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Research paper

Edaravone, a Cytoprotective Drug, Enhances Transgene Expression Mediated by Lipoplexes in HepG2 Cells and Mice

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ABBREVIATIONS:

ALT, alanine transaminase; AST, aspartate transaminase; ATRA, all-*trans* retinoic acid; DMEM, Dulbecco's modified Eagle's medium; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; EDLP, edaravone-loaded liposome; EE, encapsulation efficiency; FBS, fetal bovine serum; Lipoplex, cationic liposome/pDNA complex; NAC, *N*-acetylcysteine; PBN, *N*-*tert*-butyl-α-phenylnitrone; PDI, polydispersity index; pDNA, plasmid DNA; PEI, polyethyleneimine; PI, propidium iodide; RLU, relative light unit; ROS, reactive oxygen species; TE, transfection efficiency

Abstract

A requirement of gene therapy is efficient nucleic acid delivery. However, the application of cationic liposomes to gene therapy is restricted by their inefficient transfection capacity, which may be caused by cytotoxicity. This cytotoxicity is highly dependent on cationic lipid-induced reactive oxygen species (ROS). Here, to provide cellular protection, we used edaravone, an efficacious anti-oxidative drug, to scavenge ROS during transfection using cationic liposome/plasmid DNA complexes (lipoplexes). Both free edaravone and edaravone-loaded liposomes (EDLPs) enhanced transgene expression in the human hepatoma cell line, HepG2, while EDLPs decreased the effective dose of edaravone. The cellular protective effect of edaravone was found to decrease the cytotoxicity of cationic liposomes. Edaravone was also effective in the commercial product, Lipofectamine® 3000, which may expand the application of edaravone to promote transfection efficiency. Compared with free edaravone, EDLPs also showed superior transgene expression in mice. Our findings will promote the development of efficient and safe gene therapy.

Keywords:

Edaravone; Reactive oxygen species; Transfection; Cytoprotection; Cationic liposomes; Non-viral vector

1. Introduction

Gene therapy is a medical approach with great promise, especially for genetic disorders and cancers, because it is difficult to develop chemical pharmacotherapies for genetic disorders. For gene therapy, the development of effective and safe nucleic acid delivery systems is required. Non-viral vectors are safer platforms than viral vectors because of lower immunogenicity and more reproducibility and because of easy processing and chemical modification. Among non-viral vectors, cationic liposomes can be safely prepared with biocompatibility and biodegradability (Huang et al., 2014). Therefore, cationic liposome-mediated transfection has great potential for clinical use.

Compared with viral vectors, transfection efficiency (TE) of cationic liposomes is low and clinically insufficient. One reason for this is reactive oxygen species (ROS) generation, which induces cytotoxicity (Yun et al., 2016) and affects intracellular signaling pathways (Mikhed et al., 2015). It has been suggested that the cytotoxicity of cationic carriers, especially 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)-based cationic liposomes, is caused by ROS generation. The high surface density of positive charges on cationic liposomes is critical for ROS generation (Chen et al., 2008). 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 (Glasauer and Chandel, 2014). Moreover, ROS regulate cell differentiation, proliferation, migration, and apoptosis. In addition, they affect classical regulation of gene expression, including the stability of mRNAs and their transport in the cytosol (Adcock et al., 2004).

Cationic liposomes loaded with nucleic acids enter tumor cells and release therapeutic genes mostly via endocytosis (Rejman et al., 2005). During endocytosis, there are two possible sites that trigger ROS generation, namely where cationic

liposomes interact with cytoplasmic and endosomal membranes (Yan et al., 2008). If nanoparticles are degraded in lysosomes, instead of transferring therapeutic genes to nuclei for gene transfection, ROS generation is also triggered (Yan et al., 2008). Although the amount of ROS generated by cationic liposomes is limited, they may trigger ROS production in mitochondria by the activity of NADPH (nicotinamide-adenine dinucleotide phosphate, reduced form) oxidase or lipoxygenase (Yan et al., 2008). Mitochondrial dysfunction caused by cationic liposomes has been shown previously (Roursgaard et al., 2016), which also aggravates cellular damage. Such cytotoxicity has even been used for cancer therapy by cargo-free cationic liposomes (Yun et al., 2016).

As a free radical scavenger and cytoprotective drug, edaravone has been widely used for neurological recovery following acute brain ischemia and subsequent cerebral infarction by reducing ROS and inhibiting apoptosis (Kikuchi et al., 2012). Because the specific ROS generated by cationic liposomes are still unclear, a strategy to scavenge both hydrophilic and lipophilic ROS is reasonable. As an amphiphilic compound, edaravone has a similar ability as α-tocopherol for lipid hydroperoxide radical scavenging and with ascorbic acid for inhibiting hydro-soluble peroxide radicals (Abe et al., 2004). Indeed, edaravone protects HT22 cells from H_2O_2-induced injury by inhibiting the production of ROS and activation of the mitogen-activated protein kinase signaling pathway (Zhao et al., 2013). Moreover, edaravone protects osteoblastic cells/osteoblasts through its ROS-scavenging capacity by repressing dexamethasone-induced opening of mitochondrial permeability transition pores and reduction of the mitochondrial membrane potential (Sun et al., 2015). We hypothesized that the anti-oxidative effects of edaravone might regulate oxidative stress during transfection to prevent cellular damage and improve TE.

In the present study, we investigated the effects of edaravone on cationic liposome-induced gene transfection. According to our results, the addition of free edaravone or edaravone-loaded liposomes (EDLPs) increased TE several fold in human hepatoma HepG2 cells and mice. This enhancement by edaravone was dose-dependent. Because ROS generation-mediated cellular damage cannot be avoided during cationic lipid-based gene transfection, edaravone may be useful to protect cells from harmful ROS, thereby enhancing transgene expression.

2. Materials and methods

2.1. Materials and reagents

DOTAP chloride was obtained from NOF Corporation (Tokyo, Japan). Edaravone was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). CellROX™ Deep Red was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Propidium iodide (PI) was obtained from Wako Pure Chemical Industries (Osaka, Japan). All organic solvents of analytical grade were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cholesterol and other inorganic chemicals were obtained from Nacalai Tesque (Kyoto, Japan). Purified water was prepared using a Direct-Q UV (Merck Millipore, Merck KGaA, Darmstadt, Germany). All cell culture media were purchased from Thermo Fisher Scientific.

2.2. Preparation of plasmid DNA (pDNA)

Amplification of pDNAs pCpGfree-lucia (InvivoGen, CA, USA), pZsGreen1-N1 (Clontech, Shiga, Japan) and pCMV-luciferase [constructed previously (Fumoto et al., 2016)], was performed using Escherichia coli strains GT115, DH5α and DH5α, respectively. Isolation and purification were carried out using an EndoFree® Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). Purified pDNA was dissolved in 5% glucose and stored at -20°C.

2.3. Preparation of cationic liposomes and EDLPs

The calcium acetate gradient method (Hironaka et al., 2011)was chosen to prepare EDLPs because edaravone is partially hydro-soluble. Cationic liposomes consisting of DOTAP and cholesterol (8:1 molar ratio) were prepared by a thin lipid film hydration method with 120 mM calcium acetate to produce a 16 mg/ml lipid dispersion using a

vortex mixer. After hydration, submicron-sized liposomes were prepared using a commercially available instrument (Mini-Extruder; Avanti Polar Lipids, Inc., AL, USA) with a size-controlled polycarbonate membrane (0.1 μm pore size). The liposomes were dialyzed in Hank's balanced salt solution with 0.01 M 2-morpholinoethanesulfonic acid (pH 6.0) at 4°C to create a gradient. Some of the liposomes were used as plain cationic liposomes. Remote loading was performed by co-incubation with edaravone solution to prepare EDLPs at 37°C for 10 min.

