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RESEARCH PAPER

Cationic Liposomal Co-delivery of Small Interfering RNA and a MEK Inhibitor for Enhanced Anticancer Efficacy

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Abstract

Purpose To test whether co-delivery of anticancer small interfering RNA (siRNA) and a chemical MEK inhibitor using cationic liposomes enhances anticancer activity *in vitro* and *in vivo*.

Method MEK inhibitor PD0325901 was encapsulated in lipid layers of N',N''-dioleylglutamide-based cationic liposomes (DGL). Mcl1-specific siRNA (siMcl1) was complexed to DGL or PD0325901-loaded liposomes (PDGL). Efficiency of cellular siRNA delivery was tested using fluorescent double-stranded RNA. Silencing of target proteins was evaluated using Western blotting and real-time quantitative polymerase chain reactions. *In vivo* anticancer activity was tested using xenografted mice.

Results Size and zeta potential of PDGL were similar to DGL. PDGL could deliver double-stranded RNA into cells with efficiencies comparable to DGL. Cellular co-delivery of siMcl1 and PD0325901 reduced expression of Mcl1 and pERK1/2 proteins and more effectively reduced tumor cell survival than other treatments. In mice, siMcl1 and PD0325901 co-delivered by PDGL inhibited growth of tumors 79%. Substantial apoptosis of tumor cells was observed following PDGL-mediated co-delivery of siMcl1, but not in other groups.

Conclusions PDGL-mediated co-delivery of siMcl1 and MEK inhibitor, PD0325901, could serve as a potential strategy for combination chemogene anticancer therapy.

KEY WORDS co-delivery · combination therapy · liposome · MEK inhibitor · siRNA

ABBREVIATIONS

DG	N',N''-dioleylglutamide
DGL	DG-based cationic liposomes
DOPE	dioleyl-sn-glycero-3-phosphoethanolamine
dsRNA	double-stranded RNA
ERK	extracellular signal-related kinase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
L2K	Lipofectamine 2000
Mcl1	myeloid cell leukemia sequence 1
MEK	mitogen-activated protein/extracellular signal-regulated kinase kinase
MTT	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide
PCR	polymerase chain reaction
PDGL	PD0325901-loaded cationic liposomes
pERK1/2	phospho-ERK1/2
siGL2	luciferase-specific siRNA
siMcl1	Mcl1-specific siRNA
siRNA	small interfering RNA

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INTRODUCTION

Small interfering RNAs (siRNAs) offer considerable potential as anticancer therapeutics; however, the polygenic and complex nature of cancer requires the development of co-treatment regimens combining chemical anticancer drugs and siRNAs. Several groups have reported significant improvement with combination cancer therapies employing potential anticancer siRNAs and chemical drugs (1–3). In these studies, anticancer siRNAs and chemical drugs have typically been administered as separate formulations.

Co-delivery approaches in which anticancer siRNAs and chemicals are entrapped in the same nanocarriers have drawn recent attention (4–7). Poly (lactide-co-glycolide) nanoparticles have been used to co-deliver p-glycoprotein siRNA and paclitaxel (4). Mesoporous silica nanoparticles have been used for co-delivery of doxorubicin with siRNA against Bcl-2 (5) or p-glycoprotein (6). A guanidinium-containing cationic lipid-based liposome has been used for co-delivery of c-Myc siRNA and doxorubicin (7). Although progress has been made in combination therapy, further investigation into the synergistic co-delivery of siRNA and chemical drugs is required to achieve more effective combination paradigms. It has recently been suggested that more efficacious anticancer combinations will focus on altering multiple pathways rather than targeting single proteins (8).

The mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK) inhibitor PD0325901 is one such multi-pathway inhibitor, blocking the Raf/MEK/extracellular signal-related kinase (ERK) pathway rather than inhibiting a single target protein (9,10). In cancer biology, the Raf/MEK/ERK pathway is known to be involved in proliferation and resistance to apoptosis (11,12). Inhibition of Raf/MEK/ERK signaling using a MEK inhibitor reduces phospho-ERK1/2 (pERK1/2) levels and suppresses downstream pathways (13). PD0325901 reduces the growth of various cancers, including thyroid carcinoma (14), melanoma (11), and prostate cancers (15).

