Comparison of Cell Proliferation and Toxicity Assays Using Two Cationic Liposomes

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Received September 9, 1993; accepted March 21, 1994

The present study compares different cytotoxicity and cell proliferation assays including cell morphology, mitochondrial activity, DNA synthesis, and cell viability and toxicity assays. CaSki cells were exposed to two cationic liposomal preparations containing dimethyldioctadecyl-ammonium bromide (DDAB), dioleoylphosphatidylethanolamine (DOPE) and a commercial transfection-reagent DOTAP (N[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium-methylsulfate). The results provided by these assays were similar. However, the lactate dehydrogenase assay was more sensitive in measuring early damages of cell membranes than the Trypan blue assay. Also, cell morphology showed early toxic changes, such as cytoplasmic vacuolization and cell shrinking, and it should be included with such toxicity evaluations. DDAB:DOPE was more toxic than DOTAP. The cells treated with DOTAP at 10 μM were surviving as well as the control cells, while DOTAP at 40 uM and DDAB: DOPE at 10 μM had slight toxic effects on CaSki cells. The most toxic effects were seen in CaSki cells after treatment with DDAB: DOPE at 40 μM.

KEY WORDS: cell toxicity assays; cell proliferation assays; cationic liposomes; CaSki cells.

INTRODUCTION

Cell replication is commonly assessed by demonstrating [^{3}H]thymidine incorporation into DNA (1). An equivalent nonradioactive method is based on 5-bromo-2′-deoxyuridine (BrdU) incorporation, assessing DNA synthesis and cell proliferation (2). Mitochondrial activity can be evaluated by measuring the activity of mitochondrial dehydrogenases of viable cells, which reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (3) to an insoluble blue formazan product. Subsequently, two tetrazolium salts, i.e., sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate (XTT), and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), were introduced, which yield a water-soluble formazan product in the presence of phenazine methosulfate (PMS), obviating solubilization before spectrophotometrical analysis (4,5).

Cell toxicity is usually studied with a ^{51}Cr-release assay (6), while a LDH assay is a non-radioactive alternative to measure cytotoxicity, especially intactness or damage of cell membranes (7). LDH release during cell lysis is measured by a coupled enzymatic assay, where 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium cloride (INT) is converted into a red formazan product. Traditionally, cell viability and cytotoxicity have been assessed with the Trypan blue exclusion test, where damaged and dead cells are identified by their uptake of Trypan blue dye.

The MTT and the [^{3}H]thymidine assays (8–11), the MTT, XTT and MTS assays (5,12), and the BrdU and [^{3}H]thymidine assays (13) have been compared previously. However, no comparative studies including all the above assays have been published.

Intracellular delivery of DNA, RNA, and antisense oligonucleotides can be improved significantly with the use of cationic liposomes (14–18), which however are toxic to the cells (14,19–21). A cationic liposomal preparation containing positively charged lipid, N-[1-(2,3,-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) was introduced first (14), soon followed by DOTAP (22). Rose et al. (23) developed a new cationic liposome preparation, DDAB: DOPE, which is highly efficient for DNA, RNA or antisense oligonucleotides transfection (23,24), but its toxicity has not been compared to that of other cationic liposomes. The present study was designed to compare different cell proliferation and toxicity assays for the evaluation of the cationic liposomes DDAB:DOPE and DOTAP. For the experiments CaSki cells, a human cervical cancer cell line was used.

MATERIALS AND METHODS

Cell Culture

Adherent CaSki cells, a human cervical cancer cell line containing human papilloma virus 16 DNA, were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS, Gibco, Paisley, Scotland, U.K.) at 37°C in 5% CO_2. MTS, MTT, BrdU, LDH and [^{3}H]Thymidine assays were performed on 96-well plates (Nunc, Roskilde, Denmark) with 10^4 cells/well in a 100 ul volume and Trypan blue assay was performed on 24-well plates (Nunc, Roskilde, Denmark) with 5.2×10^4 cells/well in a 500 μl volume. For morphological study 5.2×10^4 cells/well were seeded onto round glass microscope slides in 24-well plates in a 500 μl volume. Liposomes were added 12 hours later after the seeding of the adherent cells and they were incubated for one, two, three or four days. The control cells were treated with the equivalent amount of sterile water (5 or 25 μl on 96- or 24-well plates, respectively). All assays were performed in triplicate.

