

Note

Protective Effect of Edaravone against Cationic Lipid-Mediated Oxidative Stress and Apoptosis

Takeshi Terada,^{*,a} Jayesh A Kulkarni,^b Ariel Huynh,^c Yuen Yi C. Tam,^d and Pieter Cullis^d

^aPharmaceutical Research Department, Mitsubishi Tanabe Pharma Corporation; 3–16–89 Kashima, Yodogawa-ku, Osaka 532–8505, Japan; ^bDepartment of Medical Genetics, University of British Columbia; Vancouver, BC, V6T 1Z3, Canada; ^cDepartment of Pharmaceutical Sciences, University of British Columbia; Vancouver, BC, V6T 1Z3, Canada; and ^dDepartment of Biochemistry and Molecular Biology, University of British Columbia; Vancouver, BC, V6T 1Z3, Canada.

Received August 25, 2020; accepted October 7, 2020

Liposomes containing ionizable cationic lipids have been widely used for the delivery of nucleic acids such as small-interfering RNA and mRNA. The utility of cationic lipids with a permanent positive charge, however, is limited to *in vitro* transfection of cultured cells due to its dose-limiting toxic side effects observed in animals. Several reports have suggested that the permanently charged cationic lipids induce reactive oxygen species (ROS) and ROS-mediated toxicity in cells. We therefore hypothesized that the concomitant use of ROS inhibitor could reduce toxicity and improve drug efficacy. In this study, suppression of the cationic toxicity was evaluated using an ROS scavenger, edaravone, which is a low-molecular-weight antioxidant drug clinically approved for acute-phase cerebral infarction and amyotrophic lateral sclerosis. Cell viability assay in the mouse macrophage-like cell line RAW264 indicated that the concomitant use of edaravone were not able to suppress the cytotoxicity induced by cationic liposomes comprised of monovalent cationic lipid *N*-(1-[2,3-dioleoyloxy]propyl)-*N,N,N*-trimethylammonium chloride (DOTMA) over a short period of time. Cationic lipids-induced necrosis was assumed to be involved in the cytotoxicity upon short-term exposure to cationic liposomes. On the other hand, the significant improvement of cell viability was observed when the short treatment with cationic liposomes was followed by exposure to edaravone for 24 h. It was also confirmed that apoptosis inhibition by ROS elimination might have contributed to this effect. These results suggest the utility of continuous administration with edaravone as concomitant drug for suppression of adverse reactions in therapeutic treatment using cationic liposomes.

Key words liposome; edaravone; cationic lipid; reactive oxygen species

INTRODUCTION

For gene therapy, the development of effective and safe nucleic acid delivery systems is an essential requirement. Non-viral vectors including liposomes are superior platforms than viral vectors because of low immunogenicity, high reproducibility, and availability of established, streamlined manufacturing techniques.^{1–4)} Application of liposome technology to genetic drugs required the development of methods to achieve efficient encapsulation of RNA and DNA polymers into liposome systems. The introduction of cationic lipids provided a way forward as they have strong electrostatic associations with RNA and DNA polymers.⁵⁾ Since their discovery, they have been widely used to deliver nucleic acids into targeted cells and offered potential utility in the clinic.⁶⁾ Although the benefits brought by cationic lipids in drug delivery have gained much attention and considerable efforts have been devoted to investigating better versions, the issue of toxicity has always been the main barrier in *in vivo* application.⁷⁾ Even the most widely used lipoplexes, which are complexes of mixed cationic lipids with nucleic acid polymers, are limited to *in vitro* transfection due to issues such as inflammatory toxicity, hepatotoxicity, leukopenia and thrombocytopenia *in vivo*.^{8–11)} To address this problem, ionizable cationic lipids with apparent pK_a values below 7 have widely been developed, allowing for interaction with nucleic acid polymers below the pK_a and the maintenance of a relatively neutral liposome surface

charge at physiological pH values.¹²⁾

A cause for the toxicities mediated by cationic lipids is reactive oxygen species (ROS) generation, which induces cytotoxicity and affects intracellular signaling pathways.^{13,14)} The high surface density of positive charges on cationic liposomes is believed to be critical for ROS generation.¹⁵⁾ ROS are continuously generated by ROS producers and eliminated through ROS-scavenging systems to maintain redox homeostasis under physiological conditions. Upon disruption of the equilibrium, cell damage occurs.¹⁶⁾ Moreover, ROS regulate cell differentiation, proliferation, migration, and apoptosis.