Mcl1 (myeloid cell leukemia sequence 1) is a member of the Bcl-2 family of apoptosis-regulating proteins (16). Mcl1-specific siRNA (siMcl1) is known to enhance the sensitivity of cancer cells to anticancer chemotherapeutics, and a previous study has shown that cancer cells overexpressing Mcl1 are less sensitive to the apoptosis-inducing effects of chemotherapeutic agents (17). Consistent with this, a combination chemogene regimen including siMcl1 was reported to enhance the chemosensitivity of pancreatic carcinoma to gemcitabine (18). Moreover, co-treatment with siMcl1 was shown to increase the chemosensitivity of cisplatin-resistant ovarian carcinoma cells (2).

For combined treatment of chemical drugs and siRNA, it is essential to develop efficient and biocompatible delivery systems that allow effective co-delivery to cancer cells (19–21). We previously reported that a new cationic liposome based on N',N"-dioleylglutamide (DG) delivered siRNA and provided effective target-gene silencing *in vivo* (22). In the current study, we formulated cationic DG-containing liposomes for co-delivery of siMcl1 and the MEK inhibitor PD0325901, and tested whether cationic liposomal co-delivery of siMcl1 and PD0325901 enhanced anticancer activity *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials

N-[(2R)-2,3-dihydroxypropoxy]-3,4-difluoro-2-[(2-fluoro-4-iodophenyl)amino]benzamide (PD0325901) was purchased from Selleck Chemicals LLC (Houston, TX, USA). Dioleyl-sn-glycero-3-phosphoethanolamine (DOPE) was from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol and 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were from Sigma-Aldrich (St. Louis, MO, USA). A fluorescent double-stranded RNA (Block-iT) and TRIZOL reagent were from Invitrogen (Carlsbad, CA, USA). Luciferase-specific siRNA (siGL2) and siMcl1 were from ST Pharm (Seoul, Korea). The Maxim RT PreMix Kit was from Intron Biotechnology (Seongnam, South Korea). The BCA protein assay kit was from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Anti-human Mcl1 antibody (ab32087) was from Abcam (Cambridge, United Kingdom). Anti-β-actin antibody (sc-47778), anti-pERK1/2 antibody (sc-7383), and alkaline phosphatase-conjugated anti-IgG antibody were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Polycarbonate membrane filters were from Millipore Corp. (Billerica, MA, USA).

Preparation of PD0325901-Loaded Cationic Liposomes

PD0325901-loaded cationic liposomes (PDGL) were prepared by dissolving PD0325901 in methanol and mixing with the cationic lipid DG, DOPE, and cholesterol at a molar ratio of 0.2:3:1:1; DG was synthesized as described previously (22). Empty DG-based cationic liposomes (DGL) without PD325901 were prepared by mixing DG, DOPE, and cholesterol in chloroform at a molar ratio of 3:1:1. The lipid mixtures were dried in an evaporator, and the resulting thin lipid films were hydrated with 20 mM HEPES buffered-saline (pH 7.4) and extruded three times through 0.2 μm polycarbonate membrane filters using an extruder (Northern Lipids Inc, Vancouver, Canada).

Gel Retardation Assays

Formation of complexes from cationic liposomes and siRNA was confirmed using gel retardation assays. DGL or PDGL were mixed with scrambled siRNA in diethylpyrocarbonate-treated water at N/P ratios ranging from 0.5:1 to 20:1 and incubated for 20 min at room temperature. Each mixture was loaded onto a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide, and separated by electrophoresis in tris-borate-ethylenediamine tetraacetic acid buffer. After electrophoresis, gels were visualized using a Gel Doc System (Bio-Rad Lab., Hercules, CA, USA).

Measurements of Sizes and Zeta Potentials

The sizes of DGL or PDGL alone or in siRNA complexes were determined using dynamic light scattering. The samples were diluted with 20mM HEPES-buffered saline (pH 7.4) and the hydrodynamic diameters of particles were measured by dynamic He-Ne laser (10 mW) light scattering at an angle of 90° at 24.1°C using an ELS-8000 instrument (Photal, Osaka, Japan). Zeta potentials were determined by laser Doppler microelectrophoresis at an angle of 22°. The software package (ELS-8000 software) supplied by the manufacturer was used to analyze the data.

