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Stimulation of Phospholipase D in HepG2 Cells After Transfection Using Cationic Liposomes

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Lipid events in liposome-mediated transfection (lipofection) are largely unknown. Here we studied whether phospholipase D (PLD), an important enzyme responsible for phospholipid breakdown, was affected during lipofection of HepG2 cells with a luciferase plasmid. Synthetic cholesterol (Chol) derivatives, including 3β[L-ornithinamide-carbamoyl]Chol, [polyamidoamine-carbamoyl]Chol and 3β[*N*-(*N′,N′*-dimethylaminoethane)-carbamoyl]Chol, and a cationic lipid, *N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride were mixed with a helper lipid dioleoylphosphatidylethanolamine to form respective cationic liposomes. All cationic liposomes were found to stimulate PLD. Although orders of magnitude effects of the cationic liposomes on PLD stimulation did not consistently match those on cytotoxicity and luciferase expression, a causal relationship between PLD activation and cytotoxic effect was remarkable. PLD stimulation by the cationic liposomes was likely due to their amphiphilic characters, leading to membrane perturbation, as supported by similar results obtained with other membrane-perturbing chemicals such as oleate, melittin, and digitonin. Our results suggest that lipofection induces cellular lipid changes such as a PLD-driven phospholipid turnover.

Key Words : Phospholipase D, Cationic liposomes, Cholesterol, Membrane perturbation

Introduction

Numerous researches on development and optimization of nonviral vectors for gene therapy have been accumulated because viral vector systems are known to bring about immunogenic and oncogenic response in spite of high transfection efficiency.[1,2] Successful nonviral vectors must offer several advantages including proper safety, biocompatibility and biodegradability. So far, nonviral vector systems including polycationic polymers and cationic lipids have been extensively studied to overcome various limitations to gene therapy.[3,4] In particular, gene transfer applications of DNA/liposome complexes (lipoplexes) using cationic liposomes have attracted much attention due to their potential therapeutic possibilities.[5,6]

A cationic liposome composed of 3β[*N*-(*N′,N′*-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol) and dioleoylphosphatidylethanolamine (DOPE) was shown to be a potent vector for treatment of several diseases such as melanoma, breast cancer, and cystic fibrosis.[7-9] Several derivatives of hydrophobic cholesterol (Chol) having cationic headgroups such as polyamine and guanidinium have been synthesized and tested for their utility.[10,11] We have also developed the cationic Chol derivatives that are linked to L-lysinamide, L-ornithinamide and polyamidoamine by solid-phase synthesis method.[12-14]

There have been a significant number of studies on cationic liposomes in terms of synthesis, self-assembly, and conformational changes upon a complex formation with DNA. However, their effects on host cellular membranes remain largely unknown although liposome-mediated transfection (lipofection) involves a merger of target cellular membranes, which is accompanied with dynamic changes in lipid shapes. Accordingly, it is likely that cellular lipid composition is altered during lipofection, facilitating the fusion of liposomes and cellular membranes.

Phospholipase D (PLD), widely distributed in mammalian cells, plants, and bacteria, catalyzes the hydrolysis of phospholipids such as phosphatidylcholine (PC) at their terminal phosphodiester bond to produce phosphatidic acid (PA) and headgroup.[15] Many studies have shown that PLD plays an important role in critical membrane events such as phagocytosis, endocytosis, and actin cytoskeletal rearrangement.[16-18] Previously, we observed that melittin and mastoparan, α-helical polypeptides that extensively interact with liposome and cellular membranes remarkably activated PLD in intact L1210 cells.[19,20] Membrane permeabilization induced by a steroid glycoside digitonin, a non-ionic detergent, and addition of unsaturated fatty acids also stimulated the PLD activity.[20,21] It seemed that the PLD stimulation by all these chemicals was closely related to their membrane-perturbing capability.

Not only the chemicals but also cationic Chol lipids have amphiphilic structures having hydrophobic backbones and hydrophilic headgroups to promote electrostatic interaction with negatively charged DNA. DOPE that has been widely used as a fusogenic helper lipid had superiority over dioleoylphosphatidylcholine in gene transfer using a cationic

lipid, dioleyldimethylammonium chloride (DODAC), which seemed to correlate with a difference in the ability to disrupt cellular membranes.[22] This suggests that cationic liposomes are likely to induce membrane perturbation. Consequently, we hypothesized that cationic Chol liposomes may also affect PLD activity. Here, we report that cationic Chol liposomes having cytotoxicity stimulate PLD activity.