2.4. Characterization of EDLPs

To determine the encapsulation efficiency (EE) of edaravone in EDLPs, free edaravone was separated by centrifugal ultrafiltration at 5,000 × g for 25 min at 4°C (molecular weight cut-off: 10,000; Merck Millipore). To measure the total amount of edaravone, 50 μl of the EDLP dispersion was added to 5 ml mobile phase, sonicated for 5 min, and then centrifuged at 12,000 × g for 10 min before analysis. According to Japan Pharmacopeia (JP17), the edaravone content was analyzed by high performance liquid chromatography with a UV detector (SPD-10A, Shimadzu, Kyoto, Japan) under the following conditions: C18 column (Cosmosil-Pak; 4.6 × 150 mm; particle diameter: 5 μm) with methanol, water, and acetic acid (100:100:1) as the mobile phase, at a wavelength of 240 nm and 25°C. The total run time was 15 min. The EE of EDLPs was calculated by the following equation:

$$\mathrm{EE}\ \% = \frac{(E_{total} - E_{free})}{E_{total}} \times 100\ \%,$$

where E_{total} is the total edaravone amount in EDLPs and E_{free} is the free edaravone amount in the EDLP dispersion.

2.5. Preparation of lipoplexes

The lipoplexes were prepared with equal volumes of pDNA and cationic liposomes, then incubated at 37°C for 30 min with a charge ratio (molar ratio of cationic lipids to pDNA phosphate residues) of 3.5. For *in vitro* experiments, we used pCpGfree-lucia encoding the secretable form of the synthetic luciferase gene. For *in vivo* experiments, pCMV-luciferase encoding the non-secretable form of the firefly luciferase gene was chosen. The particle size and ζ potential of liposomes and lipoplexes at 25°C were measured using a Zetasizer Nano ZS (Malvern, Worcestershire, UK).

2.6. Cell culture

HepG2 cells were obtained from RIKEN (Tokyo, Japan). The cells were grown under standard conditions in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin G (100 U)/ streptomycin (100 μg/ml) in a humidified atmosphere with 5% CO_2 at 37°C.

2.7. *In vitro* TE measurement

HepG2 cells were seeded into 24-well plates at a density of 4×10^4 cells/cm^2 in 0.5 ml medium and cultured for 24 h. The medium was replaced with FBS-free DMEM containing edaravone, EDLPs, or H_2O_2 with lipoplexes (1 μg/well pCpGfree-lucia). After transfection for 4 h, the cells were washed with phosphate buffered saline (PBS; pH 7.4) and then incubated with 10% FBS/DMEM at 37°C for another 4 h. TE in cells was assessed by mixing 5 μl medium with 100 μl substrate (Renilla Luciferase Assay System containing luciferase substrate coelenterazine; Promega, USA) and immediate measurement of the bioluminescence level by a luminometer (Lumat LB 9507; Berthold Technologies, Bad Wildbad, Germany). Luciferase activity is indicated as relative light units (RLU)/ml of medium.

2.8. Flow cytometric analysis

HepG2 cells were seeded into 24-well plates at a density of 4×10^4 cells/cm^2 in 0.5 ml medium and cultured for 24 h. The medium was replaced with FBS-free DMEM containing lipoplexes at 1 μg/well pZsGreen1-N1 with or without edaravone (100 μM). After transfection for 4 h, the cells were incubated with CellROX™ Deep Red for 30 min according to the manufacturer's protocol. The cells were washed with PBS three times and then incubated with 10% FBS/DMEM at 37°C for another 12 h. The cells were harvested and centrifuged at 1,500 ×g for 5 min at 4°C. After washing twice with PBS containing 5% FBS, cells were re-suspended in the same solution and analyzed by flow cytometry (LSRFortessa X-20, BD Biosciences, CA, USA).

2.9. Gene transfection by polyethyleneimine (PEI) and Lipofectamine® 3000

Similar to the conditions for cationic liposomes, HepG2 cells were seeded into 24-well plates at a density of 4×10^4 cells/cm^2 in 0.5 ml medium and pre-incubated for 24 h. Medium containing 1 μg/well pCpGfree-lucia/PEI polyplexes (PEI HCl MAX, linear, molecular weight 40k (22k in free base), Polysciences, Inc., PA, USA) with an N/P ratio (the ratio of moles of the amine groups of PEI to those of the phosphate groups of pDNA) of 10 with or without free edaravone was incubated for 4 h, and then washed and transferred to normal DMEM and incubated for another 4 h. The process for Lipofectamine® 3000 (Thermo Fisher Scientific)-mediated transfection was similar to that for PEI and followed the manufacturer's protocol. TE in cells was assessed by mixing the medium and substrates as described above, and then measuring the bioluminescence level using a luminometer (Lumat LB 9507; Berthold Technologies). Luciferase activity is also indicated as RLU/ml of medium.

2.10. Cell metabolic activity, cell death, and TE

For evaluation of cell metabolic activity, cells were seeded in 96-well plates at a density of 3.6×10^4 cells/cm^2 and pre-incubated for 24 h. Free edaravone or lipoplexes with or without 100 μM edaravone were added to the wells, followed by incubation for 4 h. After incubation for a further 4 h in drug-free DMEM, the medium was collected for TE measurement. TE was measured as described in section **2.7**. After collection of medium, each well was washed with PBS, then 100 μl DMEM containing 10 μl of cell counting kit-8 (CCK-8; Dojindo, Kumamoto, Japan) was added and the cells incubated for 1 h. The absorbance was measured at 450 nm using a microplate photometer (Multiskan™ FC; Thermo Fisher Scientific). Blank wells and untreated cells served as negative and positive controls, respectively.

For cell death measurement, HepG2 cells were seeded into 24-well plates at a density of 4×10^4 cells/cm^2 in 0.5 ml medium and cultured for 24 h. Medium containing lipoplexes at different lipid concentrations with or without 100 μM edaravone was added to the wells, followed by incubation for 4 h. The cells were washed with PBS, and then incubated with 10% FBS/DMEM at 37°C for another 4 h. Cells were harvested and centrifuged at 1,500 ×g for 5 min at 4°C. Then, cells were washed twice and re-suspended in 5% FBS in PBS. After incubation with PI (0.5 μg/ml) for 5 min at room temperature, the cells were analyzed using a Tali™ Image-Based Cytometer (Thermo Fisher Scientific).

2.11. Animals

Five-week-old male ddY mice (Japan SLC, Inc., Shizuoka, Japan) weighing 25-27 g were used in experiments and fed a standard laboratory diet in air-conditioned

chambers at a constant temperature and humidity. All experiments were approved by the Institutional Animal Care and Use Committee of Nagasaki University.

2.12. *In vivo* gene transfer

To measure *in vivo* TE, lipoplexes with various edaravone dosages were administered via the tail vein of mice. Ninety micrograms of pCMV-luciferase in 150 µl of a 5% glucose solution was mixed with the same volume of cationic liposomes and incubated at 37°C for 30 min with a charge ratio of 3.5. Then, 25 µl of the various dosages of EDLPs (8 µg/µl) was added and mixed well. Administration of the complexes was carried out through the tail vein. The measurements were finished after 6 h, followed by immediate euthanasia of the animals. Lungs and livers were dissected, washed with saline, and then homogenized on ice after adding 4 µl/mg tissue lysis buffer (0.1 M Tris-HCl, pH 7.8, 0.05% Triton X-100, 2 mM EDTA) (Fumoto et al., 2016). After the homogenates were centrifuged at 15,000 ×*g* for 5 min at 4°C, 20 µl of tissue homogenate supernatant was mixed with 100 µl luciferase assay substrate (PicaGene®, Toyo Ink Mfg. Co., Ltd., Tokyo, Japan). The light produced was measured using a luminometer (Lumat LB 9507; Berthold Technologies). The luciferase activity is represented as RLU/g of tissue.

2.13. Measurement of serum transaminase activities

Blood was collected from mice 24 h after intravenous injection of 5% glucose, lipoplexes, lipoplexes with 600 µg free edaravone, or EDLPs containing 30 µg edaravone. After the blood had clotted, samples were centrifuged at 15,000 ×*g* for 5 min and the supernatants were collected to obtain serum. Serum AST and ALT activities were measured with a transaminase C II test kit (Wako Pure Chemical Industries).

2.14. Statistical analysis

Statistical comparisons were performed using Student's unpaired *t*-test for comparisons of two groups, and one-way analysis of variance followed by Dunnett's post hoc test for multiple comparisons with a control group or Tukey's post hoc test for multiple comparisons among different groups. Values of $P < 0.05$ were considered to indicate statistical significance.