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is a potent scavenger of free radicals. It is highly efficient in quenching hydroxyl radicals and also prevents oxidative stress-induced cellular damage.¹⁷⁾ The ROS scavenging ability of edaravone is believed to protect various cells such as endothelial cells, neurons and myocardial cells, against damages caused by oxidative stress conditions.¹⁸⁾ In 2001, Radicut® (an aqueous based parenteral preparation of edaravone) developed by Mitsubishi Tanabe Pharma Corporation (Japan), was approved to treat patients of acute-phase cerebral infarction in Japan. Subsequently in 2015, Radicut® was approved in Japan for amyotrophic lateral sclerosis treatment.

We hypothesized that a ROS scavenger such as edaravone could be used as a concomitant drug to suppress toxic effects induced by cationic liposomes. In the present study, we evaluated the protective effect of edaravone by treating

*To whom correspondence should be addressed. e-mail: terada.takeshi@mk.mt-pharma.co.jp

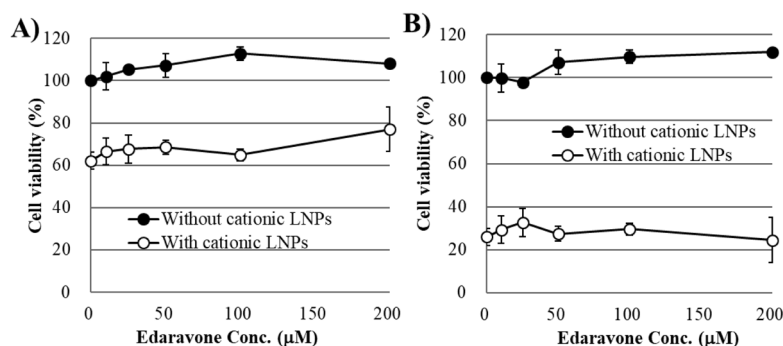


Fig. 1. Effect of Edaravone on Acute Cytotoxicity Induced by Cationic Liposomes (DOTMA:Chol at 50:50) Treatment in RAW264.7 Cells

Cell viability (% of control) was measured by MTT assay, following the treatment of 100 μM cationic liposomes in the presence of edaravone at 0–200 μM for 3 h (A) and 6 h (B). Results indicate mean ± standard deviation (S.D.) ($n = 3$).

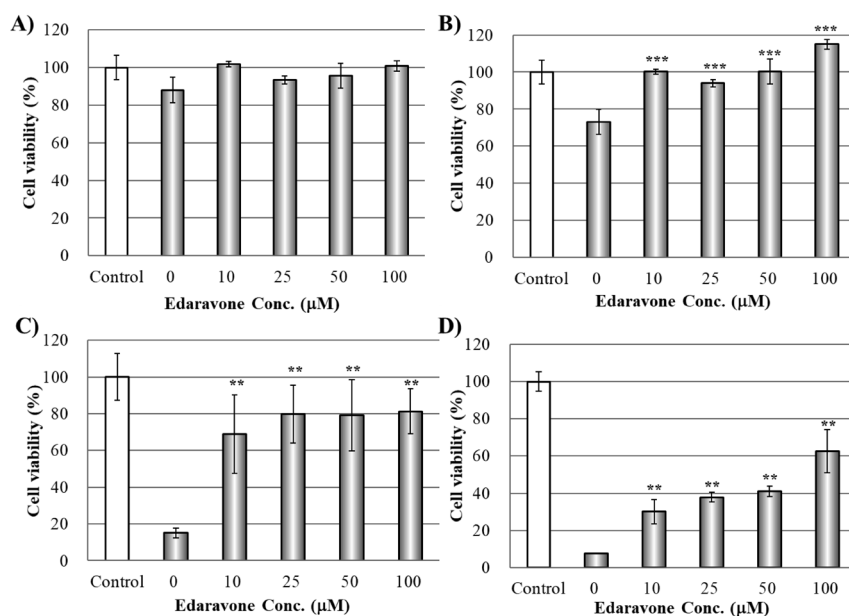


Fig. 2. Effect of 24h Incubation with Edaravone against the Cytotoxicity Induced by Cationic Liposomes (DOTMA:Chol at 50:50) Treatment in RAW264.7 Cells

MTT assay was performed on cells treated with edaravone at 0–100 μM for 24h, following the treatment with 100 μM cationic liposomes in the presence of edaravone for 0.5 (A), 1 (B), 3 (C), and 5h (D). Results indicate mean ± S.D. ($n = 3$). ** $p < 0.01$, *** $p < 0.001$, compared with cationic liposomes-treated cells in the absence of edaravone.