Experimental Section

Materials. [9,10-^{3}H(N)]Palmitic acid ([^{3}H]PAL) was purchased from Dupont NEN (Boston, MA). PC and DC-Chol were purchased from Avanti Polar Lipids (Alabaster, AL). Minimum essential medium (MEM), fetal bovine serum (FBS), and Lipofectin were obtained from Invitrogen (Carlsbad, CA). Precoated TLC (silica gel 60F_{254}) and digitonin were from Merck (Darmstadt, Germany). Synthesized melittin was obtained from Peptron (Daejeon, Korea). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

Synthesis of Cationic Cholesteryl Derivatives. Cationic Chol lipids, 3β[L-ornithinamide-carbamoyl]Chol (O-Chol) and [polyamidoamine-carbamoyl]Chol (PAM-Chol), were synthesized by conjugating the headgroup to Chol backbone using solid-phase synthesis method according to the previous reports.[13,14]

Preparation of Cationic Liposomes and Lipoplexes. Cationic liposomes and lipoplexes were prepared by the previous procedures.[13,14] In brief, synthetic Chol derivatives were resuspended in serum-free MEM and stored at 4 °C before use. A stock solution of DOPE dissolved in chloroform was evaporated to dryness under N_2 gas. The Chol derivatives solubilized were added to the dried DOPE at a weight ratio of 1:1. The mixtures were incubated overnight at 4 °C and sonicated in a water bath. Plasmid DNA (2 μg per 24-well or 10 μg per 6-well) diluted in serum-free MEM was then added to each cationic liposome at a weight ratio of DNA/liposome (1:6). The charge ratio of liposome/DNA was set to 1.7, at which transfection efficiency was optimal. The lipoplexes were left for 30 min at room temperature.

Lipofection of HepG2 Cells. HepG2 (a human liver carcinoma cell line) cells were grown in HEPES (20 mM)-buffered MEM medium supplemented with heat-inactivated 10% FBS, 2 mg/mL sodium bicarbonate, 0.12 mg/mL penicillin G, and 0.2 mg/mL streptomycin sulfate. Cells were routinely maintained in monolayer on culture dishes at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. A plasmid of pGL3-Luc encoding firefly luciferase as a reporter was amplified in *Escherichia coli* and purified with a Plasmid Maxi Kit (Qiagen, Germany) according to the supplier's protocol. Transfection of HepG2 cells was carried out by the published method.[13] HepG2 cells were seeded into a 6-well plate (7.5×10^5 cells/well) overnight. Each lipoplex in a volume of 0.6 mL was added to the cells in a 3 mL of serum-free MEM. A commercially available cationic liposome referred to as Lipofectin, a 1:1 mixture of *N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA) and DOPE, was used as a positive control. After transfection was performed for 4 h, culture media were replaced by a complete growth medium.

Measurement of PLD Activity. PLD activity was determined by measuring phosphatidylethanol (PEt) in the presence of ethanol as described previously.[20,21] Briefly, HepG2 cells were seeded into 6-well plates in 2 mL of serum-free MEM containing 0.3% (w/v) fatty acid-free bovine serum albumin and labeled with [^{3}H]PAL (3 μCi/mL) for 3 h before transfection. After treatment in the presence of 1.5% ethanol, cells were immediately trypsinized and total lipids were extracted by an addition of 2 mL of chloroform/methanol (1:1). [^{3}H]PEt produced were separated on a TLC plate using a solvent system of ethylacetate/isooctane/acetic acid/water (13/2/3/10, v/v). For the detection of [^{3}H]PEt, cold (non-isotope labeled) PEt prepared from PC using cabbage PLD was added to the cellular lipid extracts.[23]

Luciferase Expression and Cytotoxicity. HepG2 cells were seeded to a 24-well plate (1.5×10^5 cells/well) one day prior to lipofection. Each lipoplex (0.12 mL) was added to cells in a 0.6 mL of serum-free MEM. At 48 h post-transfection, cells washed with PBS were lysed in a Reporter Lysis Buffer and luciferase activity was assayed with a Luciferase Assay Kit (Promega, Madison, WI) according to supplier's protocol. Protein amounts were quantified with a bicinchoninic acid reagent (Pierce, Rockford, IL). Luminescence intensities were measured using a Lumat LB 9507 luminometer (Berthold, Germany) and presented as RLU/ng protein. For measurement of cytotoxicity, cells were plated into a 96-well (1.0×10^4 cells/well) and cell cytotoxicity was determined using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay as described previously.[14] Absorbance of the reduced MTT was measured at 570 nm using a spectrophotometer.

