10 μM PFT-α significantly increased the survival of glutamate-treated neurons (Fig. 4B, MK, CN, MK+CN, and PFT-α). In our hippocampal cultures, concentrations of PFT-α greater than 10 μM (20–100 μM) were toxic, whereas lower concentrations (0.5–7 μM) were not significantly protective. Neuroprotection afforded by 1 μM p53 AsON in DOPC/DOPG liposomes (Fig. 4B, AL−dAs) was greater than that by either MK-801, CNQX, or PFT-α and comparable with that by MK-801+CNQX.

Anionic Liposomes Facilitate Widespread ON Delivery and Transgene Expression in Neurons and Other Cell Types—After
liposomes.

pEGFP

FIG. 5. Anionic liposomes facilitate widespread oligonucleotide delivery and transgene expression in neurons. AL-Cy3ON, uptake of Cy3ONs encapsulated in anionic liposomes by neurons. Neurons were incubated with AL-Cy3ONs for 1 h at 37°C in the presence of serum. Note the punctate fluorescence in the cytoplasm and the diffuse nuclear label. AL-pEGFP, expression of EGFP in neurons. Neurons were treated for 48 h with 1 μg of pEGFP encapsulated in anionic liposomes. pEGFP, neurons treated with 1 μg of pEGFP alone. Representative confocal images from 3 independent experiments are shown along with the corresponding bright field images. Scale bar, 10 μm.

Table III

Comparison of the uptake of Cy3ONs delivered by anionic liposomes and cationic lipids

| Cell type                           | Percent of cells with intracellular Cy3 fluorescence |
|-------------------------------------|-----------------------------------------------------|
|                                     | Cy3ONs encapsulated in anionic DOPC/DOPG liposomes   | Cy3ONs complexed with cationic DC-Chol/DOPE liposomes | Cy3ONs without delivery system |
|-------------------------------------|-----------------------------------------------------|-----------------------------------------------------|--------------------------------|
| Primary rat hippocampal neurons     | 100                                                 | 9                                                   | 6                              |
| Chinese hamster ovary cell line (CHO-K1) | 99 ± 1.7                                            | 41.5                                               | 17                             |
| Human cervical carcinoma (HeLa)     | 100                                                 | 50.3                                               | 51.5                           |
| Human hepatoma (HuH-7)              | 97.8 ± 2                                            | 31.5                                               | 18.3                           |
| Canine kidney cell line (MDCK)      | 98.2 ± 1.8                                          | 16.5                                               | 5                              |
| Mouse embryonic fibroblasts (MEF-1) | 99.7 ± 0.6                                          | 33.5                                               | 20                             |

a Mean ± S.D. of three independent experiments.
b Mean of two independent experiments.

Anionic liposomes are held to be inefficient ON delivery vectors, primarily because of poor ON encapsulation, reported previously (30). Earlier studies (31, 32) used phosphate-buffered saline with 150 mM NaCl as the hydration buffer and obtained 5–10% ON encapsulation in liposomes containing 20% w/w anionic lipid. Increasing the ionic strength increases lamellarity in the bilayer (33), thus decreasing solute entrapment (34). This inverse relationship between salt concentration and DNA encapsulation was also observed in our experiments and has recently been confirmed (35). In anionic liposomes, encapsulated nucleic acids exist in two pools, one population associated with the bilayer and the other in the aqueous compartment of the vesicle (34). Greater than 20 mol % anionic lipid in the bilayer decreases lipid-nucleic acid interactions, which might explain the low ON encapsulation in liposomes with 30 and 60 mol % anionic lipid. The lower encapsulation of phosphorothioate ONs can be attributed to increased repulsion between anionic lipid and the sulfur atom of phosphorothioate ONs compared with the oxygen atom of phosphodiester ONs.

Although electrostatic interactions between cationic lipids and DNA have been extensively studied, recent reports have raised the intriguing possibility of DNA binding to zwitterionic lipids. Molecular dynamics simulation of interactions between a binary mixture of cationic dimyristoyltrimethylammonium propionate and zwitterionic dimyristoylphosphatidylcholine (DMPC) and DNA showed a substantial population of DMPC headgroup nitrogens in close proximity to a DNA phosphate (36). This study also predicted equal probabilities for the existence of TAP or PC groups around DNA phosphates. Stable binding of DNA to PC bilayers due to the tethering of DNA by the PC headgroup has also been reported (37). Interestingly,
this binding was drastically reduced by a brief exposure to 1 M NaCl because of the screening of the attractive forces between DNA phosphates and PC nitrogens by high concentrations of monovalent ions. Moreover, charge pairing and intermolecular hydrogen bonding between phosphatidylcholine and phosphatidylglycerol headgroups in mixed bilayers have been reported to effect partial screening of negative charges of lipid phosphate groups (38). Lipid-lipid and lipid-DNA interactions therefore play a major role in the encapsulation of nucleic acids in anionic liposomes, and these interactions can be modulated, among other factors, by ionic strength.