3. Results

3.1. Preparation and characterization of EDLPs

The ultimate goal of the present study was *in vivo* transfection. Therefore, we first formulated EDLPs. To trap edaravone in liposomes by remote loading, we decreased the cholesterol proportion relative to DOTAP from a DOTAP:cholesterol ratio of 1:1 to enhance the EE of EDLPs. The molar ratio of DOTAP to cholesterol was optimized after single factor assessments of EE, particle size, and the polydispersity index (PDI). The particle size and PDI of EDLPs before and after incubation were almost unchanged (data not shown). A molar ratio of DOTAP:cholesterol of 8:1 was selected as the optimal formulation. The highest EE of approximately 67% after incubation for 10 min at 37°C was obtained at a certain concentration of edaravone by the calcium acetate gradient method. The final edaravone concentration was 2 mg/ml. Before and after dialysis, the size, PDI, and ζ potential of liposomes were maintained at around 100 nm, 0.07, and 84 mV, respectively. After incubation with pDNA, the size doubled (209.7 ± 0.7 nm), while the ζ potential halved (45.5 ± 1.0 mV) with an increase in PDI (0.240 ± 0.003).

3.2. *In vitro* TE by free edaravone and EDLPs

As shown in Fig. 1A, 100 μM edaravone had the strongest ability to promote TE (6.1 times higher) compared with the control group. This efficiency was highly dependent on the applied dose of edaravone. Below 100 μM, the TEs were slightly increased from the control group but less effective than 100 μM. When the edaravone concentration was more than 250 μM, the TE tended to decrease with increasing concentration, especially at 1,000 μM. After liposome formation, EDLPs containing only 2 μM edaravone showed highly improved TE (7-fold) compared with the control

group (Fig. 1B). The relationship between the edaravone concentration and TE was bell-shaped.

3.3. Relationship between TE and ROS levels in cells

We analyzed the relationship between TE and cellular ROS levels by flow cytometry. The number of gene expression-positive cells was increased by co-incubation with 100 μM edaravone (Fig. 2B) compared with the control group (Fig. 2A) with a significant difference in mean fluorescence intensity (Fig. 2D). Lipoplexes enhanced cell ROS levels in comparison with the basal level (Fig. 2A, C and E). The cell ROS level was decreased by the addition of edaravone (Fig. 2A, B and E). As expected, the gene expression-positive cells in the control and edaravone groups were both centralized in the ROS-negative quadrant (Fig. 2A and B).

3.4. TE in a gradient H_2O_2 environment

To assess the effect of ROS on TE, we added H_2O_2. As shown in Fig. 3, TE decreased with increasing concentration of H_2O_2 and was sharply reduced at 1,000 μM in the absence of edaravone. Edaravone effectively enhanced TE at all cellular ROS levels, and this enhancement was very high when the H_2O_2 concentration was more than 250 μM. These results are in line with those in Fig. 1A; i.e. suppression of ROS increased TE.

3.5. Dependency of cell metabolic activity, cell death, and TE on edaravone concentration

To check the effects of edaravone itself on HepG2 cells, we measured their metabolic activity in edaravone solution without lipoplexes (Fig. 4A). Approximately

100% cell metabolic activity was observed with edaravone at less than 500 μM. Even at 1,000 μM edaravone, the metabolic activity of HepG2 cells was still >90%. However, the cytotoxicity of cationic lipoplexes was obvious in terms of cell metabolic activity (Fig. 4B). The CCK-8 assay showed a lipid concentration-dependent decrease in HepG2 cell metabolic activity, especially at concentrations of >50 μM (Fig. 4B). In Figs. 1, 2 and 3, the lipid concentration was 23.4 μM. At concentrations of 20 and 50 μM, the lipoplexes without edaravone showed slight inhibition of cell metabolic activity, indicating partial toxicity. Edaravone rescued the cells with partial toxicity. Dehydrogenase-based assays, such as the CCK-8 assay, reflect cell conditions that depend on several elements including NAD(H), NADP(H), and mitochondrial activity. Because cellular ROS levels might affect the CCK-8 assay results, we also measured cell death using PI (Fig. 4C). Cell death was low below 10 μM. Increasing lipid concentration resulted in increased cell death. Again, edaravone rescued the cells from death at moderate lipid concentrations between 20 and 50 μM. TE reached a plateau at a lipid concentration of 50 μM, at which edaravone significantly enhanced the TE (Fig. 4D). At a lipid concentration of 100 μM, cell death was obvious with or without edaravone (Fig. 4C), and there was no significant difference in TE (Fig. 4D). The concentration of edaravone (100 μM) might be insufficient at such high lipid concentration.

3.6. Effect of edaravone on transfection mediated by PEI and Lipofectamine® 3000

Because edaravone had a promising effect on the DOTAP-based non-viral transfection system, we applied edaravone to other non-viral vectors. Edaravone had a significant effect on Lipofectamine® 3000 transfection (Fig. 5A). Compared with

normal conditions, the TE of Lipofectamine® 3000 was enhanced by more than 3-fold by co-incubation with 250 μM edaravone. As a cationic lipid-based transfection system, the relationship between TE and edaravone concentration was almost identical to the former results (Fig. 1). Unfortunately, ROS scavenging by edaravone scarcely improved TE of linear PEI polyplexes with a maximum 1.6-fold increase compared with the control at 100 μM and even produced decreased TE at edaravone concentrations higher than 250 μM (Fig. 5B).

3.7. *In vivo* TE

EDLPs effectively increased TE at certain doses, especially in the 30 μg group, which demonstrated 20 times higher TE than the control group in the lungs (Fig. 6A). Such enhancement also occurred in the liver (Fig. 6B). Concerning the difference in the doses giving the highest TE between the lungs (30 μg) and liver (40 μg), the amount of EDLPs containing 40 μg edaravone in the lungs may have been saturated, providing more edaravone for the liver than the 30 μg dose to scavenge ROS. Similar to the *in vitro* conditions, the dose-dependency of edaravone was also observed to be bell-shaped in mice. Furthermore, pulmonary TE was several times higher than hepatic TE in all mice because cationic liposomes were mainly trapped in the lungs. Free edaravone solution with a similar dose (30 μg) did not increase TE in either organ (Fig. 6C and D). Increasing the dose of free edaravone to 270 μg and 600 μg slightly improved TE in the lungs. For the liver, only 270 μg increased TE efficiency, indicating that 600 μg of edaravone might be an overdose as it produced no TE enhancement.

3.8. The effect of edaravone on cationic lipoplex-induced hepatic toxicity

To assess the cytoprotective effects of edaravone *in vivo*, we measured serum AST

and ALT levels as markers of hepatic toxicity. Serum AST and ALT levels in the vehicle group were 35.5 ± 9.3 and 18.0 ± 8.2 IU/L, respectively. These levels were greatly increased 24 h after lipoplex injection compared with the vehicle group (Fig. 7). This
375 indicated that the lipoplex treatment induced acute hepatic injury. The addition of free edaravone or EDLPs effectively decreased the serum transaminase activity levels. EDLPs with only 30 μg edaravone protected against acute hepatic toxicity to a similar degree as 600 μg free edaravone.

4. Discussion

The positive charge of carriers in non-viral transfection is a double-edged sword. It promotes cellular uptake and endosome escape for gene transfer, but generates harmful ROS during membrane fusion in these processes (Yan et al., 2008). Several strategies can decrease ROS production and increase TE. For example, acetylation of branched PEI reduces ROS production (Calarco et al., 2013), L-carnosine (antioxidant) (Dey et al., 2011) and α-lipoic acid (Dharmalingam et al., 2017) conjugations eliminate ROS produced by cationic materials. In this study, direct use of an antioxidant, rather than synthetic modification, simplifies the system and we show that the antioxidant, edaravone, improves cationic lipid-mediated transfection in HepG2 cells (Fig. 1, 2, 4 and 5) and mice (Fig. 6).