Cellular Uptake of siRNA

The cellular uptake of siRNA was monitored using a fluorescent double-stranded RNA (Block-iT) and assessed by fluorescence microscopy and flow cytometry. KB human epithelial carcinoma cells (American Type Culture Collection, Manassas, VA, USA) were seeded onto 24-well plates at a density of 8×10^4 cells/well. The following day, at which time the cells had reached 60-70% confluence, the cells were treated with 300 μl of medium containing siRNA (0.02 μg) complexed to DGL or PDGL entrapping 0.22 μg of PD at an N/P ratio of 10:1. After a 24-h incubation without a medium change, the cells were observed under a fluorescence microscope (Leica DM IL, Wetzla, Germany). In some cases, fluorescence-positive cells were quantified using a BD FACSCalibur and the Cell Quest Pro software (BD Biosciences, San Jose, CA, USA).

In Vitro siRNA-Mediated Gene Silencing

Target-gene silencing by siRNA complexed to DGL or PDGL was measured at both mRNA and protein levels. For real-time quantitative polymerase chain reaction (PCR), KB cells were plated in 24-well plate (8×10^4 cells/well) and incubated for 24 h. The plated cells were treated with 300 μl of medium containing siRNA (0.02 μg) complexed to DGL or PDGL entrapping 0.22 μg of PD. Twenty-four hours later, total mRNA was extracted using the TRIZOL reagent, and cDNA was synthesized from the mRNA template using a Maxim RT PreMix Kit. Real-time PCR was conducted using SYBR Green I Master Mix and a LightCycler 2.0 (Roche Applied Science, Mannheim, Germany). For Mcl1, the sequence of the sense primer was 5′-AGCTGCATCGAACCATTAGC-3′, and that of the antisense primer was 5′-GCTCCTACTCCAG-CAACACC-3′. Mcl1 mRNA levels were normalized to those of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

For Western blotting, lysates of whole cells (100 μg total protein) were prepared at 48 h after treatment of cells using siRNA. Cells were added with lysis buffer (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 mg/ml leupeptin, and 1 mM phenylmethyl-sulfonyl fluoride) on ice. Protein in lysates, quantified using a BCA protein assay kit as described by the manufacturer, were separated by SDS-PAGE on 10% gels. Western blotting was performed using antibodies specific for Mcl1 (1:1000), pERK1/2 (1:1000), and β-actin (1:2500). Bands were visualized using an alkaline phosphatase-conjugated anti-IgG antibody.

In Vitro Anticancer Efficacy

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assays and crystal violet staining were used to measure *in vitro* anticancer effects of various treatments. KB cells were seeded in 48-well plates at an initial density of 2×10^4 cells/well in culture medium and grown overnight. Cells were next treated with 300 μl of medium containing siRNA (0.02 μg) complexed to DGL or PDGL entrapping 0.22 μg of PD at an N/P ratio of 10:1. In some experiments, cells were treated with various concentrations of free PD0325901 dissolved in dimethyl sulfoxide. After a 24-h incubation, viability was quantitatively measured by MTT assay or visualized by crystal violet staining. For MTT assays, 20 μl of MTT solution (5 mg/ml in sterile phosphate-buffered saline, pH 7.4) was added to each well. The cells were incubated for an additional 2 h in the presence of MTT. The medium was then removed and 200 μl of a 0.04 N HCl/isopropanol solution was added to each well. Absorbance was measured at 570 nm using a microplate reader (Sunrise-Basic TECAN, Männedorf, Switzerland). The viability of KB cells was expressed as a percentage of control values. For crystal violet staining, the cells were washed twice with phosphate-buffered saline, and treated with 200 μl of staining solution (0.5% crystal violet and 20% methanol). The stained cells were observed under a phase-contrast microscope.

In Vivo siRNA-Mediated Gene Silencing

Silencing of target genes *in vivo* was measured in 4-week-old female BALB/c mice (Orient Bio, Inc., Seungnam, South Korea) subcutaneously injected in the right flank with 3×10^6 KB cells. When tumors had grown to diameters of 6–7 mm, mice were intratumorally injected twice every other day with 0.7 mg/kg of siRNA, naked or in complexes with DGL or PDGL containing 0.72 mg/kg of PD0325901. Two days after the second injection, tumor tissues were extracted, and Mcl1 mRNA and protein levels were analyzed as described above.