RAW264.7 mouse macrophage-like cells with liposomes composed of cationic lipid *N*-(1-[2,3-dioleoyloxy]propyl)-*N,N,N*-trimethylammonium chloride (DOTMA), which has been reported to cause ROS-mediated cytotoxicity.¹⁹⁾

MATERIALS AND METHODS

Materials DOTMA was purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Cholesterol, edaravone, 2', 7'-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Hoechst dye was purchased from Invitrogen (Burlington, ON, Canada). RAW264.7 mouse macrophage-like cell line was obtained from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS).

Preparation of Cationic Liposomes Cationic liposomes were prepared as previously described.^{20,21)} Briefly, lipid components composed of DOTMA/Chol (50/50 mol%) were dissolved in ethanol at 20 mM of total lipid. The lipids etha-

nol solution was mixed with distilled water through a staggered herringbone micromixer (SHM) at a total flow rate of 2 mL/min, and a flow rate ratio of 3 (v/v) (water/ethanol ratio). The resulting suspension was subsequently dialyzed against at least 1000-fold volume of distilled water overnight to remove the residual ethanol. Cationic liposomes solution was then filtered through a 0.2 μm filter.

Analysis of Cationic Liposomes Particle size and z-potential analysis was carried out by dynamic light scattering with Malvern Zetasizer (Worcestershire, U.K.). Total lipids were determined by measuring the cholesterol content using the Cholesterol E assay (Wako Pure Chemical Corporation, Richmond, VA, U.S.A.).

Cell Viability Analysis Cell viability was determined by performing the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT, Sigma-Aldrich) assay on cultured RAW264.7 cells as described previously.²²⁾ Briefly, 15000 cells/well suspended in 10% FBS/DMEM were seeded 96-well plates, grown overnight. In Fig. 1, cells were preincubated with FBS-free DMEM containing edaravone for 1 h, followed

by the treatment of 100 μ M cationic liposomes in the presence of 0–200 μ M edaravone in FBS-free DMEM for 3 and 6 h at 37°C. In Fig. 2, after treatment of 100 μ M cationic liposomes with edaravone in FBS-free DMEM for 0.5–5 h, the medium was removed and the cells were incubated with 10% FBS/DMEM in the presence of 0–100 μ M edaravone for 24 h at 37°C. After each treatment, cell viability was measured as follows: 30 μ L of 5 mg/mL MTT in phosphate buffered saline (PBS) was added to cells in the presence of 125 μ L cell culture medium. After 2 h incubation at 37°C, 125 μ L of 20% sodium dodecyl sulfate dissolved in dimethylformamide:water (1:1) at pH 4.7 was added, and the absorbance at 570 nm was measured after the overnight incubation. Results were expressed as percentages of control.

Fluorescent Analysis of Intracellular ROS RAW264.7 cells suspended in 10% FBS/DMEM were seeded at 15000 cells per well in 96-well plates, grown overnight. Cells were incubated with 10% FBS/DMEM containing 10 μ M edaravone and 250 μ M cationic liposomes for 24 h at 37°C. After the incubation, the treatment mediums were removed, and cells were incubated with 10 μ M DCFH-DA dye (ROS staining) and 0.7 μ g/mL Hoechst dye (nuclear staining) in PBS for 30 min in darkness. After being washed 1 time with PBS, fluorescence images were analyzed using a Cellomics ArrayScan VTI HCS Reader (Thermo Scientific, Pittsburgh, PA, U.S.A.).²³⁾