Liposomes

For the comparison of different toxicity and proliferation assays CaSki cells were either exposed to a commercially available Transfection-reagent DOTAP (Boehringer Mannheim Biochemica, Pentzberg, Germany) or a cationic liposomal preparation containing dimethyldioctadecyl-ammonim bromide (DDAB) and dioleoylphosphatidyl-ethanolamine (DOPE) (2/5 w/w) as described previously

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0724-8741/94/0800-1127$07.00/0

(23). DDAB (1.32 mg) and DOPE (3.31 mg) in chloroform were evaporated to dryness in a rotating evaporator. Three ml of sterile water was added, and after one hour the mixture was sonicated for 10 minutes (Branson bath sonicator, Model 2200, Danbury, CT, USA). The size distribution of lipid dispersions was determined by quasielastic light-scattering (Nicomp Submicron Particle Sizer, Model 370, Santa Barbara, CA). The mean size of DOTAP was 70 nm and 98% particles were less than 135 nm in diameter. The mean size of DDAB:DOPE liposomes was 35 nm and 96% vesicles were below the size of 65 nm. In all experiments the final concentrations of DOTAP and DDAB in cell culture were 10 or 40 μM.

Morphology

CaSki cells were washed twice with phosphate buffered saline (PBS), fixed in 4% formaldehyde and stained with toluidine blue for analysis on light microscope.

Trypan Blue Assay

Trypan blue staining was performed according to routine procedures. Of each sample, four fields were counted in Bürker's chamber. The assay was performed in triplicate. The cells were counted, and the stained cells were expressed as a percentages of viable cells.

MTT Assay

MTT (Amresco, Ohio, USA) was dissolved in PBS at 5 mg/ml. The solution was filtered through a 0.22 μm filter to remove formazan crystals and 20 μl of MTT solution was added into each well. The cells were incubated for three hours at 37°C in 5% CO_2. Then medium with MTT solution was flicked out and 100 μl of acid-isopropanol (0.04 M HCl in isopropanol) was added. The cells were shaken for 30 minutes at room temperature to ensure that all crystals were dissolved. Plates were read on a multiwell scanning spectrophotometer using the wavelength of 570 nm.

MTS Assay

We used a commercially available kit (CellTiter 96 Aqueous, Promega, Madison, WI, USA). Shortly, 100 μl of PMS solution were added to the 2.0 ml of MTS solution immediately before addition to the cells. 20 μl of MTS/PMS solution was pipetted into each well. The cells were incubated one and a half hour at 37°C in 5% CO_2. Plates were read on a multiwell scanning spectrophotometer (Multiskan® Plus Elisa reader, Labsystems, Helsinki, Finland) using the wavelength of 492 nm.

[^{3}H]Thymidine Uptake Assay

The cells were pulsed with 0.5 μCi [^{3}H]thymidine per well for six hours, then detached from the culture plate using trypsin-EDTA. Detaching was controlled by microscopy. The [^{3}H]thymidine uptake was quantified after automatic collection on a cell harvester (1295-001 Cell Harvester, LKB Wallac, Turku, Finland) by liquid scintillation in a β emission counter (Liquid Scintillation Counter, Wallac 1409, Pharmacia, Uppsala, Sweden).