In Vivo Antitumor Efficacy

The anticancer effect of siMcl1 and PD0325901 co-delivered using PDGL was studied using KB tumor-xenografted mice. When tumors had grown to diameters of 6–7 mm, mice were intratumorally injected with 0.7 mg/kg of siRNA in complexes with DGL or PDGL containing 0.72 mg/kg PD0325901 every other day (five injections total). Tumor tissues were extracted 17 days after tumor inoculation and fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at a thickness of 6 mm. Tissue sections were stained with hematoxylin and eosin, and observed by optical microscopy.

Statistical Analysis

Statistical analysis of data was performed using Student's *t*-test or ANOVA with post hoc Student–Newman–Keuls test. SigmaStat software (version 3.5, Systat Software, Richmond, CA, USA) was used for all analyses, and a *p*-value less than 0.05 was considered significant.

RESULTS

Characterization of siRNA Lipoplexes

The physicochemical properties of complexes of siRNA with PDGL were similar to those of siRNA with DGL. Gel retardation assays showed that both DGL and PDGL inhibited the electrophoretic mobility of siRNA at N/P ratios as low as 5:1 (Fig. 1a). Zeta potential values decreased after forming complexes with siRNA, but did not significantly differ between DGL and PDGL (Fig. 1b). Zeta potential values of PDGL decreased from 37.7 ± 2.1 mV to 16.5 ± 2.0 upon siRNA complexation. The sizes of siRNA lipoplexes were 229.5 ± 2.6 nm for DGL and 230.6 ± 5.8 nm for PDGL.

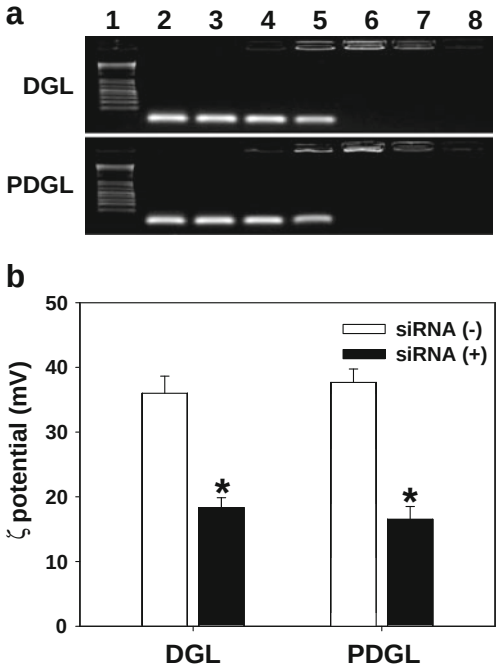

Fig. 1 Gel retardation and zeta potential of siRNA lipoplexes. DGL or PDGL were complexed with siRNA at various N/P ratios and electrophoresed on a 1.5% agarose gel. Mobility of siRNA was visualized by ethidium bromide staining (**a**). Lane 1: 1-kb-plus ladder; lane 2: siRNA alone; lane 3: N/P ratio, 0.5:1; lane 4: N/P ratio, 1:1; lane 5: N/P ratio, 2:1; lane 6: N/P ratio, 5:1; lane 7: N/P ratio, 10:1; lane 8: N/P ratio, 20:1. Zeta potential values (**b**) of liposome alone or complexed with siRNA were measured by laser Doppler microelectrophoresis at an angle of 22°. (*$p<0.001$ *vs.* group with no siRNA complexation; *t*-test).

Efficiency of dsRNA Cellular Uptake by PDGL

The presence of PD0325901 in PDGL did not affect the dsRNA-delivery efficiency of DGL. Fluorescence microscopy revealed negligible cellular fluorescence after treatment with naked fluorescent dsRNA. In contrast, DGL- and PDGL-mediated delivery of fluorescent dsRNA increased the intensity of intracellular fluorescence (Fig. 2a). FACS analyses showed that the fluorescence-positive cell fraction increased after delivery of dsRNA using DGL or PDGL (Fig. 2b). Compared with naked dsRNA, DGL and PDGL dsRNA lipoplexes produced 36.7-fold and 35.0-fold increases in fluorescence-positive fractions, respectively (Fig. 2c).