Results and Discussion

Figure 1 shows the structures of cationic Chol derivatives of DC-Chol, O-Chol, and PAM-Chol, and those of another cationic lipid DOTMA and a helper lipid DOPE, which were employed for liposome formation in this study. We previously characterized the formation, shapes, and particle sizes of the cationic liposomes by agarose gel electrophoresis and atomic force microscopy.[13,14] The various cationic headgroups were conjugated to hydrophobic Chol backbone that allowed the Chol derivatives to be soluble and to bind the negatively charged DNA. When the cationic Chol lipids were resuspended in a serum-free medium, they formed a micellar solution.

We investigated whether PLD activity was changed during cationic liposome-mediated gene transfer. PA is the endogenous lipid product of PLD, but PA can be degraded to diacylglycerol by PA phosphohydrolase activity or produced by diacylglycerol kinase activity from diacylglycerol.[15] PLD mediates the unique transphosphatidylation reaction in the presence of ethanol, catalyzing the generation of PEt at the expense of PA production. Hence, PEt formation from HepG2 cells in a culture medium containing 1.5% ethanol was monitored as readout of PLD activity.[19-21] The isotope-

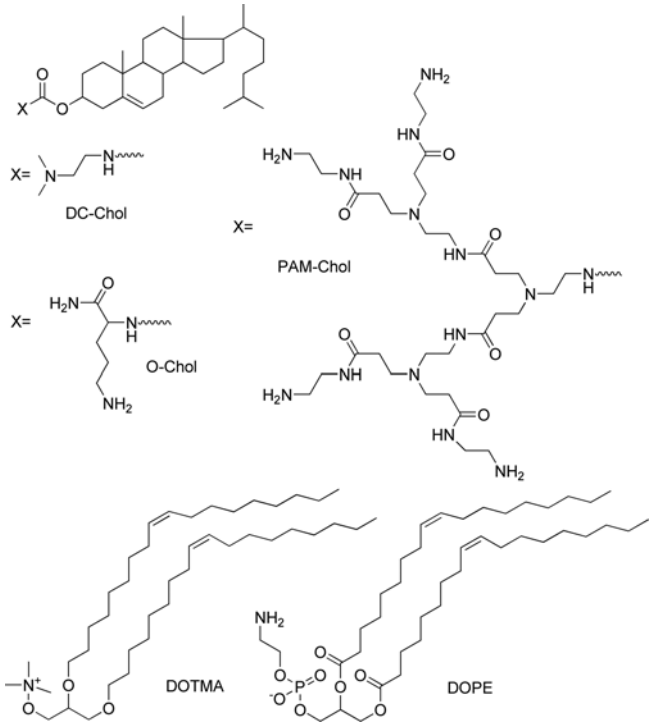

Figure 1. Chemical structures of lipids used in this study.

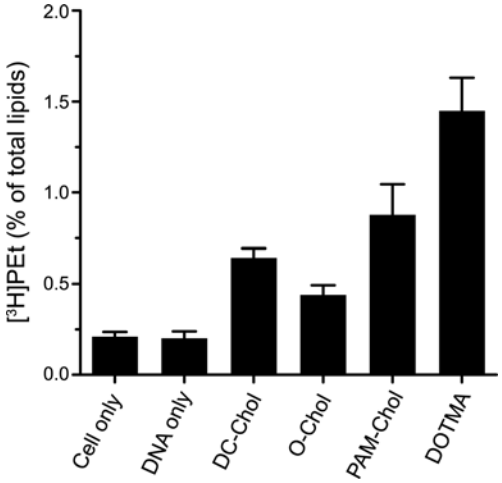

Figure 2. PLD stimulation following gene delivery using cationic lipoplexes. Cationic Chol derivatives and DOTMA as indicated were mixed with DOPE. [^{3}H]PAL-labeled HepG2 cells were transfected with mixtures of the respective liposomes and a luciferase plasmid DNA. Controls included resting cells (cell only) or cells treated with DNA in the absence of liposome (DNA only). After 4 h transfection in the presence of 1.5% ethanol, the [^{3}H]PEt formed was measured by TLC and the radioactivity was expressed as percentage of total lipid radioactivity. The data were presented as percentage of radioactivity in PEt with respect to the total lipid radioactivity. Values are expressed as means ± SD for 3-4 measurements performed in duplicate.