BrdU Assay

A commercially available kit (BrdU Labeling and Detection Kit III, Boehringer Mannheim Biochemica, Pentzberg, Germany) was used according to manufacturer's instructions. Shortly, 10 μl of BrdU labeling solution (final concentration 10 μmol/l) was added per well and incubated for four hours at 37°C. After incubation, the cells were washed twice with PBS containing 10% FCS and fixed in 0.5 M HCl with 70% ethanol for 30 minutes. After three washings, nuclease solution was added and incubated for 30 minutes at 37°C. The cells were washed three times before adding anti-BrdU-antibody solution (monoclonal antibody, Fab fragments from mouse) conjugated with peroxidase was added. The cells were incubated for 30 minutes at 37°C. After three washings peroxidase substrate was added and incubated for 30 minutes. Plates were read on a multiwell scanning spectrophotometer using wavelength of 405 nm.

LDH Assay

This assay was performed by using a commercial kit according to manufacturer's instructions (CytoTox 96, Promega, Madison, WI, USA). As a control of maximal LDH release, lysis solution was added onto the cells followed by incubation in 5% CO_2 at 37°C for 45 minutes. The plate was then centrifuged at low speed (700 g) for two minutes. Fifty μl aliquots from all wells were transferred to a fresh 96 well. Reconstituted substrate mixture was added, the plates were covered with foil and incubated at room temperature for 30 minutes. The reaction was stopped with stop solution. The plates were read using a wavelength of 492 nm without one hour. The addition of lysis solution or liposomes into medium did not interfere with the background absorbance. The percent cytotoxicity was calculated as follows: first the culture medium background was subtracted from the average of different absorbanse values. Percentage cytotoxicity was calculated using the formula: [(Experimental − Control Cells):(Max LDH Release of Control Cells − Control Cells)] × 100.

RESULTS AND DISCUSSION

In preliminary experiments several seeding densities were studied on a 96-well plate to find the optimal seeding density for cell growth. At days 1, 2, 3 and 4 the cells were counted, and the optical density of the cells was measured with the MTT assay. The relation of cell number and optical density was linear during the four days procedure, when the seeding density was 10^4 cells/well.

When the effects of liposomes on CaSki cells were studied, all tests showed similar trends, although the absolute values were different. DOTAP at 10 μM concentration was non-toxic in any assay. The cells exposed with DOTAP at 40 μM survived nearly as well as the control cells. DDAB: DOPE at 10 μM were slightly toxic against CaSki cells, while toxicity was apparent at concentration of 40 μM. The results are summarized in Figure 1.

The MTT assay gave similar results as the thymidine and the MTS assays. However, there were some differences between the BrdU and the thymidine assays, although both are measuring DNA synthesis. The difference was most