Reduced Target Gene Expression After Co-delivery of siMcl1 and PD0325901 via PDGL

The cellular co-delivery of siMcl1 and PD0325901 using PDGL reduced Mcl1 expression and pERK1/2 levels. Treatment of cells with siGL2 using DGL did not alter Mcl1 or pERK1/2 levels (Fig. 3). The mRNA expression

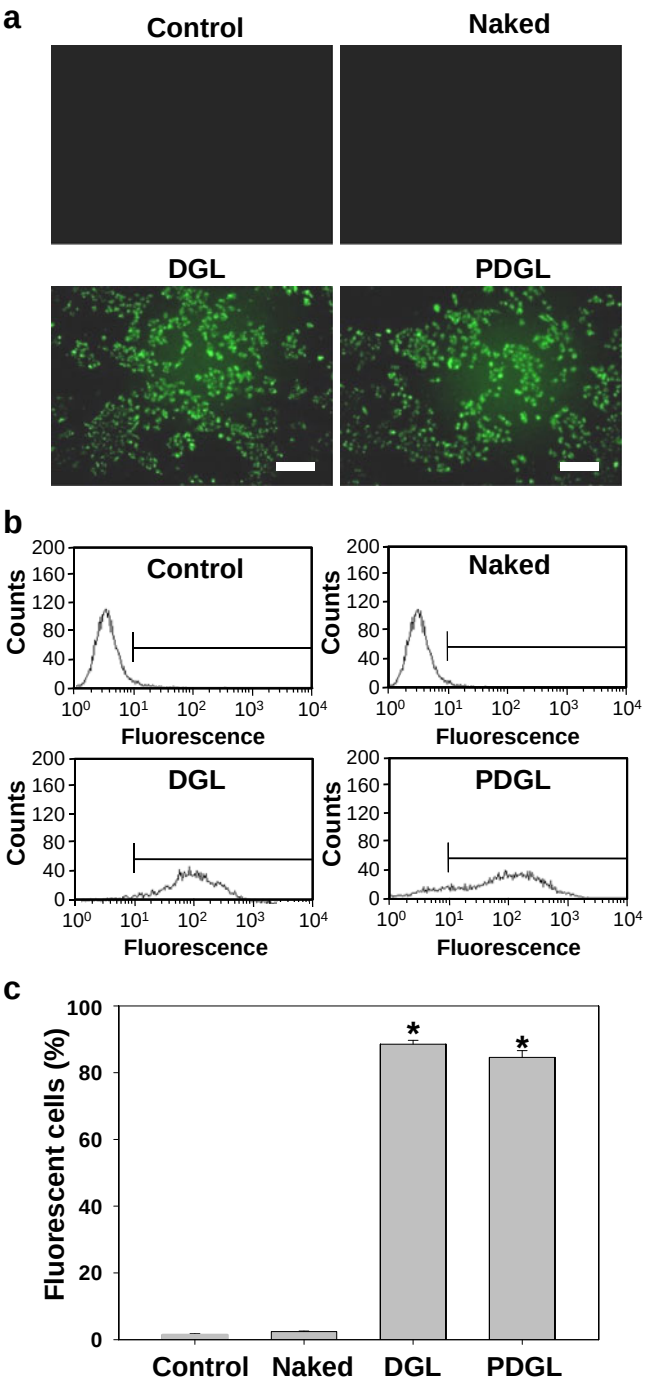

Fig. 2 Cellular uptake of fluorescent dsRNA using PDGL. KB cells were treated with fluorescent marker-labeled dsRNA in naked form or as lipoplexes. Untreated KB cells were used as controls. After 24 h, cellular fluorescence was observed under a fluorescence microscope (**a**) and measured by flow cytometry (**b**, **c**). Scale bar: 100 μm. Representative flow cytometry data (**b**). Fluorescence intensity data (**c**) are expressed as means ± SEMs (* $p < 0.001$ *vs.* control and naked siRNA-treated groups; $n = 4$; ANOVA and Student–Newman–Keuls).