labeled cells were incubated with the lipoplexes of cationic Chol derivatives and a luciferase plasmid for 4 h. As shown in Figure 2, all cationic liposomes significantly stimulated the PLD activity. The effect of DOTMA liposome on PLD activity was the highest (7-fold over the control). PAM-Chol

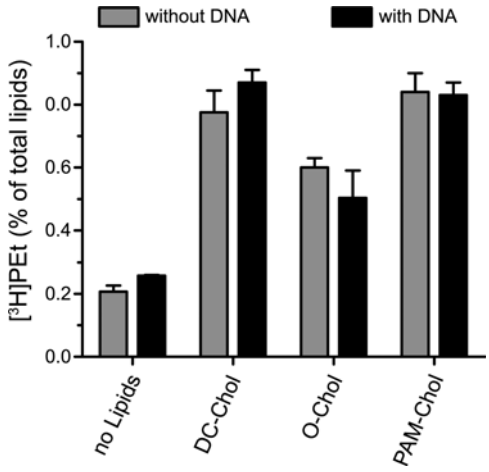

Figure 3. Comparison between the effects of cationic liposomes and lipoplexes on PLD activity. [^{3}H]PAL-labeled HepG2 cells were incubated with liposomes (without DNA) of cationic Chol derivatives and DOPE or with their respective lipoplexes (with DNA) as indicated conditions. As a control, liposome-untreated cells were tested. After 4 h incubation in the presence of 1.5% ethanol, the [^{3}H]PEt formed was measured by TLC. The radioactivity of [^{3}H]PEt formed was measured and presented as described in the legend of Figure 2.

liposomes also showed a remarkable stimulatory effect (4-fold), which was next ranked by DC-Chol (3-fold) and O-Chol (2-fold) liposomes. When cells were incubated with only DNA, the PLD stimulation was negligible in comparison with control cells that contained neither liposome nor DNA. We then examined a possibility of differential effects between liposomes and lipoplexes on PLD activation. For this, cells were incubated with the cationic Chol liposomes in the absence or presence of DNA. The stimulating effects of DC-Chol, PAM-Chol, and O-Chol liposomes on PLD activity were similar to those of respective lipoplexes (Figure 3), indicating that the cationic liposome alone was the main factor for PLD stimulation.

Introduction of DNA into mammalian cells using cationic liposomes has been demonstrated to be an effective method, comparable to other transfection methods. We compared the transfection efficiency of the cationic Chol liposomes. For this purpose, we used a luciferase plasmid as a reporter gene and measured luminescence intensities. O-Chol liposome showed the highest transfection efficiency, which was five times higher than that of DC-Chol liposome (Figure 4). The effectiveness of a commercially available DOTMA liposome was also significant. PAM-Chol liposome was less effective than DC-Chol liposome. As expected, luminescence was hardly detectable in the absence of liposome irrespective of the presence of the reporter gene.

Lipofection is subject to decrease cell viability to some extent because of its cytotoxic effect. Cell cytotoxicity of the cationic Chol liposomes was determined in the absence of a plasmid DNA by measuring relative cell viability with a MTT assay. We observed the concentration-dependent cytotoxic effects of the cationic liposomes but the resulting cytotoxicity seemed somewhat different among them (Figure 5).

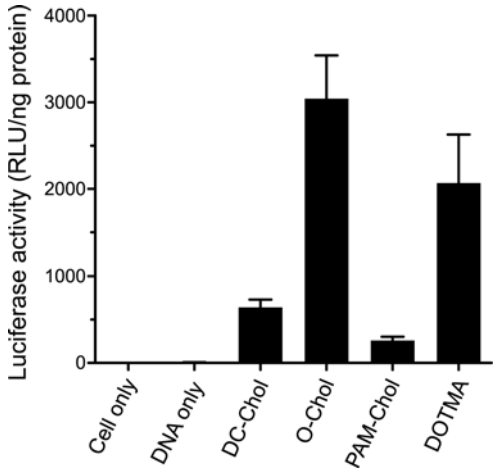

Figure 4. Test of transfection efficiency by cationic liposomes. Transfection of HepG2 cells with a luciferase gene was carried out using the indicated cationic Chol liposomes for 4 h. As controls, cells were incubated without or with the plasmid in the absence of Chol liposome. The luciferase activity was determined with a commercial assay kit at 48 h posttransfection and normalized against cell lysate amounts. The data were presented as means ± SD for 3-4 measurements performed in triplicate.