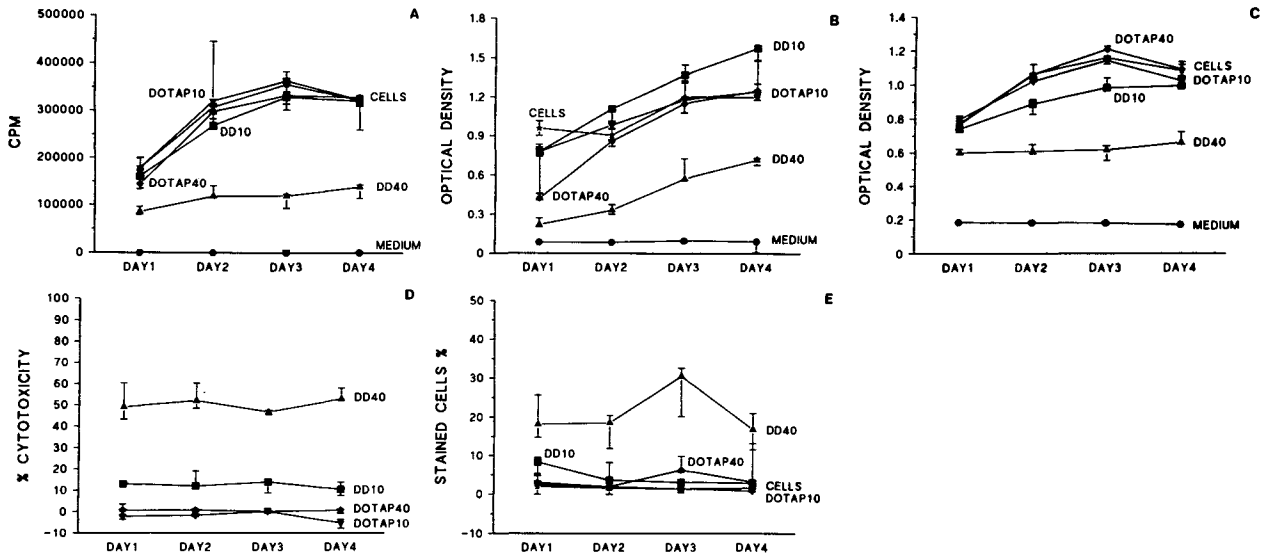

Fig. 1. Results of the assays. The CaSki cells were incubated with DDAB:DOPE (DD) or DOTAP. The concentrations of DDAB or DOTAP were 10 or 40 μM. "Cells" are control cells treated with water. The values of the assays were measured at day 1, 2, 3, and 4. All assays were made in triplicate, and the bars represent the ranges of the experiments. a) Thymidine assay. The vertical axis shows the counts per minute. b) BrdU assay. The vertical axis shows the optical density. c) MTS assay. The vertical axis shows the optical density. d) LDH assay. The vertical axis shows the toxicity, which is calculated from the formula described in materials and methods. e) Trypan blue assay. The vertical axis shows the percentages of stained cells.

marked, when the cells were exposed to DDAB:DOPE at a concentration of 40 μM. Whereas [^{3}H]thymidine incorporation was strongly suppressed (1a), the BrdU assay suggested a 3–4 folds increase in DNA content (1b). However, at the beginning of the study the basic level of DNA synthesis was much lower in the BrdU experiments than in [^{3}H]thymidine experiments. When the effects of oligonucleotides on cells are studied, one disadvantage of the [^{3}H]thymidine and the BrdU assays is that nonspecific intracellular degradation of antisense oligonucleotides may lead to a release of thymidine and uridine. This results in competition of the binding with [^{3}H]thymidine or BrdU during the assays (25).

The technically simple MTS assay requires only one solution (MTS with PMS) added onto the cells. After incubation, the results are read with a multiwell scanning spectrophotometer. The commercially available kits are relatively expensive for large-scale use, but the alternative use of MTT dye, for example, is more economical (3,8–11). Modified MTT protocols where formazan crystals are dissolved more efficiently by dimethyl sulphoxide or dimethylformamide and sodium dodecyl sulphate have been introduced (26,27).

There were also differences between the LDH and the Trypan blue assays. The results were similar when the cells were treated with DOTAP. After exposing the cells to DDAB:DOPE at 10 μM the LDH assay suggested higher cytotoxicity than the Trypan blue assay. The most divergent results were seen when the cells were exposed to DDAB:DOPE at 40 μM. The Trypan blue assay stained 30 percent of the cells, while the cytotoxicity was 50 percent in the LDH assay. The LDH assay appears to be more sensitive in measuring the earlier damages of cell membrane than the Trypan blue assay. In the Trypan blue assay, only dead cells are stained. The variation of the results obtained by Trypan blue staining indicates that the assay is less inaccurate than the other assays. The Trypan blue staining seems to be valid only for checking the overall cell viability, while the LDH assay is a more sensitive test for cytotoxicity measurements. The disadvantage of this assay is that phenol red in culture medium results in a high background.

Inter-experiment variability was smallest in the MTS and MTT assays (less than 10%). In the LDH, (^{3}H)thymidine and BrdU assays some results showed more than 10% variation. The Trypan blue test had the longest variations, frequently more than 20%.