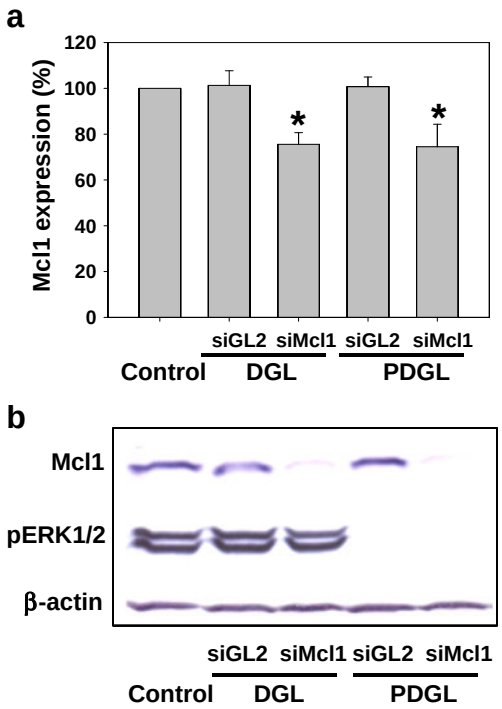

Fig. 3 Mcl1 expression and pERK1/2 levels after co-delivery of siMcl1 with MEK inhibitor using PDGL. KB cells were treated with siGL2 or siMcl1 in a complex with DG and PDGL. (**a**) After 24 h, mRNA expression levels of Mcl1 were measured by real-time PCR ($n = 4$). (*$p < 0.05$ *vs.* control, and group with siGL2 complexation; ANOVA and Student–Newman–Keuls). (**b**) After 48 h, Mcl1 protein and pERK1/2 levels were analyzed by Western blotting.

levels of Mcl1 did not significantly differ between the groups treated with siGL2/DGL and with siGL2/PDGL (Fig. 3a). Unlike siGL2, siMcl1 delivered using DGL or PDGL reduced the mRNA (Fig. 3a) and protein (Fig. 3b) expression levels of Mcl1 protein. pERK1/2 levels were affected by PDGL regardless of the presence of complexed siRNA (Fig. 3b). Although PD0325901 did not alter the silencing effect of siMcl1 delivered in lipoplexes, it did affect proteins involved in the Raf/MEK/ERK signaling pathway, with PD0325901 delivered using PDGL significantly reducing the levels of pERK1/2. Treatment of KB cells with DGL did not reduce pERK1/2 levels, regardless of the presence of siRNA.

In Vitro Antitumor Effects After Co-delivery of siMcl1 and PD0325901 via PDGL

siMcl1 and PD0325901 co-delivered using PDGL exerted greater antitumor effects than siMcl1 or PD0325901 administered alone (Fig. 4). When KB cells were treated with various concentrations of free PD0325901, significant reduction of cell viability was observed from 0.96 μg/ml (Fig. 4a). After treatment with 24.1 and 48.2 μg/ml of free PD0325901, the cell viability was 49.0%±6.2% and 33.0%±6.1%, respectively (Fig. 4a). MTT assays revealed