DNA Fragmentation Analysis RAW264.7 cells suspended in 10% FBS/DMEM were plated on a 60 mm dish at a density of 1×10^6 cells/21 cm², grown overnight. Briefly, cells were treated with FBS-free DMEM containing 100 μ M cationic liposomes (DOTMA:Chol at 50:50) and 10 μ M edaravone for 3 h at 37°C. The medium was replaced with 10% FBS/DMEM in absence and presence of 10 μ M edaravone. After the incubation for 24 h at 37°C, the supernatant cells and adherent cells were collected. After centrifugation, the cells (5×10^5 cells) were collected, and DNA was extracted according to the method of Wang *et al.*²⁴⁾ The extracted DNA was dissolved in TE buffer (10 mM Tris buffer, pH 7.4, containing 1 mM ethylenediaminetetraacetic acid (EDTA)) and subjected to 1.5% agarose gel electrophoresis with 1 kb DNA Ladder (Invitrogen, Burlington, ON, Canada).²⁵⁾

Statistical Analysis All values were indicated as mean $\pm$ standard deviation (S.D.). Statistical comparisons were performed using one-way ANOVA followed Dunnett's *post hoc* test for multiple comparisons. Values of $p < 0.05$ were considered to indicate statistical significance.

RESULTS AND DISCUSSION

Effect of Edaravone on Acute Cytotoxicity Induced by Cationic Liposomes To determine the effect of edaravone on cationic liposomes-mediated cytotoxicity, particles were prepared using the cationic lipid DOTMA and cholesterol at a molar ratio of 50:50. Particles were determined to have a diameter of 45.9 ± 0.9 nm (mean $\pm$ S.D.) and a positive z-potential of $+51.6 \pm 0.4$ (mean $\pm$ S.D.). Various concentrations of these cationic liposomes were incubated with RAW264.7 cells for 1–6 h. As shown in Fig. S1, cytotoxicity (as measure by cell viability using MTT) in RAW264.7 cells was observed as soon as 1 h post liposomes treatment in a concentration- and time-dependent manner. Indeed, it has been reported that DOTMA-based cationic liposomes rapidly increase ROS gen-

eration in treated cells in a concentration-dependent manner, reaching the plateau at 40–60 min.¹⁹⁾

The reaction rates of edaravone to eliminate ROS have been determined to be 10^7 M⁻¹s⁻¹ or higher, indicating that edaravone enables rapid ROS elimination.²⁶⁾ We, therefore, hypothesized that short-term co-administration of edaravone with cationic liposomes could protect cells against cationic lipids-mediated cytotoxicity. In this study, cell viability was measured following the treatment of 100 μ M of cationic liposomes in the presence of edaravone at 0–200 μ M for 3 and 6 h. As shown in Fig. 1, cationic lipid-mediated cytotoxicity was not suppressed at any edaravone concentration. This is not unexpected as a previous report indicated that HepG2 cell death induced by 100 μ M of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)-liposome was evident with or without edaravone since high cationic lipid content caused destabilization of the plasma membrane and cell collapse.²⁷⁾ It is possible that membrane solubilization, poration, and lysis triggers acute cell necrosis before the occurrence of ROS-induced cell apoptosis.

Effect of Incubation with Edaravone against Cationic Liposomes-Induced Cytotoxicity It has been reported that typical cationic carriers such as cationic liposome, chitosan and polyethylenimine (PEI) can trigger cell apoptosis^{28,29)} and apoptotic cells often appear after approximately 24–48 h exposure to the cationic liposomes. Moreover, the protective effect of edaravone against paraquat, hyperosmolarity, and hypoxia-induced ROS production was also observed after 24 h of the treatment.^{30–32)} It is therefore possible that long-term exposure to edaravone may be necessary to suppress ROS-induced apoptosis after treatment with cationic liposome.

In order to clarify the protective effect of edaravone against cationic liposomes mediated-cytotoxicity, RAW264.7 cells were incubated with cationic liposomes for 0.5 to 5 h, followed by treatment of edaravone for 24 h, and then measurement of cell viability by MTT assay. As shown in Fig. 2, the cationic liposomes-induced cytotoxicity was markedly alleviated by edaravone at as low as 10 μ M ($p < 0.01$ or $p < 0.001$, compared with cationic liposomes-treated cells in the absence of edaravone).