To choose the best anti-oxidant for TE enhancement, we tested six antioxidants, i.e., edaravone, all-trans retinoic acid (ATRA), α-tocopherol, ascorbic acid, *N*-acetylcysteine (NAC) and *N*-*tert*-butyl-α-phenylnitrone (PBN) (Fig. 1A and supplementary Fig. S1). Edaravone produced the strongest enhancement of TE among the tested anti-oxidants. Partition coefficient (LogP) is critical for cellular uptake and intracellular disposition. Edaravone, an amphiphilic compound with a moderate LogP value (supplementary Table S1), has a similar ability as α-tocopherol to inhibit lipid peroxide radicals and a similar ability as ascorbic acid to inhibit hydro-soluble peroxide radicals (Abe et al., 2004). Although PBN has a similar LogP value (supplementary Table S1) and a similar TE-enhancing ability as edaravone (Fig. 1A and supplementary Fig. S1), edaravone is effective at much lower concentrations than PBN. Overall, we consider that edaravone was more suitable to scavenge free radicals during transfection with lipoplexes than other anti-oxidants. We, therefore, chose edaravone for further experiments.

The concentration of edaravone was critical for TE enhancement (Fig. 1). Considering the balance of ROS levels between cellular health and damage caused by ROS generation during gene transfection, moderate reduction of ROS levels by edaravone was efficacious, as shown in Fig. 1A. Edaravone effectively decreased cellular ROS levels, which was highly enhanced by lipoplexes (Fig. 2). In addition, the population of gene expression-positive cells was significantly increased in the edaravone group (Fig. 2A, B and D). It is clear that the gene expression-positive cells of both control and edaravone groups were centralized in the ROS negative quadrant by flow cytometry (Fig. 2A and B). Furthermore, the percentage of cells with high ROS levels and without gene expression was decreased by edaravone, while the percentages of double negative and double positive quadrants were almost unchanged (Fig. 2A and B). These findings indicate that ROS generation during transfection was harmful for gene expression. Thus, the TE enhancement by edaravone was possibly attributed to scavenging of the ROS generated during transfection. However, when edaravone content was more than 250 μM there was a decrease in TE, possibly because the antioxidant capacity may reduce ROS to an extremely low level (Fig. 1A). This inverse correlation between higher concentrations of edaravone content and TE might be attributed to extreme ROS reduction that worsens the health of HepG2 cells. It has been reported that ROS reduction by antioxidant MCI-186 (edaravone) results in cell cycle arrest without a decrease in viability (Suzuki et al., 2005). In cell cycle arrest, nuclear membranes are generally intact with low permeability, thereby inhibiting transfection. Hence, retention of the cell redox balance maintains the status and condition of cells, which affects the TE.

As mentioned above, the TE enhancement by EDLPs (Fig. 1B) was also possibly attributed to the scavenging of generated ROS. EDLPs increased TE with a much lower

content of edaravone (Fig. 1A and B). Therefore, modulation of ROS levels in the microenvironment by EDLPs might be better than that in the macroenvironment by free edaravone. Because free drug can be easily metabolized and distributed to non-targeted organs, the formation of EDLPs is preferable for *in vivo* delivery. Although EDLPs could increase TE more than seven times with much lower content, the adoption of EDLPs, i.e. edaravone-loaded cationic liposomes decreased the overall TE (Fig. 1A and B). The relationship between charge ratio (CR) and gene transfection efficiency has been described as peak shaped, with an increase followed by a decrease (Elisabete G et al., 2004). In our experiment, the optimized charge ratio (3.5) was located on the anti-correlation side of the peak value (data not shown), which explains why TE was decreased by the addition of cationic lipids.

Similar to *in vitro* conditions, the TE enhancement in mice was also dose-dependent; TE increased and then decreased with the increase in edaravone dose (Fig. 6A and B). The TE decrease at higher edaravone doses was possibly caused by interruption of redox homeostasis, which induced an extreme reduction in ROS (Glasauer and Chandel, 2014). Maintenance of the redox equilibrium may be important for edaravone-mediated enhancement of *in vivo* TE. In addition, the ROS generation by cationic liposomes not only disrupted the redox equilibrium to induce toxicity but also transmitted stress signals to the immune system. Accumulation of cationic lipoplexes can cause lung toxicity expressed as pulmonary inflammation. Such responses induced by cationic liposomes and lipoplexes have been observed and characterized by significant enhancement of several proinflammatory cytokines (Xue et al., 2014). To overcome the inflammatory toxicity of cationic liposome-mediated gene transfer, the sequential injection method (Tan et al., 2001) and modified cationic lipids (Elouahabi et al., 2003) have been developed. The inhibition of inflammatory responses by these

strategies significantly enhance *in vivo* TE. ROS play a central role in inflammasome activation (Harijith et al., 2014), and the scavenging of ROS by edaravone might restrain subsequent immunogenic responses. Systemic toxicities of lipoplexes by generating ROS include hepatic injury with elevated levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (Xue et al., 2014). In this study, edaravone and EDLPs ameliorated the acute hepatic injury caused by lipoplexes (Fig. 7). This effect of edaravone might be attributed to its ability to directly scavenge for hydroxyl radicals (Zong et al., 2014). Hence, the reduction of inflammation induced by cationic lipoplexes may be another factor that contributes to enhanced TE by edaravone administration *in vivo*.

It is noteworthy that free edaravone barely improved TE in mice at the same dosage with EDLPs (Fig. 6). The lipoplexes and EDLPs may have formed a complex by electrostatic interactions with negatively charged blood proteins after intravenous injection. Five minutes after intravenous administration of EDLPs, nearly 70% of edaravone accumulated in the lungs (supplementary Fig. S2). In contrast, free edaravone injection resulted in undetectable accumulation in the lungs. Accumulation of edaravone in the liver of free and EDLPs groups was comparable. Obviously, the biodistribution properties of edaravone in EDLPs was similar to those of liposomes, which were completely different from those of the free drug. ROS generation may be dependent on the accumulated amount of cationic lipoplexes. Free edaravone, unlike EDLPs, could not efficiently accumulate in organs. Therefore, a much higher dose of free edaravone is necessary to achieve TE enhancement (Fig. 6). EDLPs could achieve the synchronous distribution of edaravone and lipoplexes, and decrease the application dose of edaravone. Moreover, EDLPs (with only 30 μg of edaravone) could decrease serum AST and ALT levels as effectively as 600 μg of free edaravone (Fig. 7). There is

another possibility that co-delivery of lipoplexes and edaravone by the same endocytosis pathways may enhance the influence of edaravone. Edaravone would be released from EDLPs at the time of membrane fusion when ROS generation may occur, followed by rapid and direct ROS scavenging.

We measured the cytotoxicity of lipoplexes with or without edaravone (Fig. 4) and established an unbalanced ROS environment model (Fig. 3). Transfection with the cationic liposomes resulted in high cell death rates at liposome concentrations of >50 μM (Fig. 4C). These results support findings from another study that showed cytotoxicity to be one of the most significant disadvantages of cationic-based transfections (Yan et al., 2008). The cellular protection by edaravone via removal of generated ROS may be critical for higher cell viability (Fig. 4B and C). The cytotoxicity of cationic liposomes can be separated into internalization (uptake)-dependent and -independent toxicities (Soenen et al., 2009). In addition, ROS generation is lipid-dose-dependent and then reaches a steady state level at concentrations higher than approximately 50-100 μM (Yun et al., 2016). Thus, in large amounts, cationic liposomes cause extreme destabilization of the plasma membrane and cell collapse, partly explaining the inefficiency of edaravone with high lipid content (100μM) (Fig. 4B, C and D). To investigate oxidative stress responses of cells and cytoprotective effects of antioxidant agents, cells are usually exposed to H_2O_2 in the culture medium. We tested the effect of an H_2O_2 concentration gradient on TE enhancement by edaravone (Fig. 3). Mammalian cell lines enter temporal growth arrest at >100 μM H_2O_2 and permanent growth arrest at >250 μM (Gulden et al., 2010). When the concentration is higher than 1,000 μM, it results in necrotic cell death (Gulden et al., 2010). Edaravone enhanced TE at all H_2O_2 concentrations, possibly because of its capacity of ROS scavenging. These results strongly indicate the effectiveness of edaravone for TE enhancement.