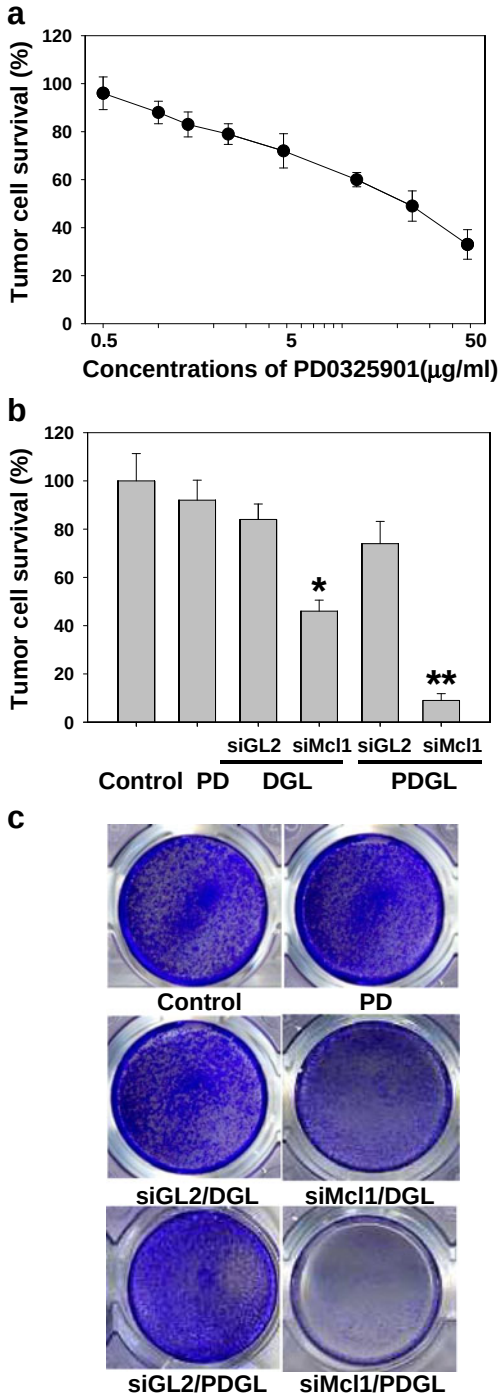

Fig. 4 *In vitro* anticancer effect of siMcl1 and MEK inhibitor co-delivered using PDGL. KB cells were treated with siGL2 or siMcl1 in complexes with DGL or PDGL. Untreated cells were used as a control. For comparison, cells were treated with free PD0325901 (PD) with various concentrations (**a**). After 24 h, survival fraction of KB cells was measured using MTT assays (**b**). (*$p < 0.001$ *vs.* siGL2-treated groups, ** $p < 0.001$ *vs.* other groups; $n = 4$; ANOVA and Student–Newman–Keuls). Viable cells were visualized by crystal violet staining (**c**).

that 46.0%±5.1% of cells were viable after delivery of siMcl-1 using DGL (Fig. 4b). After treatment of cells with siGL2 complexed to PDGL, 74.0%±9.2% of cells survived. In contrast, only 8.9%±2.8% of cells treated with siMcl1 complexed to PDGL survived. Consistent with MTT assay results, crystal violet staining showed the fewest number of stained cells after co-delivery of siMcl1 with PD0325901 using PDGL (Fig. 4c).

In Vivo Silencing of Target Expression After Co-delivery of siMcl1 Using PDGL

Similar to *in vitro* studies, Mcl1 expression and pERK1/2 levels were affected by siMcl1 and PD0325901, respectively, in KB tumor-bearing mice. Mcl1 mRNA levels were significantly reduced by treatment of cells with siMcl1, but not with siGL2. Moreover, the use of PDGL did not affect the expression levels of Mcl1. There was no significant difference between DGL and PDGL after delivery of siMcl1 (Fig. 5a). Western blotting showed that pERK1/2 levels were significantly decreased by the MEK inhibitor, PD0325901, but not by siMcl1 (Fig. 5b). The levels of pERK1/2 protein were similar among untreated, siGL2 in DGL complex, and siMcl1 in DGL complex. Similarly, the expression of Mcl1 was not affected by PD0325901. The band densities of Mcl1 proteins in groups treated with siGL2 were similar regardless of lipoplexes. Both Mcl1 expression and pERK1/2 levels were decreased only after co-delivery of siMcl1 complexed to PDGL.

In Vivo Antitumor Effects of siMcl1 and PD0325901 Co-delivered via PDGL

siMcl1 with PD0325901 co-delivered using PDGL provided significantly greater inhibition of tumor growth than other treatments. At 17 days after tumor inoculation, siMcl1 in DGL complexes and siGL2 in PDGL complexes suppressed tumor size by 47% and 13%, respectively, compared with the control group. Notably, treatment of mice with siMcl1 in PDGL complexes suppressed tumor size by 79% compared with the control group (Fig. 6a). A histological analysis of tumor sections revealed destroyed tumor cells in the group co-treated with siMcl1 and PD0325901 using PDGL (Fig. 6b).