The pattern of neuronal loss that occurs after excitotoxicity is an apoptotic-necrotic continuum, depending on mitochondrial function and the severity of the insult. Pharmacological interventions to prevent neurodegeneration have recently shifted their focus from the “classical” receptor blockade strategies to approaches that target downstream intracellular mediators (39) like p53, which promote cell death via both apoptotic and necrotic pathways (40). The rationale behind these approaches is validated in the present study, where sequence-specific down-regulation of p53 and concomitant neuroprotection was achieved by p53 AsONs delivered in anionic DOPC/DOPG liposomes. Moreover, the increase in neuronal survival due to p53 AsONs was comparable with glutamate receptor antagonists and the p53 inhibitor, pifithrin-α. The anionic lipid moiety (DOPG or DOPS) in the liposomes influenced the extent of neuroprotection achieved with p53 AsONs. Phosphatidylserine is a known activator of protein kinase C (41), which has been implicated in excitotoxic neuronal loss (42) and is also known to phosphorylate p53 and induce its sequence-specific DNA binding activity (43). Thus, neuroprotection by p53 AsONs delivered by DOPC/DOPS liposomes was possibly offset by increased activation of protein kinase C, resulting in lower biological response compared with that of DOPC/DOPG liposomes.

Cationic lipids, often used to transiently express reporter genes or down-regulate specific proteins, have been successful with transformed cell lines where the cells are relatively healthy and no other manipulations (except the addition or removal of cationic lipid-DNA complexes) are performed. However, conclusive evidence of the ability of cationic lipids to effectively deliver nucleic acids in a “rescue” paradigm is absent, due in large part to their inherent toxicity as seen in this and other studies (7, 8). Indeed, complexes of p53 AsONs with cationic DC-Chol/DOPE liposomes were unstable colloids and ineffective in rescuing glutamate-treated neurons. An important observation of our studies was the 5–10-fold reduction of phosphorothioate AsON dose required to achieve maximal neuroprotection when delivered by anionic DOPC/DOPG liposomes. Thus, anionic liposomes not only increase the efficacy of phosphorothioates but may also minimize their non-sequence-specific effects.

Oligonucleotides labeled with Cy3 and encapsulated in anionic liposomes were taken up by neurons in a fairly rapid manner, and Cy3 fluorescence was visible in all neurons exposed to the anionic liposomes. We have experimental evidence that anionic liposomes are internalized into neurons by receptor-mediated endocytosis. The remarkable enhancement of biological activity seen with p53 AsONs delivered by anionic liposomes can be explained in part by the widespread delivery of the oligonucleotides to the entire neuronal population, which would in turn ensure a uniform down-regulation of the p53 protein.

Recent reports suggest that expression of foreign genes in neurons using nonviral vectors is best achieved by cationic lipid-DNA complexes carrying an overall negative charge (9, 19–21). In agreement with these studies, anionic liposome-
mediated delivery of the EGFP plasmid resulted in the expression of the reporter protein in hippocampal neurons. As stated previously, an important limitation of cationic lipids for DNA delivery is their inactivation in serum-containing medium. Both Cy3ON delivery and EGFP transfection by anionic liposomes occurred in the presence of serum, indicating that the DOPC/DOPG vector system is fully functional in serum-containing medium. Finally, we demonstrated that Cy3ONs delivered by anionic liposomes were taken up by a variety of cell lines. The cell types used in our experiments (CHO, HeLa, HuH-7, Chinese hamster ovary, and MDCK cell lines) were chosen because of their utility as popular model systems for basic research and potential clinical significance. The rapid uptake of AL-Cy3ONs by all the cells independent of the tissue of origin underscores the universal applicability of the anionic DOPC/DOPG liposomes for DNA delivery.

In conclusion, by studying the physicochemical features that influence lipid-mediated DNA delivery, we were successful in developing an anionic liposomal vector that overcomes the considerable limitations of cationic lipids. The unique properties of the anionic liposomes allowed for rapid intracellular delivery of oligonucleotides and the generation of a sensitive biological response in a variety of cell types, supporting the inactivation of cationic lipids in serum-containing medium. Both Cy3ON delivery and EGFP transfection by anionic liposomes occurred in the presence of serum, indicating that the rapid uptake of AL-Cy3ONs by all the cells independent of the tissue of origin underscores the universal applicability of the anionic DOPC/DOPG liposomes for DNA delivery.

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