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Co-administration of liposomal l-OHP and PEGylated TS shRNA-lipoplex: A novel approach to enhance anti-tumor efficacy and reduce the immunogenic response to RNAi molecules

Eman Alaaeldin[a,b], Amr S. Abu Lila[a,d,f], Hidenori Ando[a,c], Masakazu Fukushima[c], Cheng-Long Huang[e], Hiromi Wada[e], Hatem A. Sarhan[b], Khaled A. Khaled[b], Tatsuhiro Ishida[a,c,*]

[a] Department of Pharmacokinetics and Biopharmaceutics, Institute of Biomedical Sciences, Tokushima University, 1-78-1, Sho-machi, Tokushima 770-8505, Japan
[b] Department of Pharmaceutics, Faculty of Pharmacy, Minia University, Minia 61519, Egypt
[c] Department of Cancer Metabolism and Therapy, Institute of Biomedical Sciences,Tokushima University, 1-78-1, Sho-machi, Tokushima 770-8505, Japan
[d] Department of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmacy, Zagazig University, Zagazig 44519, Egypt
[e] Department of Thoracic Surgery, Faculty of Medicine, Kyoto University, Kyoto, Japan
[f] Department of Pharmaceutics, College of Pharmacy, Hail University, Hail 81442, Saudi Arabia

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ABSTRACT

Many therapeutic strategies have been applied in efforts to conquer the development and/or progression of cancer. The combination of chemotherapy and an RNAi-based approach has proven to be an efficient anticancer therapy. However, the feasibility of such a therapeutic strategy has been substantially restricted either by the failure to achieve the efficient delivery of RNAi molecules to tumor tissue or by the immunostimulatory response triggered by RNAi molecules. In this study, therefore, we intended to investigate the efficacy of using liposomal oxaliplatin (liposomal l-OHP) to guarantee the efficient delivery of RNAi molecules, namely shRNA against thymidylate synthase (TS shRNA) complexed with cationic liposome (TS shRNA-lipoplex), to solid tumors, and to suppress the immunostimulatory effect of RNAi molecules, TS shRNA, following intravenous administration. Herein, we describe how liposomal l-OHP enhanced the intra-tumor accumulation of TS shRNA-lipoplex and significantly reduced the immunostimulatory response triggered by TS shRNA. Consequently, such enhanced accumulation of TS shRNA-lipoplex along with the cytotoxic effect of liposomal l-OHP led to a remarkable tumor growth suppression (compared to mono-therapy) following systemic administration. Our results, therefore, may have important implications for the provision of a safer and more applicable combination therapy of RNAi molecules and anti-cancer agents that can produce a more reliable anti-tumor effect.

1. Introduction

RNA interfering molecules (RNAi) [siRNA, oligodeoxynucleotides (ODNs), microRNA, and short-hairpin RNA] are potent tools for regulating and exploring gene expression [1–3]. These agents can alter molecules upon which tumor cells depend, and act together with anticancer drugs to enhance treatment [4,5]. However, several barriers are known to restrain the full utilization of RNAi technology [6]. Potent RNAi is of no utility if it is not efficiently delivered to its target tissue. Reduced cellular uptake, short half-life, rapid systemic clearance, and RNase degradation of naked RNAi molecules, all hinder an efficient level of intra-tumor accumulation, so that achieving therapeutically adequate levels becomes a major challenge [7,8]. Cationic liposome has been complexed electrostatically with RNAi (RNAi-lipoplex) to provide enhanced cell penetration and increased protection against serum enzymes [9]. In addition, PEGylation of RNAi-containing cationic liposome (PEGylated RNAi-lipoplex) is known to prolong the circulation time of RNAi molecules via a reduction in the recognition of the lipoplex by the mononuclear phagocyte system [10,11], and to enhance PEGylated RNAi-lipoplex accumulation in solid tumors via an enhanced