Previously, Wei *et al.* have demonstrated that cathepsin-B (marker of necrosis) was detected in A549 cells incubated with cationic particles for 30 min.³³⁾ In contrast, activation of caspase-3 (a marker of apoptosis) was not detected at 30 min, but at 24 h after incubation with cationic particles. Therefore, our results, which showed that the cationic liposomes-induced cytotoxicity was not affected by short-term exposure to edaravone but was attenuated by long-term exposure, are reasonable in view of the mechanism of this cytotoxicity. However, the short half-life (5.4 min) of edaravone upon intravenous administration is not suitable for clinical use.¹⁴⁾ Some studies are investigating edaravone loaded in alginate-based nanocomposite hydrogel or liposomes for sustained release of edaravone.^{34–36)} It is expected that such sustained release drug-delivery systems would be more amenable to patient use.

Effect of Edaravone against ROS Generation The inhibitory effect of edaravone on cationic liposomes-induced ROS generation was evaluated by dichloro-dihydro-fluorescein diacetate (DCFH-DA) fluorescence assay, a quantitative assessment for oxidative stress in cells. DCFH-DA passively diffuses into cells, where the two acetate groups are cleaved by intracellular esterases to yield cell-impermeable DCFH,

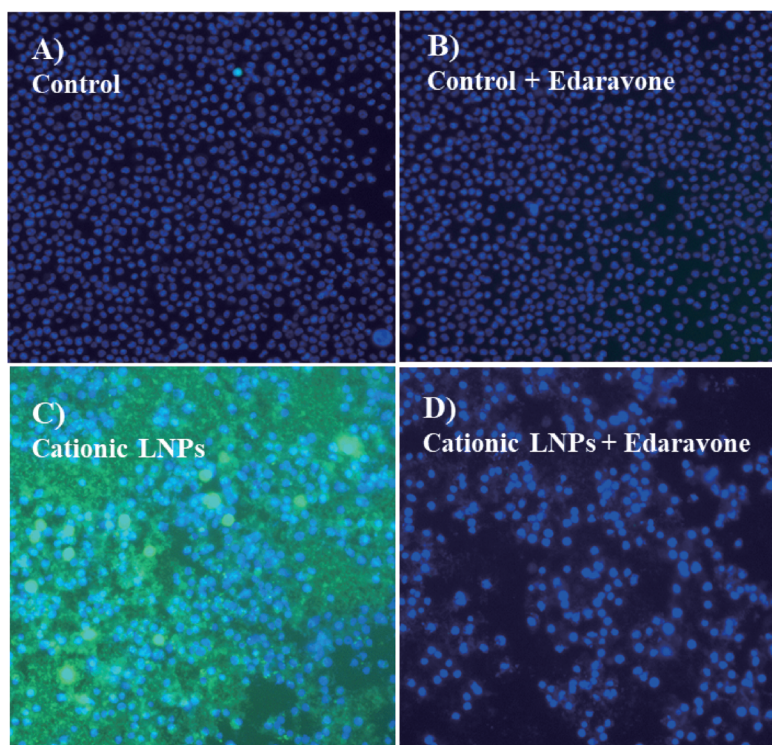


Fig. 3. Effect of Edaravone against ROS Generated by Cationic Liposomes (DOTMA:Chol at 50:50) in RAW264.7 Cells

ROS production was analyzed by double staining with Hoechst dye (blue color: nuclear staining) and DCFH-DA dye (green color: ROS staining). Cells were incubated for 24 h with no edaravone or liposomes (A), 10 μ M of edaravone (B), 250 μ M of cationic liposomes (C) and the concomitant treatment of 250 μ M of cationic liposomes with 10 μ M of Edaravone (D), followed by staining with Hoechst and DCFH-DA.

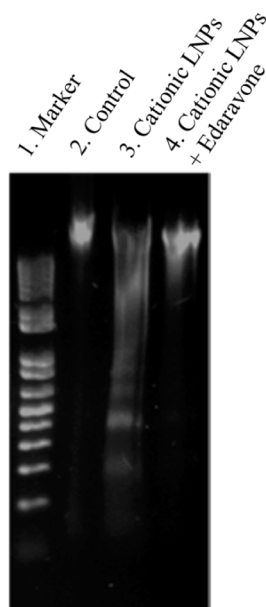


Fig. 4. Effect of Edaravone on Cationic Liposomes-Induced DNA Fragmentation in RAW264.7 Cells

Cells were treated with cationic liposomes (DOTMA:Chol at 50:50) for 3 h followed by 24 h incubation with and without edaravone and then analyzed for DNA fragmentation by gel electrophoresis. From the left, Lane 1: 1Kb DNA marker, Lane 2: without treatment, Lane 3: treatment with 100 μ M of cationic liposomes, Lane 4: treatment with 100 μ M of cationic liposomes followed by incubation with 10 μ M of edaravone.

which is subsequently oxidized by ROS to form the highly fluorescent product DCF. RAW264.7 cells were treated with cationic liposomes in the presence or absence of edaravone for 24 h followed by DCFH-DA staining. Our results indicate

that ROS fluorescent staining was detected in RAW264.7 cells treated with cationic liposomes alone (Fig. 3), while edaravone co-treatment eliminated the stimulated ROS generation in the cells.