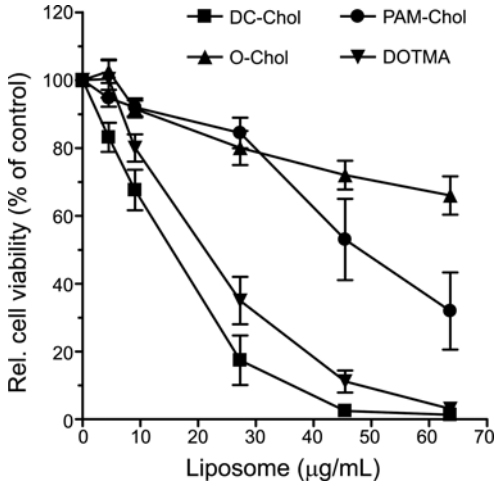

Figure 5. Effects of cationic liposomes on cell viability. HepG2 cells were incubated with various concentrations of cationic Chol liposomes without DNA for 4 h. Cell viability was then measured using a MTT assay after 48 h. Relative cell viability was calculated as a percentage to the control resting cells.

DC-Chol liposome was the most cytotoxic of all liposomes, which was followed by DOTMA liposome. When the concentration in which relative cell viability reached 50% comparing to the control (designated as IC_{50}), IC_{50} values of DC-Chol and DOTMA liposomes were calculated approximately 15.5 and 21.2 μg/mL, respectively. In case of PAM-Chol liposome, IC_{50} value was approximately 48.0 μg/mL and O-Chol liposome showed the least cytotoxic effect.

We tested whether other cytotoxic chemicals such as oleate (an unsaturated fatty acid), melittin (an amphiphilic polypeptide), and digitonin (a well-known membrane permeabilizing reagent) could also exert stimulatory effects on PLD in HepG2 cells. Although molecular characteristics of

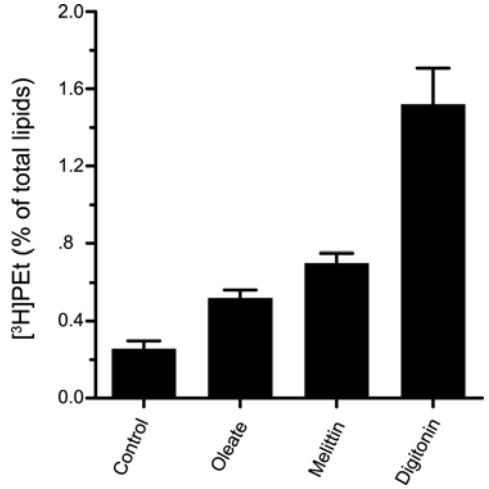

Figure 6. Effects of membrane-perturbing chemicals on PLD activity in HepG2 cells. [^{3}H]PAL-labeled HepG2 cells were untreated or treated with oleate (0.5 mM), melittin (5 mM), or digitonin (24 mM) in the presence of 1.5% ethanol. After 20 min incubation, [^{3}H]PEt formed was measured and presented as described in the legend of Figure 2.

the chemicals are different each other, there is a considerable common property among them in that they extensively interact with membranes leading to morphological changes such as micelle formation, membrane fusion, or membrane pore formation.[24-26] When HepG2 cells were treated with them, substantially increased PEt formation was observed with oleate (2-fold), melittin (3-fold), and digitonin (6-fold) (Figure 6). Melittin is known to have hemolytic and antimicrobial activity and perturb lipid bilayer structures.[25,27] The exposure of mouse proximal tubular cells to exogenously added oleic acid evoked severe cell injury and oleic acid disordered the physical properties of membranes.[28,29] Treatment of L1210 cells with digitonin, a well-known membrane permeabilizing reagent, evoked the extensive changes in cellular membrane lipids like mastoparan 7, an amphiphilic peptide that disorganizes membrane structures through lipid binding.[30] The considerable similarity in PLD activation between cationic liposomes and the cytotoxic chemicals tested suggests that membrane destabilization induced by cationic liposomes may activate PLD.