Assessment of cell morphology is a sensitive method. DOTAP at a concentration of 10 μM had no effect on cell morphology. DOTAP at 40 μM and DDAB:DOPE at 10 μM caused minor cellular changes, such as cell shrinking, reduced number of mitoses, and vacuolization of the cytoplasm (Figure 2b). In contrast the MTT, MTS, and LDH assays revealed no toxicity with DOTAP at 40 μM concentration, and the BrdU and thymidine assays showed toxicity only after the 1 day exposure. The morphological changes were most prominent after exposure to DDAB:DOPE at 40 μM concentration. More intensely stained cells, karyopyknosis and hyperchromasia were observed. Vacuolization of the cytoplasm was present in almost every cell, and also several apoptotic cells were seen (Figure 2c). The morphological changes were found already after one day incubation becoming more prominent during the following days.

In our previous experiments, DDAB:DOPE at 10 μM concentration was more toxic to the CaSki cells (24) than in these experiments at the same concentration. We have tried to find out the possible reason for this discrepancy (e.g. the passages, the phase and the activity of cells). In this study the different lot of DDAB:DOBE was used, which might be the most important factor.

The ability to measure cytotoxicity and cell proliferation is important in cell biology and increasingly important in

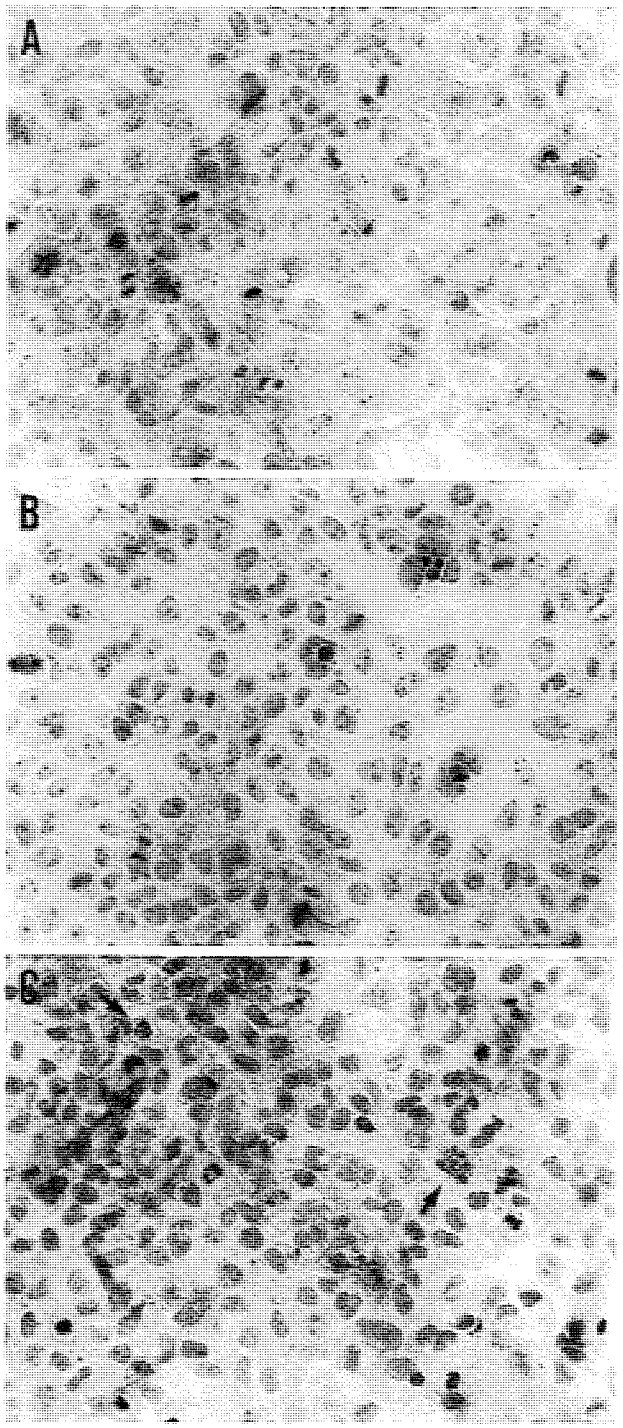

Fig. 2. Morphology of the cells. Morphology of CaSki cells after exposure to DDAB:DOPE for three days (original magnification × 250). a) The control cells treated with water. b) CaSki cells after DDAB;DOPE treatment. The concentration of DDAB was 10 μM. The slight morphologic changes are present, some cells are rounded, number of mitosis is reduced, also vacuolization of cytoplasm is seen in a few cells. c) CaSki cells after exposure to DDAB:DOPE for three days. The concentration of DDAB was 40 μM. Morphological changes are seen: hyperchromasia, karyopyknosis and abundant vacuolization of cytoplasm. Some cells are apoptotic (arrows).

drug delivery studies. Although cytotoxicity and cell proliferation in vitro are not directly related to the situation in vivo, such assays provide important data on the activity of the different compounds at cellular level. Although the thymidine and BrdU assays measure the DNA synthesis and the MTT and MTS assays measure mitochondrial activity, the results obtained from these assays were similar. The LDH assay was an applicable and sensitive test to measure cytotoxicity. Also the investigation of cell morphology is a very sensitive method, and assessment of cell morphology should be included in all assays. Our results suggest that DDAB:DOPE is more toxic than DOTAP for Caski cells. Therefore DDAB:DOPE might not replace DOTAP in drug delivery studies.