Effect of Edaravone on Cationic Liposomes-Mediated Apoptosis The apoptotic process is intimately connected to orderly DNA fragmentation, in which the key apoptotic proteases (caspases) initiate specific DNA cleavage.³⁷⁾ To verify the protective effect of edaravone on cationic lipids-mediated apoptosis, we assessed the patterns of DNA fragmentation in RAW264.7 cells following the treatment of cationic liposomes with and without edaravone. As shown in Fig. 4, DNA fragmentation was clearly observed when the cells were treated with cationic liposomes for 3 h followed by incubation without edaravone for 24 h. As expected, the incubation with edaravone for 24 h inhibited the DNA fragmentation, revealing the protective effect of edaravone against cationic lipids-mediated apoptosis. It should be noted that DNA fragmentation was not observed when the cells were treated with cationic liposomes for 3 h without additional incubation for 24 h (Fig. S2) suggesting that ROS-induced apoptosis has minimal contribution to early cytotoxicity, but rather occurs slowly over time.

CONCLUSION

In this study, we showed that the long-term treatment of edaravone could suppress the cytotoxicity by cationic liposomes containing DOTMA *in vitro*. Further, the improvement of cell viability was attributed to the elimination of ROS produced by cationic liposomes-stimulated cells and inhibition of ROS-induced apoptosis, rather than to necrosis due to acute cytotoxicity. Cationic lipid-mediated cytotoxicity has

been reported to vary with physicochemical changes due to the incorporation of nucleic acids in cationic liposomes.^{38,39)} These factors may affect the protective effect of edaravone. In order to allow a more comprehensive understanding of the mechanism, further studies on the protective effect of edaravone against cationic lipid-mediated toxicities are needed to determine whether edaravone can demonstrate the significant effect *in vivo*. The present study showed the usefulness of the concomitant administration with edaravone for the therapeutic use of cationic liposomes, and it is expected to become a starting point for the clinical application.

Acknowledgments This project was partially funded by Mitsubishi Tanabe Pharma Corporation. PRC is funded by a Canadian Institutes of Health Research Foundation grant (FDN 148469). PRC is also the CEO and Scientific Director of the NanoMedicines Innovation Network (NMIN), a National Centers of Excellence of Canada (NCE). JAK is supported by a NMIN Postdoctoral Award in Gene Therapy.