Results using commercial products (Fig. 5) further proved our hypothesis that anti-oxidative effects of edaravone affect lipoplex-mediated transfection (lipofection). As a cationic lipid-based transfection agent, Lipofectamine® 3000 also generates ROS during endocytosis and membrane fusion, which explained TE enhancement at certain concentrations of edaravone (Fig. 5A). However, edaravone inefficiently improved the gene expression level for PEI polyplexes (Fig. 5B). Linear PEI polyplex-based transfection is induced by the 'proton sponge' effect with barely any ROS generation even at a high concentration (Hall et al., 2015; Nguyen and Szoka, 2012; Roursgaard et al., 2016). The cytotoxicity of polyplexes is usually caused by undegradable material instead of sharply increased ROS generation and membrane instability induced by cationic lipids (Roursgaard et al., 2016). In this case, edaravone might be inefficient at preventing PEI-induced cytotoxicity. Although edaravone may only work for lipofection, it would be able to enhance TE with high safety because edaravone itself is already used clinically.

5. Conclusion

This study demonstrated that edaravone increased TE by scavenging for superfluous ROS induced by cationic liposomes. To overcome the cytotoxicity of cationic liposomes, edaravone might eliminate the ROS generated during lipofection, which cause cellular damage. It was noteworthy that formation of EDLPs decreased the efficacious edaravone dose, especially in mice. Because ROS levels are associated with TE, modulation of cellular ROS is important for gene delivery.

Acknowledgments

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Authorship contributions

Participated in research design: Wang, Fumoto, and Nishida.

Conducted experiments: Wang.

Performed data analysis: Wang, Miyamoto, and Tanaka.

Wrote or contributed to the writing of the manuscript: Wang and Fumoto.

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Figure Captions

Fig. 1. Effect of edaravone (A) and EDLPs (B) on TE in HepG2 cells. (A) Edaravone concentrations (0–1,000 μM). (B) EDLP concentrations (0–20 μM, edaravone equivalent). pCpGfree-lucia (2 μg/ml, 1 μg/well) was complexed with cationic liposomes at a charge ratio of 3.5. Free edaravone (A) or EDLPs (B) at various concentrations were mixed with lipoplexes and then transfected for 4 h. The lipid content for all groups was equalized by plain cationic liposomes (B). Luciferase activity in the medium was determined after incubation for a further 4 h. Each bar represents the mean + SD (n=3). $^{**}P < 0.01$, compared with the control.

Fig. 2. Relationship between gene expression and cell ROS levels. pZsGreen1-N1 (2 μg/ml, 1 μg/well) was complexed with cationic liposomes at a charge ratio of 3.5. (A) 0 and (B) 100 μM free edaravone. (C) Cells without lipoplexes to detect basal ROS levels. (D) TE level. (E) ROS level. The blue line represents the basal ROS level. The numbers shown on plots represent the percentage of gated cells. Representative figures of the three experiments are shown. MFI, mean fluorescence intensity. Each bar represents the mean + SD (n=3). $^{**}P < 0.01$; $^{***}P < 0.001$, compared with the control.

Fig. 3. Effect of H_2O_2 co-incubation on TE with or without edaravone. pCpGfree-lucia (2 μg/ml, 1 μg/well) was complexed with liposomes at a charge ratio of 3.5. Various amounts of H_2O_2 (0–1,000 μM) with or without 100 μM edaravone were mixed with lipoplexes and then transfected into HepG2 cells for 4 h. Luciferase activity in the

medium was determined after incubation for an additional 4 h. Each point represents the mean + SD (n=3). *P< 0.05, compared with the control.

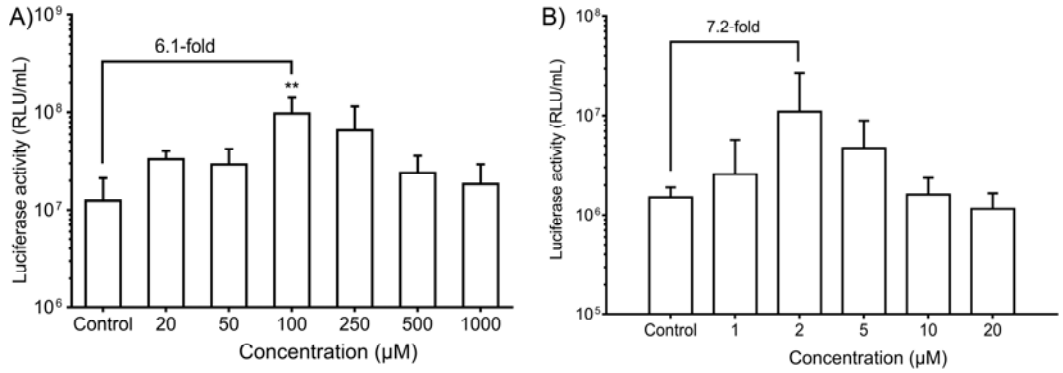
Fig. 4. Metabolic activity, cell death, and TE measurements. (A) Metabolic activity of cells treated by free edaravone (10–1,000 μM). Each bar represents the mean ± SD (n=4). There was no significant difference. Metabolic activity (B), cell death (C), and TE (D) of cells treated with lipoplexes [5–100 μM lipids, 0.43–8.6 μg/ml (0.043-0.86 μg/well for panel B and D, 0.22-4.3 μg/well for panel C, respectively) pCpGfree-lucia, charge ratio 3.5] with (filled square) or without (empty circle) 100 μM edaravone. HepG2 cells were treated with drugs for 4 h. Cell metabolic activity, cell death and TE were analyzed after incubation for an additional 4 h. Each dot represents the mean + SD (n=3). *P< 0.05, **P< 0.01.

Fig. 5. Effect of edaravone on TE mediated by Lipofectamine® 3000 (A) and PEI (B) complexes. pCpGfree-lucia (2 μg/ml, 1 μg/well) was complexed with PEI at an N/P ratio of 10, whereas lipoplexes containing Lipofectamine® 3000 were prepared following the manufacturer's protocol (1 μg/ml pCpGfree-lusia, 0.5 μg/well). Free edaravone at various concentrations was mixed with lipoplexes (A) or polyplexes (B), and then transfected into HepG2 cells for 4 h. Luciferase activity in the medium was determined after incubation for a further 4 h. Each bar represents the mean + SD (n=3). *P< 0.05, compared with the control.

Fig. 6. TE of lipoplexes in murine lungs (A, C) and livers (B, D) with various EDLP doses (A, B) and free edaravone (C, D). pCMV-luciferase (90 μg) was complexed with liposomes at a charge ratio of 3.5. Various doses of EDLPs and free edaravone were post-mixed with lipoplexes. The lipid content for all groups was equalized by plain cationic liposomes. Luciferase activity was determined at 6 h after administration. Each bar represents the mean + SD (n=3). **P*< 0.05, compared with the control.

Fig. 7. Effect of free edaravone and EDLPs on cationic lipoplex-induced hepatic toxicity. pCMV-luciferase (90 μg/mouse) was complexed with liposomes at a charge ratio of 3.5. Lipoplexes were mixed with EDLPs (30 μg edaravone) or free edaravone (600 μg). The lipid content for all groups was equalized by plain cationic liposomes. Serum transaminase activities, AST (empty bar) and ALT (filled bar), were determined 24 h after administration. Each bar represents the mean + SD (n≥3). **P*< 0.05, ***P*< 0.01 compared among groups. ## *P*< 0.01 compared with other groups.