Abbreviations: DC-6-14, O,O′-ditetradecanoyl-*N*-(alpha-trimethyl ammonio acetyl) diethanolamine chloride; DOPE, dioleoylphospatidyl-ethanolamine; CHOL, cholesterol; INF-γ, interferon gamma; IL-6, interleukin 6; $mPEG_{2000}$-DSPE, 2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy (polyethylene glycol)-2000]; NF-κB, nuclear factor-kappa B; l-OHP, oxaliplatin; POPC, 1–1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; RNAi, RNA interference; RTV, relative tumor volume; shRNA-lipoplex, shRNA against thymidylate synthase complexed with cationic liposome; TLRs, toll like receptors (TLRs); TNF-α, tumor necrosis factor alpha; TS shRNA, shRNA against thymidylate synthase

* Corresponding author at: Department of Pharmacokinetics and Biopharmaceutics, Institute of Biomedical Sciences, Tokushima University, 1-78-1, Sho-machi, Tokushima 770-8505, Japan.
E-mail address: ishida@tokushima-u.ac.jp (T. Ishida).

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permeation and retention (EPR) effect [12]. However, the EPR effect may be insufficient for the delivery of adequate amounts of PEGylated lipoplex to solid tumors due to the heterogeneity of the tumor microenvironment, which is exemplified by hypovascularity [13].

Recent studies have introduced new approaches to improve the accumulation of nanocarrier systems in solid tumors. Combining treatments with transforming growth factor-β type 1 receptor (TβR-1) inhibitor [14] or tumor necrosis factor-α (TNF-α) [15,16] has resulted in an enhanced accumulation of nanocarriers with a subsequent pronounced anti-tumor effect of their payloads. In addition, metronomic dosing with either cyclophosphamide or S-1 can augment the intra-tumor accumulation of co-administered chemotherapeutic agent-containing PEGylated liposome via alteration of the tumor vascular permeability [13,17,18]. Nevertheless, immunogenic responses and induction of inflammatory cytokines production triggered by RNAi molecules constitute a potential barrier against the utility of RNAi [19,20].

Effective intracellular delivery of synthetically manufactured RNAi duplexes can activate the innate immune system to produce high levels of inflammatory cytokines (IL-6, TNF-α and IFNs) via the induction of RNA-sensing toll-like receptors (TLR3, 7 and 8) and other plasma proteins [21,22]. Activation of TLR-mediated immunostimulatory response and excessive production of inflammatory cytokines can increase the potential of systemic immunotoxicity [23]. Fortunately, relatively simple methods have been recently identified to conquer the immunostimulant effect of delivered RNAi molecules, such as the chemical modification of RNAi duplexes and the co-administration of immunosuppressive agents [23].

Oxaliplatin (l-OHP) is a third-generation platinum analogue with activity and safety profiles that are superior to those of other platinum derivatives, including cisplatin and carboplatin [24]. We have previously established that the sequential administration of liposomal l-OHP reduces tumor interstitial pressure, which is believed to alter the tumor microenvironment for a more enhanced delivery of subsequent doses of PEGylated liposome to solid tumors [25,26]. In addition, we and others have reported the immunosuppressive effect of some chemotherapeutic agents against the immunogenic response triggered by RNAi-lipoplex [27–29]. Therefore, in the present study, we focused on the effect of co-administering liposomal l-OHP for improving targeted delivery and abrogating the immunostimulatory response of a PEGylated TS shRNA-lipoplex in a human colorectal cancer xenograft mouse model.

2. Materials and methods

2.1. Materials

Dioleoylphosphatidylethanolamine (DOPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), and 2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy (polyethylene glycol)-2000] (mPEG$_{2000}$-DSPE) were generously donated by NOF (Tokyo, Japan). O,O′-ditetradecanoyl-*N*-(α-trimethyl ammonio acetyl) diethanolamine chloride (DC-6-14) as a cationic lipid was purchased from Sogo Pharmaceutical (Tokyo, Japan). Cholesterol (CHOL) was of analytical grade (Wako Pure Chemical, Osaka, Japan). Opti-MEM I and Lipofectamine RNAi MAX were purchased from Invitrogen (CA, USA). ^{3}H-cholesterylhexadecyl ether (^{3}H-CHE) was purchased from PerkinElmer Japan (Yokohama, Japan). All other reagents were of analytical grade.