ACKNOWLEDGMENTS

This study has been supported in part by a research from the Finnish Cancer Society, a research grant from the Savo Cancer Fund (KL), a research contract from the Medical Research Council of the Academy of Finland, and a joint research grant from Fabrique de Tabaque Reunie and British-American Tobacco Company (BAT).

REFERENCES

1. J. H. Taylor, P. S. Woods, and W. L. Hughes. The organization and duplication of chromosomes as revealed by autoradiographic studies using tritium labelled thymidine. *Biochemistry.* 43:122–128 (1957).
2. H. G. Gratzner, R. C. Leif, D. J. Ingram, and A. Castro. The use of antibody spesific for bromodeoxyuridine for the immunofluorescent determination of DNA replication in single cells and chromosomes. *Exp. Cell Res.* 95:88–94 (1975).
3. T. Mosmann. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods.* 65:55–63 (1983).
4. N. W. Roehm, G. H. Rodgers, S. M. Hatfield, and A. L. Glasebrook. An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. *J. Immunol. Methods.* 142:257–265 (1991).
5. A. H. Cory, T. C. Owen, J. A. Barltrop, and J. G. Cory. Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. *Cancer Commun.* 3:207–212 (1991).
6. K. T. Brunner, J. Mauel, M. C. Cerottini, and B. Chapuis. Quantitive assay of the lytic action of immune lymphoid cells on ^{51}Cr-labelled allogenic target cells in vitro: inhibition by isoantibody and by drugs. *Immunology.* 41:181–196 (1968).
7. C. Korzeniewski, and D. M. Callewaert. An enzyme-release assay for natural cytotoxicity. *J. Immunol. Methods.* 64:313–320 (1983).
8. T. R. Mosmann, and T. A. T. Fong. Spesific assays for cytokine production by T cells: a review. *J. Immunol. Methods.* 116:151–158 (1989).
9. F. Denizot, and R. Lang. Rapid colorimetric assay for cell growth and survival. *J. Immunol. Methods.* 89:271–277 (1986).
10. M. Ferrari, M. C. Fornasiero, and A. M. Isetta. MTT colorimetric assay for testing macrophage cytotoxic activity in vitro. *J. Immunol. Methods.* 131:165–172 (1990).
11. K. Heeg, J. Reimann, D. Kabelitz, C. Hardt, and H. Wagner. A rapid colorimetric assay for the determination of IL-2-producing helper T cell frequencies. *J. Immunol. Methods.* 77:237–246 (1985).
12. D. A. Scudiero, R. H. Shoemaker, K. D. Paull, A. Monks, S. Tierney, T. H. Nofziger, M. J. Currens, D. Seniff, and M. R. Boyd. Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensivity in culture using human and other tumor cell lines. *Cancer Res.* 48:4827–4833 (1988).
13. A. Raza, C. Spiridonidis, K. Ucar, G. Mayers, R. Bankert, and