As lipoplex always passes through target cellular membranes, it is thought that such cytotoxicity, not necessarily but at least partially, influences on cellular membranes. Previously, gene transfer using a cationic DODAC/DOPE liposome in BHK cells, a disruption of membrane integrity by the liposome was proposed as a relevant mechanism.[22] In addition, a study on gene delivery using amphiphilic polypeptides in COS-7 cells, the membrane-perturbing activity of polypeptides was regarded as an important factor for efficient transfection.[31] Cationic lipids have generally adopted both hydrophobic and hydrophilic groups for promoting gene transfer. Cationic lipids such as *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium (DOTAP) were shown to bind phospholipids and affect physical properties of liposome bilayer structure.[32] Due to their amphiphilic structures, cati-

onic liposomes are expected to strongly interact with target cellular membranes. Moreover, liposomes and lipoplexes made of DOTAP analogs and Chol or DOPE became more stable and lipid composition of the liposomes critically determined gene transfer efficiency.[33]

The PLD stimulation by lipoplexes was repeated in the absence of DNA, suggesting that cationic liposomes themselves could perturb the HepG2 cellular membranes (Figure 3). This result was in good agreement with the cytotoxicity test performed in the absence of DNA (Figure 5). The results of PLD stimulation by the cytotoxic Chol liposomes might be originated from their ability to induce membrane perturbation (Figures 2 and 5). Melittin, oleate, and digitonin also stimulated PLD activity (Figure 6). Thus, the actions of cationic liposomes are likely parallel to those of the membrane-perturbing chemicals. Perturbation of cellular membranes induced by chemical or physical stimuli modulated various biological events including membrane fluidity, gene expression, and heat shock response.[34,35] These observations raise the possibility that membrane-perturbing action of the cationic Chol liposomes can cause PLD-dependent change in lipid composition.

Conclusion

In summary, we report that cationic liposomes comprising DC-Chol, O-Chol, and PAM-Chol together with DOPE significantly stimulated PLD activity in HepG2 cells. These observations support an idea that cytotoxic and membrane-perturbing effects induced by the Chol liposomes play a primary role in the upregulation of PLD activity. Although biochemical and biophysical properties of cationic lipids have been systematically studied with model membranes, their potential to affect intact cellular membranes has not been well understood. This study provides an insight into a new character of cationic liposome for its potential to alter cellular lipid composition. In this regard, our results suggest that PLD-catalyzed phospholipid breakdown can be considered as an index for evaluating a biological property of cationic liposomes.

