International Journal of Pharmaceutics 548 (2018) 173–181

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Edaravone, a cytoprotective drug, enhances transgene expression mediated by lipoplexes in HepG2 cells and mice

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ARTICLE INFO

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

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.

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

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

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https://doi.org/10.1016/j.ijpharm.2018.06.068
Received 8 March 2018; Received in revised form 26 June 2018; Accepted 29 June 2018
Available online 30 June 2018
0378-5173/

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 M 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 5000 × *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:

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

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