Author Contributions The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

REFERENCES

- Thomas CE, Ehrhardt A, Kay MA. Progress and problems with the use of viral vectors for gene therapy. *Nat. Rev. Genet.*, **4**, 346–358 (2003).
- Baum C, Kustikova O, Modlich U, Li Z, Fehse B. Mutagenesis and oncogenesis by chromosomal insertion of gene transfer vectors. *Hum. Gene Ther.*, **17**, 253–263 (2006).
- Bessis N, GarciaCozar FJ, Boissier MC. Immune responses to gene therapy vectors: influence on vector function and effector mechanisms. *Gene Ther.*, **11** (Suppl. 1), S10–S17 (2004).
- Wachler R, Russell SJ, Curiel DT. Engineering targeted viral vectors for gene therapy. *Nat. Rev. Genet.*, **8**, 573–587 (2007).
- Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM, Danielsen M. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 7413–7417 (1987).
- Wang Y, Miao L, Satterlee A, Huang L. Delivery of oligonucleotides with lipid nanoparticles. *Adv. Drug Deliv. Rev.*, **87**, 68–80 (2015).
- Lv H, Zhang S, Wang B, Cui S, Yan J. Toxicity of cationic lipids and cationic polymers in gene delivery. *J. Control. Release*, **114**, 100–109 (2006).
- Hortobagyi GN, Ueno NT, Xia W, *et al.* Cationic liposome-mediated E1A gene transfer to human breast and ovarian cancer cells and its biologic effects: a phase I clinical trial. *J. Clin. Oncol.*, **19**, 3422–3433 (2001).
- Zhang JS, Liu F, Huang L. Implications of pharmacokinetic behavior of lipoplex for its inflammatory toxicity. *Adv. Drug Deliv. Rev.*, **57**, 689–698 (2005).
- Dass CR. Lipoplex-mediated delivery of nucleic acids: factors affecting *in vivo* transfection. *J. Mol. Med. (Berl)*, **82**, 579–591 (2004).
- Tousignant JD, Gates AL, Ingram LA, Johnson CL, Nietupski JB, Cheng SH, Eastman SJ, Scheule RK. Comprehensive analysis of the acute toxicities induced by systemic administration of cationic lipid:plasmid DNA complexes in mice. *Hum. Gene Ther.*, **11**, 2493–2513 (2000).
- Cullis PR, Hope MJ. Lipid nanoparticle systems for enabling gene therapies. *Mol. Ther.*, **25**, 1467–1475 (2017).
- Yun CH, Bae CS, Ahn T. Cargo-free nanoparticles containing cationic lipids induce reactive oxygen species and cell death in HepG2 Cells. *Biol. Pharm. Bull.*, **39**, 1338–1346 (2016).
- Mikhed Y, Gorlach A, Knaus UG, Daiber A. Redox regulation of genome stability by effects on gene expression, epigenetic pathways and DNA damage/repair. *Redox Biol.*, **5**, 275–289 (2015).
- Chen W, Yan W, Huang L. A simple but effective cancer vaccine consisting of an antigen and a cationic lipid. *Cancer Immunol. Immunother.*, **57**, 517–530 (2008).
- Glasauer A, Chandel NS. Targeting antioxidants for cancer therapy. *Biochem. Pharmacol.*, **92**, 90–101 (2014).
- Jiao L, Zhang J, Li Z, Liu H, Chen Y, Xu S. Edaravone alleviates delayed neuronal death and long-dated cognitive dysfunction of hippocampus after transient focal ischemia in wistar rat brains. *Neuroscience*, **182**, 177–183 (2011).
- Higashi Y, Jitsuiki D, Chayama K, Yoshizumi M. Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one), a novel free radical scavenger, for treatment of cardiovascular diseases. *Recent Pat. Cardiovasc. Drug Discov.*, **1**, 85–93 (2006).
- Yun CH, Bae CS, Ahn T. Cargo-free nanoparticles containing cationic lipids induce reactive oxygen species and cell death in HepG2 cells. *Biol. Pharm. Bull.*, **39**, 1338–1346 (2016).
- Belliveau NM, Huft J, Lin PJ, Chen S, Leung AK, Leaver TJ, Wild AW, Lee JB, Taylor RJ, Tam YK, Hansen CL, Cullis PR. Microfluidic synthesis of highly potent limit-size lipid nanoparticles for *in vivo* delivery of siRNA. *Mol. Ther. Nucleic Acids*, **1**, e37 (2012).
- Leung AK, Hafez IM, Baoukina S, Belliveau NM, Zhigaltsev IV, Afshinmanesh E, Tieleman DP, Hansen CL, Hope MJ, Cullis PR. Lipid nanoparticles containing siRNA synthesized by microfluidic mixing exhibit an electron-dense nanostructured core. *J. Phys. Chem. C. Nanomater. Interfaces*, **116**, 18440–18450 (2012).
- Chen S, Zaifman J, Kulkarni JA, Zhigaltsev IV, Tam YK, Ciufolini MA, Tam YYC, Cullis PR. Dexamethasone prodrugs as potent suppressors of the immunostimulatory effects of lipid nanoparticle formulations of nucleic acids. *J. Control. Release*, **286**, 46–54 (2018).
- Tam YYC, Chen S, Zaifman J, Tam YK, Lin PJ, Ansell S, Roberge M, Ciufolini MA, Cullis PR. Small molecule ligands for enhanced intracellular delivery of lipid nanoparticle formulations of siRNA. *Nanomedicine*, **9**, 665–674 (2013).
- Wang L, Hirayasu K, Ishizawa M, Kobayashi Y. Purification of genomic DNA from human whole blood by isopropanol-fractionation with concentrated NaI and SDS. *Nucleic Acids Res.*, **22**, 1774–1775 (1994).
- Gong J, Traganos F, Darzynkiewicz Z. A selective procedure for DNA extraction from apoptotic cells applicable for gel electrophoresis and flow cytometry. *Anal. Biochem.*, **218**, 314–319 (1994).
- Watanabe K, Tanaka M, Yuki S, Hirai M, Yamamoto Y. How is edaravone effective against acute ischemic stroke and amyotrophic lateral sclerosis? *J. Clin. Biochem. Nutr.*, **62**, 20–38 (2018).
- Wang S, Fumoto S, Miyamoto H, Tanaka M, Nishida K. Edaravone, a cytoprotective drug, enhances transgene expression mediated by lipoplexes in HepG2 cells and mice. *Int. J. Pharm.*, **548**, 173–181 (2018).
- Iwaoka S, Nakamura T, Takano S, Tsuchiya S, Aramaki Y. Cationic liposomes induce apoptosis through p38 MAP kinase-caspase-8-Bid pathway in macrophage-like RAW264.7 cells. *J. Leukoc. Biol.*, **79**, 184–191 (2006).
- Hasegawa M, Yagi K, Iwakawa S, Hirai M. Chitosan induces apoptosis via caspase-3 activation in bladder tumor cells. *Jpn. J. Can-*