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References

1. Ledley, F. D. *Hum. Gene Ther.* **1995**, *6*, 1129.
2. Nishikawa, M.; Huang, L. *Hum. Gene Ther.* **2001**, *12*, 861.
3. Choi, J. S.; Park, J. S. *Methods Mol. Med.* **2001**, *65*, 23.
4. Tiera, M. J.; Winnik, F. O.; Fernandes, J. C. *Curr. Gene Ther.* **2006**, *6*, 59.
5. Oku, N.; Yamazaki, Y.; Matsuura, M.; Sugiyama, M.; Hasegawa, M.; Nango, M. *Adv. Drug Deliv. Rev.* **2001**, *52*, 209.
6. Simoes, S.; Filipe, A.; Faneca, H.; Mano, M.; Penacho, N.; Duzgunes, N.; de Lima, M. P. *Expert Opin. Drug Deliv.* **2005**, *2*, 237.
7. Meidan, V. M.; Glezer, J.; Salomon, S.; Sidi, Y.; Barenholz, Y.; Cohen, J. S.; Lilling, G. *J. Liposome Res.* **2006**, *16*, 27.
8. Peters, M. T.; Brigham, K. L.; King, G. A.; Meyrick, B. O.; Gao, X.; Stecenko, A. A. *Exp. Lung Res.* **1999**, *25*, 183.
9. Reynier, P.; Briane, D.; Coudert, R.; Fadda, G.; Bouchemal, N.; Bissieres, P.; Taillandier, E.; Cao, A. *J. Drug Target.* **2004**, *12*, 25.
10. Aissaoui, A.; Oudrhiri, N.; Petit, L.; Hauchecorne, M.; Kan, E.; Sainlos, M.; Julia, S.; Navarro, J.; Vigneron, J. P.; Lehn, J. M.; Lehn, P. *Curr. Drug Targets* **2002**, *3*, 1.
11. Islam, R. U.; Hean, J.; van Otterlo, W. A.; de Koning, C. B.; Arbuthnot, P. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 100.
12. Choi, J. S.; Lee, E. J.; Jang, H. S.; Park, J. S. *J. Biochem. Mol. Biol.* **2000**, *33*, 476.
13. Choi, J. S.; Lee, E. J.; Jang, H. S.; Park, J. S. *Bioconjug. Chem.* **2001**, *12*, 108.
14. Jang, H. S.; Lee, Y.; Kim, T. I.; Park, J. S.; Choi, J. S. *Bull. Korean Chem. Soc.* **2012**, *33*, 1353.
15. Jenkins, G. M.; Frohman, M. A. *Cell. Mol. Life Sci.* **2005**, *62*, 2305.
16. Corrotte, M.; Chasserot-Golaz, S.; Huang, P.; Du, G.; Ktistakis, N. T.; Frohman, M. A.; Vitale, N.; Bader, M. F.; Grant, N. J. *Traffic* **2006**, *7*, 365.
17. Donaldson, J. G. *Biochim. Biophys. Acta* **2009**, *1791*, 845.
18. Komati, H.; Naro, F.; Mebarek, S.; De Arcangelis, V.; Adamo, S.; Lagarde, M.; Prigent, A. F.; Nemoz, G. *Mol. Biol. Cell* **2005**, *16*, 1232.
19. Lee, S. Y.; Park, H. S.; Lee, S. J.; Choi, M. U. *Arch. Biochem. Biophys.* **2001**, *389*, 57.
20. Lee, S. Y.; Park, N. G.; Choi, M. U. *FEBS Lett.* **1998**, *432*, 50.
21. Lee, S. Y.; Yeo, E. J.; Choi, M. U. *Biochem. Biophys. Res. Commun.* **1998**, *244*, 825.
22. Mui, B.; Ahkong, Q. F.; Chow, L.; Hope, M. J. *Biochim. Biophys. Acta* **2000**, *1467*, 281.
23. Jung, K.; Koh, E.; Choi, M. U. *Bull. Korean Chem. Soc.* **1989**, *10*, 595.
24. Chernomordik, L. V.; Leikina, E.; Frolov, V.; Bronk, P.; Zimmerberg, J. *J. Cell Biol.* **1997**, *136*, 81.
25. Dempsey, C. E. *Biochim. Biophys. Acta* **1990**, *1031*, 143.
26. Vitale, M. L.; Rodriguez Del Castillo, A.; Trifaro, J. M. *J. Neurochem.* **1992**, *59*, 1717.
27. Takei, J.; Remenyi, A.; Dempsey, C. E. *FEBS Lett.* **1999**, *442*, 11.
28. Antollini, S. S.; Barrantes, F. J. *J. Biol. Chem.* **2002**, *277*, 1249.
29. Lieberthal, W.; Sheridan, A. M.; Schwartz, J. H. *J. Lab. Clin. Med.* **1997**, *129*, 260.
30. Park, H. S.; Lee, S. Y.; Kim, Y. H.; Kim, J. Y.; Lee, S. J.; Choi, M. *Biochim. Biophys. Acta* **2000**, *1484*, 151.
31. Niidome, T.; Takaji, K.; Urakawa, M.; Ohmori, N.; Wada, A.; Hirayama, T.; Aoyagi, H. *Bioconjug. Chem.* **1999**, *10*, 773.
32. Campbell, R. B.; Balasubramanian, S. V.; Straubinger, R. M. *Biochim. Biophys. Acta* **2001**, *1512*, 27.
33. Regelin, A. E.; Fankhaenel, S.; Gurtesch, L.; Prinz, C.; von Kiedrowski, G.; Massing, U. *Biochim. Biophys. Acta* **2000**, *1464*, 151.
34. Carratu, L.; Franceschelli, S.; Pardini, C. L.; Kobayashi, G. S.; Horvath, I.; Vigh, L.; Maresca, B. *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 3870.
35. Los, D. A.; Murata, N. *Sci. STKE* **2000**, *2000*, pe1.