Fig. 1


A)
6.1-fold
**
Luciferase activity (RLU/mL)
Control
20
50
100
250
500
1000
Concentration (μM)
B)
7.2-fold
Luciferase activity (RLU/mL)
Control
1
2
5
10
20
Concentration (μM)

705

Fig. 2

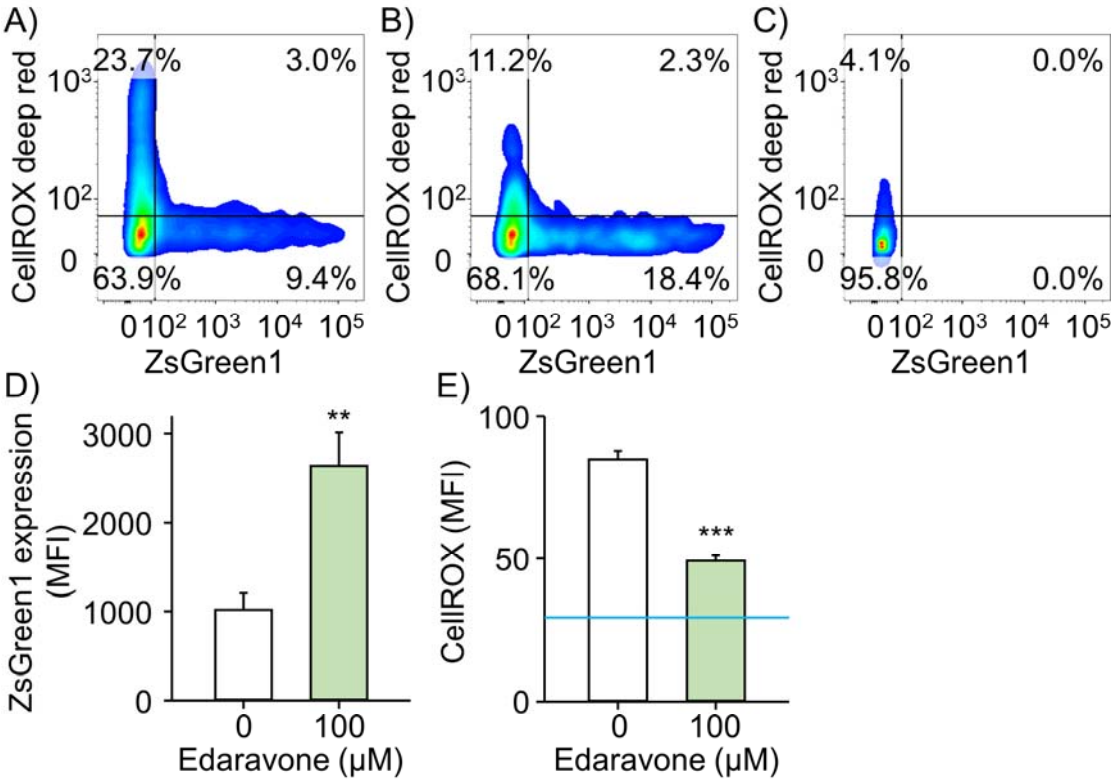
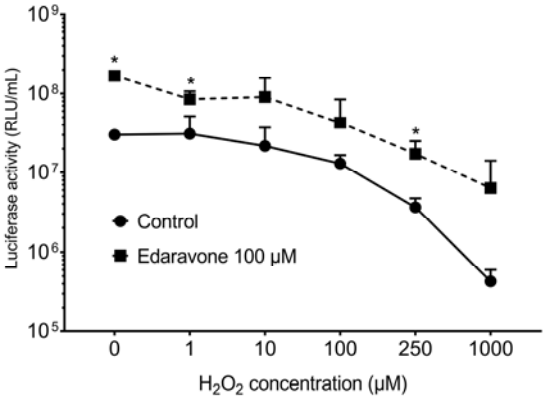
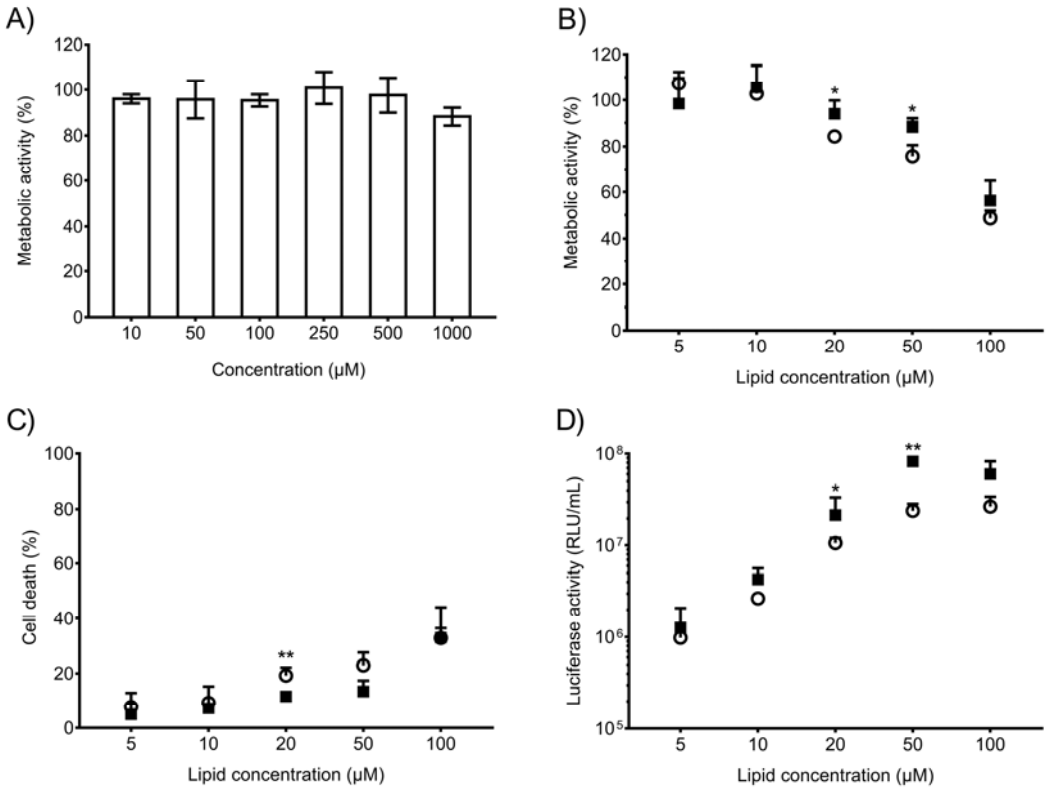

A)
B)
C)
CellROX deep red
ZsGreen1
23.7%
3.0%
63.9%
9.4%
11.2%
2.3%
68.1%
18.4%
4.1%
0.0%
95.8%
0.0%
D)
ZsGreen1 expression (MFI)
Edaravone (μM)
**
E)
CellROX (MFI)
***

Fig. 3


10^9
10^8
10^7
10^6
10^5
Luciferase acivity (RLU/mL)
*
*
*
Control
Edaravone 100 μM
0
1
10
100
250
1000
H_2O_2 concentration (μM)

710

Fig. 4


A)
Metabolic activity (%)
120
100
80
60
40
20
0
10
50
100
250
500
1000
Concentration (μM)
B)
Metabolic activity (%)
120
100
80
60
40
20
0
*
*
5
10
20
50
100
Lipid concentration (μM)
C)
Cell death (%)
100
80
60
40
20
0
**
5
10
20
50
100
Lipid concentration (μM)
D)
Luciferase activity (RLU/mL)
10^8
10^7
10^6
10^5
**
*
5
10
20
50
100
Lipid concentration (μM)