- cer Res.*, **92**, 459–466 (2001).
- 30) Shokrzadeh M, Shaki F, Mohammadi E, Rezagholizadeh N, Ebrahimi F. Edaravone decreases paraquat toxicity in A549 cells and lung isolated mitochondria. *Iran. J. Pharm. Res.*, **13**, 675–681 (2014).
- 31) Li Y, Liu H, Zeng W, Wei J. Edaravone protects against hyperosmolarity-induced oxidative stress and apoptosis in primary human corneal epithelial cells. *PLOS ONE*, **12**, e0174437 (2017).
- 32) Cao B, Chai C, Zhao S. Protective effect of edaravone against hypoxia-induced cytotoxicity in osteoblasts MC3T3-E1 cells. *IUBMB Life*, **67**, 928–933 (2015).
- 33) Wei X, Shao B, He Z, *et al.* Cationic nanocarriers induce cell necrosis through impairment of Na(+)/K(+)-ATPase and cause subsequent inflammatory response. *Cell Res.*, **25**, 237–253 (2015).
- 34) Fan Y, Wu W, Lei Y, Gaucher C, Pei S, Zhang J, Xia X. Edaravone-loaded alginate-based nanocomposite hydrogel accelerated chronic wound healing in diabetic mice. *Mar. Drugs*, **17**, 285 (2019).
- 35) Hironaka K, Inokuchi Y, Fujisawa T, Shimazaki H, Akane M, Tozuka Y, Tsuruma K, Shimazawa M, Hara H, Takeuchi H. Edaravone-loaded liposomes for retinal protection against oxidative stress-induced retinal damage. *Eur. J. Pharm. Biopharm.*, **79**, 119–125 (2011).
- 36) Shimazaki H, Hironaka K, Fujisawa T, Tsuruma K, Tozuka Y, Shimazawa M, Takeuchi H, Hara H. Edaravone-loaded liposome eyedrops protect against light-induced retinal damage in mice. *Invest. Ophthalmol. Vis. Sci.*, **52**, 7289–7297 (2011).
- 37) Didenko VV, Ngo H, Baskin DS. Early necrotic DNA degradation: presence of blunt-ended DNA breaks, 3' and 5' overhangs in apoptosis, but only 5' overhangs in early necrosis. *Am. J. Pathol.*, **162**, 1571–1578 (2003).
- 38) Romøren K, Thu BJ, Bols NC, Evensen Ø. Transfection efficiency and cytotoxicity of cationic liposomes in salmonid cell lines of hepatocyte and macrophage origin. *Biochim. Biophys. Acta*, **1663**, 127–134 (2004).
- 39) Sakurai F, Inoue R, Nishino Y, Okuda A, Matsumoto O, Taga T, Yamashita F, Takakura Y, Hashida M. Effect of DNA/liposome mixing ratio on the physicochemical characteristics, cellular uptake and intracellular trafficking of plasmid DNA/cationic liposome complexes and subsequent gene expression. *J. Control. Release*, **66**, 255–269 (2000).