H. D. Preisler. Double labeling of S-phase murine cells with bromodeoxyuridine and a second DNA-specific Probe. *Cancer Res.* 45:2283–2287 (1985).

14. P. L. Felgner, T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G. M. Ringold, and M. Danielsen. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. U.S.A.* 84:7413–7417 (1987).
15. R. W. Malone, P. L. Felgner, and I. M. Verma. Cationic liposome-mediated RNA transfection. *Proc. Natl. Acad. Sci. U.S.A.* 86:6077–6081 (1989).
16. C. F. Bennet, M.-Y. Chiang, H. Chan, J. E. E. Shoemaker, and C. K. Mirabelli. Cationic lipids enhance cellular uptake and activity of phosphorothioate antisense oligonucleotides. *Mol. Pharmacol.* 41:1023–1033 (1992).
17. F. Cumin, F. Asselbergs, M. Lartigot, and E. Felder. Modulation of human prorenin gene expression by antisense oligonucleotides in transfected CHO cells. *Eur. J. Biochem.* 212:347–354 (1993).
18. A. R. Thierry, A. Rahman, and A. Dritschilo. Liposomal delivery as a new approach to transport antisense oligonucleotides. In R. P. Erickson and J. G. Izant (eds.), *Gene Regulation: Biology of antisense RNA and DNA*, Raven Press, Ltd. 2, NY, 1992, pp. 147–161.
19. R. Leventis and J. R. Silvius. Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles. *Biochim. Biophys. Acta.* 1023:124–132 (1990).
20. E. Yoshihara, and T. Nakae. Cytolytic activity liposomes containing stearylamine. *Biochim. Bhiophys. Acta.* 854:530–546 (1986).
21. Y. A. Hannun, and R. M. Bell. Functions of sphingolipids and sphingolipid breakdown products in cellular regulation. *Science.* 243:500–507 (1989).
22. L. Stamatatos, R. Leventis, M. J. Zuckermann, and J. R. Silvius. Interactions of cationic lipid vesicles with negatively charged phospholipid vesicles and biological membranes. *Biochemistry.* 27:3917–3925 (1988).
23. J. K. Rose, L. Buonocore, and M. A. Whitt. A new cationic liposome reagent mediating nearly quantitive transfection of animal cells. *Biotechniques.* 10:520–525 (1991).
24. K. Lappalainen, A. Urtti, I. Jääskeläinen, K. Syrjänen, and S. Syrjänen. Cationic liposomes mediated delivery of antisense oligonucleotides targeted to HPV 16 E7 mRNA in CaSki cells. *Antiviral Reseach.* 23:119–130 (1994).
25. A. M. Krieg. Uptake and efficacy of phospodiester and modified antisense oligonucleotides in primary cell cultures. *Clin. Chem.* 39:710–712 (1993).
26. A. A. van de Loosdrecht, E. Nennie, G. Ossenkoppele, R. H. J. Beelen, and M. M. A. C. Langenhuijsen. Cell mediated cytotoxicity against U 937 cells by human monocytes and macrophages in a modified colorimetric MTT assay. *J. Immunol. Methods.* 141:15–22 (1991).
27. M. Hansen, S. E. Nielsen, and K. Berg. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J. Immunol. Methods.* 119:203–210 (1989).