715 Fig. 5

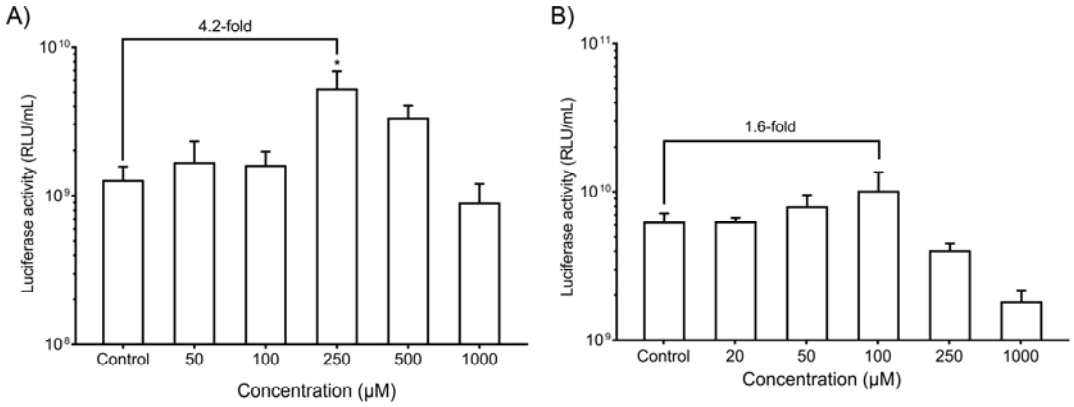
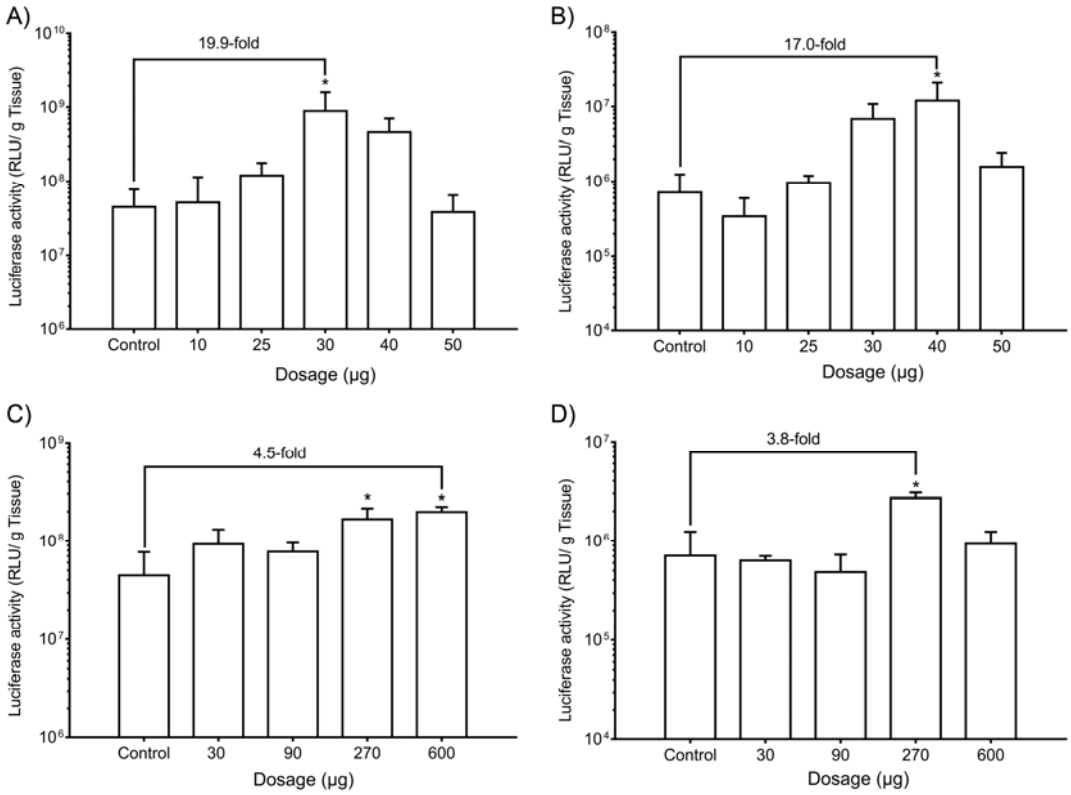
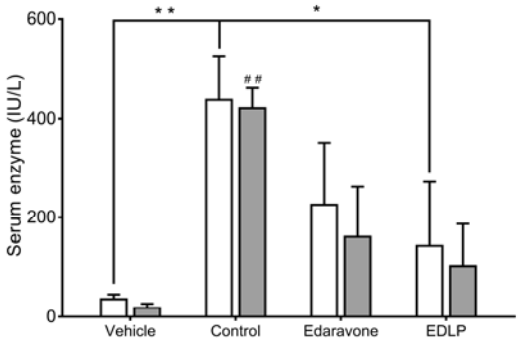

A)
4.2-fold
*
Luciferase activity (RLU/mL)
10^{10}
10^{9}
10^{8}
Control
50
100
250
500
1000
Concentration (μM)
B)
1.6-fold
Luciferase activity (RLU/mL)
10^{11}
10^{10}
10^{9}
Control
20
50
100
250
1000
Concentration (μM)

Fig. 6


A)
19.9-fold
Luciferase activity (RLU/ g Tissue)
Control
10
25
30
40
50
Dosage (μg)
B)
17.0-fold
Luciferase activity (RLU/ g Tissue)
Control
10
25
30
40
50
Dosage (μg)
C)
4.5-fold
Luciferase activity (RLU/ g Tissue)
Control
30
90
270
600
Dosage (μg)
D)
3.8-fold
Luciferase activity (RLU/ g Tissue)
Control
30
90
270
600
Dosage (μg)

720

Fig. 7


Serum enzyme (IU/L)
600
400
200
0
* *
*
##
Vehicle
Control
Edaravone
EDLP

Supplementary Data

INTERNATIONAL JOURNAL OF PHARMACEUTICS

Edaravone, a Cytoprotective Drug, Enhances Transgene Expression Mediated by Lipoplexes in HepG2 Cells and Mice

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1. Materials and methods for supplementary experiments

1.1. Materials and reagents

All-trans retinoic acid (ATRA), α-tocopherol, *N*-acetylcysteine (NAC) and ascorbic acid were obtained from Wako Pure Chemical Industries, (Osaka, Japan). *N*-tert-butyl-α-phenylnitrone (PBN) and 3-methyl-1-p-tolyl-5-pyrazolone (an internal standard, IS), were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). DiD (1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate), a lipid probe, was obtained from Thermo Fisher Scientific (Waltham, MA, USA).

All other materials were sourced as described in the main text.

1.2. Preparation of DiD-labeled cationic liposomes

DOTAP/cholesterol (8:1 molar ratio) and 0.65% DiD were dissolved in methanol, and liposomes prepared by the thin lipid film method. The lipid film was then hydrated with 5% glucose to produce 16 mg/ml liposomes using a vortex mixer. Then as described in the main text, submicron-sized liposomes were prepared using a commercially available instrument with a 0.1 μm size-controlled polycarbonate membrane. The other samples were prepared as described in the main text.

1.3. Administration of cationic liposomes, free edaravone and EDLPs

Mice were administrated samples of equal dose and formulation as the samples used in the *in vivo* transfection experiments. Samples were administered via the tail vein, and 5 min later the mice were euthanized under anesthesia. Lungs and livers were extracted, washed with saline, and then homogenized on ice after adding 4 μl/mg saline.

1.4. Sample extraction

For edaravone, 100 μl of tissue sample was spiked with 20 μl IS (175 μg/μl), 10 μl 20% formic acid, and 20 μl water (For calibration samples, water was substituted with 20 μl edaravone standard solution). The mixture was vortexed to mix, after which, 200 μl acetonitrile was added and vortex-mixed vigorously for 2 min. Then, the sample was centrifuged at 20,000 × g for 10 min. After that, 20 μl of the supernatant were injected into the HPLC system with a UV/VIS detector (SPD-10A, Shimadzu, Kyoto, Japan) for analysis.

For DiD, 100 μl of tissue sample was mixed with 900 μl methanol and vortexed for 2 min. The sample was then centrifuged at 20,000 × g for 10 min and the fluorescence intensity of the supernatant detected using a spectrofluorophotometer (RF-6000, SHIMADZU, Kyoto, Japan) with an excitation wavelength at 555 nm and an emission wavelength at 570 nm. The results were assessed using the equation:

$$\%\ dose/tissue = \frac{content\ in\ tissue}{administered\ amount} \times 100\%.$$

1.5. *In vitro* TE measurement

The cell culture conditions, experimental procedure and TE measurement were performed as described in the main text.

1.6. Partition coefficient measurement

Certain concentrations of ascorbic acid, edaravone, NAC or PBN were dissolved in 2 mL of 1-octanol-saturated PBS (0.01 M), then mixed with an equal volume of PBS-saturated 1-octanol. Samples were shaken in a 37°C water bath at 100 rpm for 24 h. The concentration alteration in the PBS phase was measured using a UV spectrophotometer (UV-1850, SHIMADZU, Kyoto, Japan).

ATRA and α-tocopherol are hydrophobic compounds; therefore, different concentrations were dissolved in 2 ml PBS (0.01 M)-saturated 1-octanol, then mixed with an equal volume of 1-octanol-saturated PBS (0.01 M) and shaken in a 37°C water bath at 100 rpm for 24 h. The concentration alteration in the 1-octanol phase was measured using a spectrofluorophotometer (RF-6000, SHIMADZU, Kyoto, Japan) with 241/488 nm and 261/325 nm as excitation/emission wavelengths for ATRA and α-tocopherol, respectively.

The results were assessed using the equation:

$$logP = log\frac{C_{1-\mathrm{octanol}}}{C_{\mathrm{PBS}}},$$

where $C_{1\text{-octanol}}$ is the concentration of the compound in 1-octanol; C_{PBS} is the compound concentration in PBS.