2.2. Short hairpin double stranded RNA(shRNA)

shRNA against thymidylate synthase (TS shRNA) and nonspecific shRNA (control shRNA), chemically synthesized and purified by HPLC, were purchased from Hokkaido System Science (Hokkaido, Japan). The sequence of shRNA against thymidylate synthase (TS) is as follows: 5′-GUAACACCAUCGAUCAUGAUAGUG CUCCUGGUUGUCAUGAAUCG AUGGUGUUACUU-3′. The sequence of the control shRNA is as follows: 5′-UCUUAAUCGCGUAUAAGGCUAGUGCUCCUGG UUGGCCUUxAU ACGCGAUUAAGAUU-3′. The final concentration of the shRNA was adjusted to 50 μM with TE buffer (10 mM Tris, 1 mM EDTA, pH 8).

2.3. Animals and tumor cell line

Male BALB/c Slc and BALB/c nu/nu mice aged 4–5 weeks (20–25 g) were purchased from Japan SLC (Shizuoka, Japan). Mice were kept under pathogen-free conditions. They had free access to water and mouse chow, and were housed under systematized environmental conditions (constant temperature, humidity, and 12-h dark-light cycle). All animal experiments were evaluated and approved by the Animal and Ethics Review Committee of the University of Tokushima. A human colon carcinoma cell line, DLD-1, was kindly provided by Taiho Pharmaceutical and was kept in RPMI-1640 medium (Wako Pure Chemical) supplemented with 10% heat-inactivated fetal bovine serum (AusGenex, Brisbane, Australia).

2.4. In vitro cytotoxicity assay and gene knockdown

The effect of a combination of liposomal l-OHP and TS shRNA on the *in vitro* viability of a DLD-1 cell line was investigated. Cells were plated at an initial density of 2×10^3 cells/well in 96-well plates 24 h before transfection using lipofectamine RNAi MAX. The cells were treated with either liposomal l-OHP (20 μg l-OHP/ml, 2.6 μmol phospholipid/ml), control shRNA (5 nM), a mixture of liposomal l-OHP (20 μg l-OHP/ml, 2.6 μmol phospholipid/ml) and control shRNA (5 nM), TS shRNA (5 nM), or a mixture of liposomal l-OHP (20 μg l-OHP/ml, 2.6 μmol phospholipid/ml) and TS-shRNA (5 nM). Following 72 h incubation at 37 °C, the cells were further incubated with 50 μl MTT reagent (5 mg/ml) for 4 h at 37 °C. Then, 150 μl of acid-isopropanol (0.04 N HCl in isopropanol) was added to each well to dissolve the formazan crystals. The absorbance of each well was read at 570 nm on a microplate reader, Wallac1420 ARVOsx (PerkinElmer Life Sciences, MA, USA). The data represent three independent experiments.

To investigate the effect of a combination of liposomal l-OHP and TS shRNA on *in vitro* TS gene expression, DLD-1 cells were cultured and treated as mentioned above. PEGylated liposome containing l-OHP (liposomal l-OHP) (100 nm, 1.01 mg l-OHP/ml) was prepared as described previously [13]. Total RNA from the treated cells was isolated using an RNeasy® Plus Universal Mini kit (QIAGEN), 48 h post treatments. Total RNA (1 μg) was reverse transcribed with a Superscript II kit (Invitrogen) according to the manufacturer's instructions. Real-time PCR reactions using a One-Step RT-PCR system (QIAGEN) were performed according to the manufacturer's protocol. TAQ DNA Polymerase (Takara) was used to amplify the produced cDNA using Oligo (dt) specific primers. The ΔΔCT method was used to evaluate the relative level of expression of each gene (Expression of GAPDH mRNA was considered an internal standard).

2.5. Preparation of liposomal l-OHP

PEGylated liposomes containing l-OHP (liposomal l-OHP) were composed of HSPC, cholesterol and mPEG$_{2000}$-DSPE (2:1:0.2, molar ratio). The liposomes were prepared using a reverse-phase evaporation method as described earlier [13]. Un-encapsulated l-OHP was removed by dialysis and the loaded l-OHP was determined using an atomic absorption photometer (Z-5700, Hitachi, Tokyo, Japan). The particle size of the prepared liposomes was 101 ± 11 nm, as determined with a NICOMP 370 HPL submicron particle analyzer (Particle Sizing System, CA, USA). The l-OHP concentration in the liposome formulation was adjusted as 1.01 mg l-OHP/ml.