2. Results

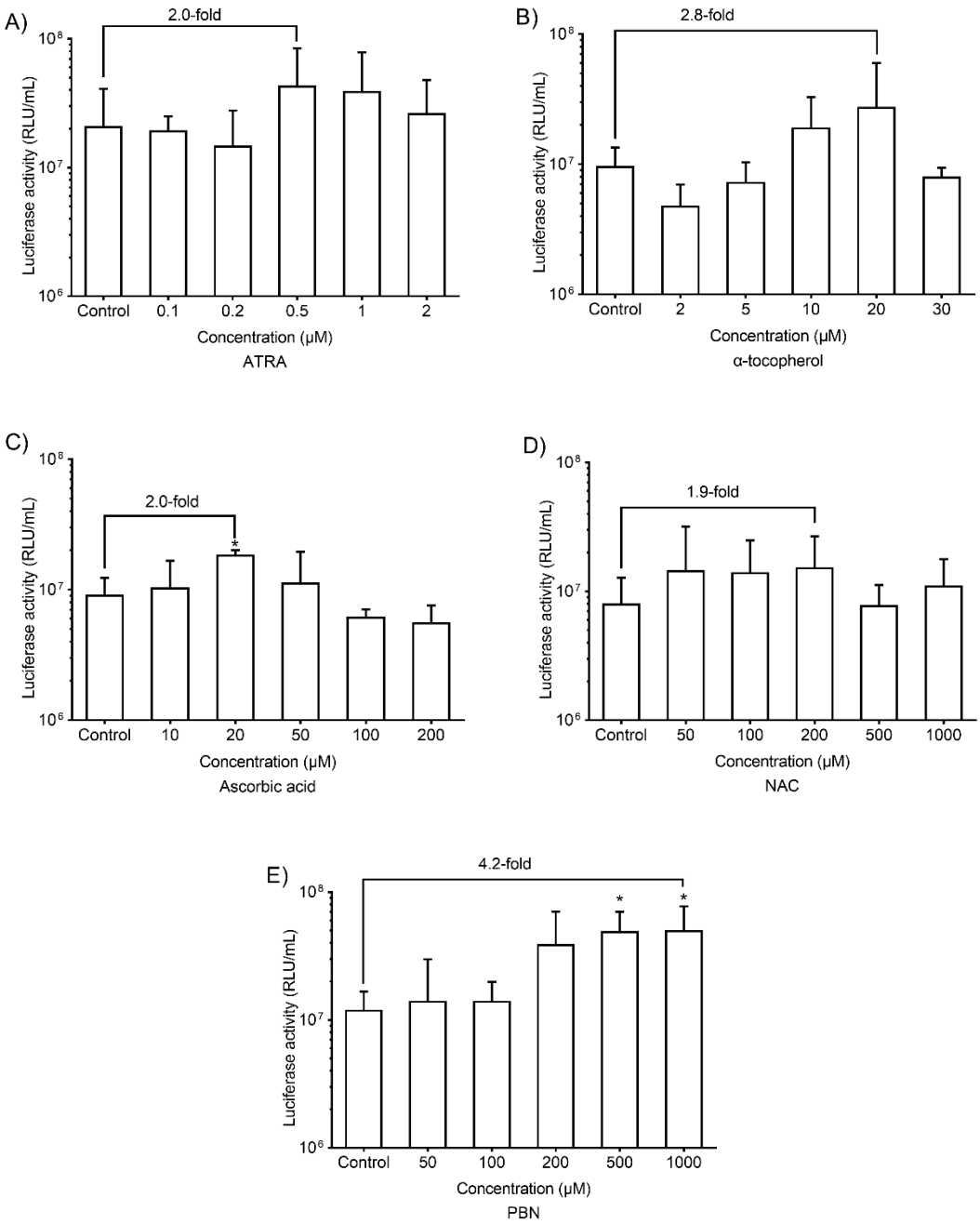

Fig. S1. Effect of ATRA (A), α-tocopherol (B), ascorbic acid (C), NAC (D) and PBN (E) on TE in HepG2 cells. (A) ATRA concentrations (0-2 μM). (B) α-tocopherol concentrations (0-30 μM). (C) Ascorbic acid concentrations (0-200 μM). (D) NAC concentrations (0-1,000 μM). (E) PBN concentrations (0-1000 μM). pDNA (2 μg/ml, 1 μg/well) was complexed with cationic liposomes at a charge ratio of 3.5. All compounds at various concentrations were mixed with lipoplexes and then transfected for 4 h. Luciferase activity in the medium was determined after incubation for a further 4 h. Each bar represents the mean + SD (n=3). **P*< 0.05, compared with the control.

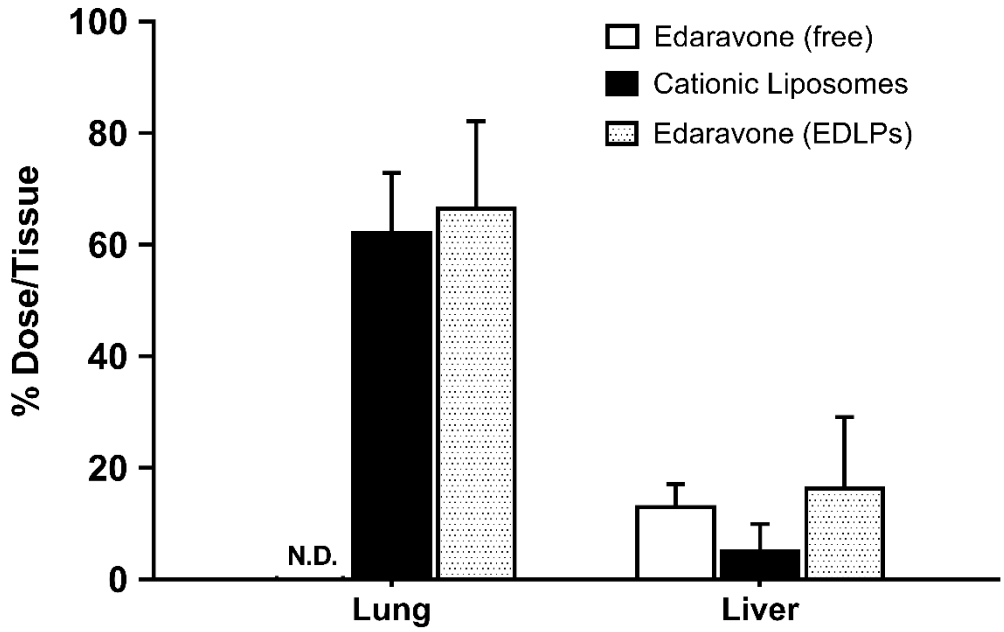

Fig. S2. Biodistribution of free edaravone, cationic liposomes and EDLPs 5 min after i.v. injection. The free edaravone group (empty bar) represents 600 μg edaravone, the cationic liposomes group (solid bar) represents lipoplexes containing plain cationic liposomes, and the EDLPs group (hatched bar) represents lipoplexes containing EDLPs (30 μg edaravone). For cationic liposomes and EDLPs, lipoplexes were prepared with a pDNA (90 μg) and liposome mixture at a charge ratio of 3.5. Liposome or edaravone content in organs was determined 5 min after administration. The results are represented as percentage of dose per tissue. Each bar represents the mean + SD (n≥3). N.D., not detected.

Table S1. Partition coefficients of anti-oxidants.

	Measured values (Log P)	Reported values (Log P)
All-*trans* retinoic acid	3.54 ± 0.35	6.3 [a, *]
α-tocopherol	4.19 ± 0.02	10 [a, *]
Ascorbic acid	-2.99 ± 0.23	-1.85 [b, *]
Edaravone	1.06 ± 0.02	1.12 [b, #]
N-acetylcysteine	-1.10 ± 0.25	-0.66 [a, *]
N-*tert*-butyl-α-phenylnitrone	1.30 ± 0.01	1.26 [b, #]

Each value represents the mean ± SD (n=3).

a: predicted value in 1-octanol/water; *b*: experimental value in 1-octanol/water.

*: PubChem, National Center for Biotechnology Information, National institutes of Health;

#: Chemistry Dashboard, United States Environmental Protection Agency.