DISCUSSION

In this study, we formulated cationic PDGL for co-delivery of siMcl1 and the MEK inhibitor, PD0325901. We demonstrated that co-delivery of siMcl1 with PD0325901 using PDGL provided significant silencing of target proteins and substantially inhibited tumor growth in xenografted mice.

Although the minimal N/P ratio for complete complexation of siRNA and liposomal formulation was found to be 5:1, the N/P ratio of 10:1 provided higher cellular

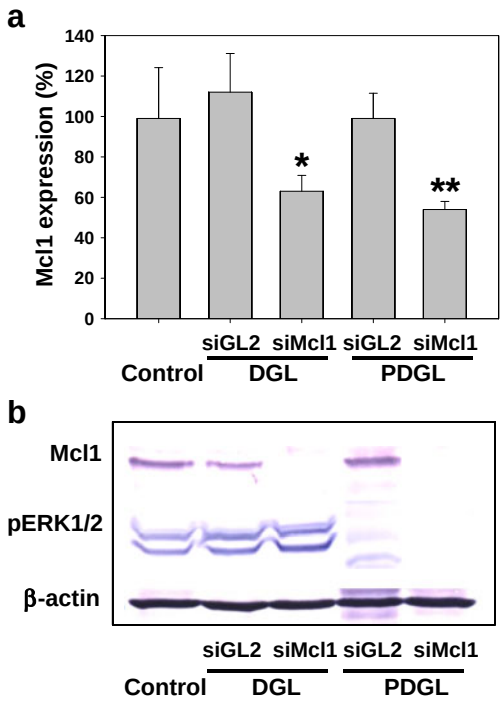

Fig. 5 *In vivo* target-gene silencing. Lipoplexes of siGL2 or siMcl1 (0.7 mg/kg) were intratumorally administered twice every other day. Two days after the second injection, mice ($n = 4$/group) were sacrificed and Mcl1 expression in tumor tissues was analyzed by quantitative real-time PCR (**a**) and Western blotting (**b**). (*$p < 0.05$ *vs.* control and siGL2/DGL-treated groups; **$p < 0.05$ *vs.* control and siGL2/PDGL-treated groups; ANOVA and Student–Newman–Keuls).

transfection of siRNA than that of 5:1 (Supplementary Material Fig. S1). The N/P ratio dependent transfection activity has been reported in plasmid DNA delivery using polyethylenimine (23). Such a dependence of transfection activity to N/P ratio could be explained as that the extra positive charges could enhance the binding of nanoparticles to negatively charged cell membranes (24). However, the N/P ratio needs to be optimized due to the increased cytotoxicity at higher N/P ratios. Thus, although both N/P ratios of 10:1 and 20:1 provided higher cellular siRNA delivery efficiencies than that of 5:1, we chose the N/P ratio of 10:1 as the optimal complexation ratio.

Consistent with previous studies demonstrating the antitumor activity of siMcl1, we found that siMcl1 delivered using either DGL or PDGL reduced the survival of KB tumor cells. Mcl1 has been reported to be involved in the growth of tumor cells and to reduce sensitivity to apoptosis (17). Apoptosis-inducing effects have reported for siMcl1 in ovarian carcinoma cells (2), and for antisense Mcl1 oligonucleotides in non-small cell lung cancer cells (17). Moreover, siMcl1 has been shown to enhance the chemosensitivity of pancreatic carcinoma cells to gemcitabine (25). Given these previous reports, the chemosensitizing potential of siMcl1 co-delivered with anticancer drugs would be expected to increase the sensitivity of cancer cells, apart from the apoptosis-inducing effects of siMcl1.

For the combined chemogene strategy with siMcl1, we entrapped PD0325901 in DG-based liposomal bilayers. DG is a newly developed cationic lipid synthesized by conjugation of the anionic amino acid glutamate to oleylamine. We previously reported that DGL could deliver siRNA and silence target genes *in vitro* and *in vivo* (22). Because PD0325901 is soluble in chloroform, we formulated it in the lipid component of DG-containing liposomes. The fact that there were no differences between DGL and PDGL in size and zeta potential values indicates that PD0325901 was compatible with the lipid components of DGL, and did not affect the integrity of liposomal structures. Moreover, the efficiency of cellular delivery of fluorescent dsRNA by PDGL supports the idea that

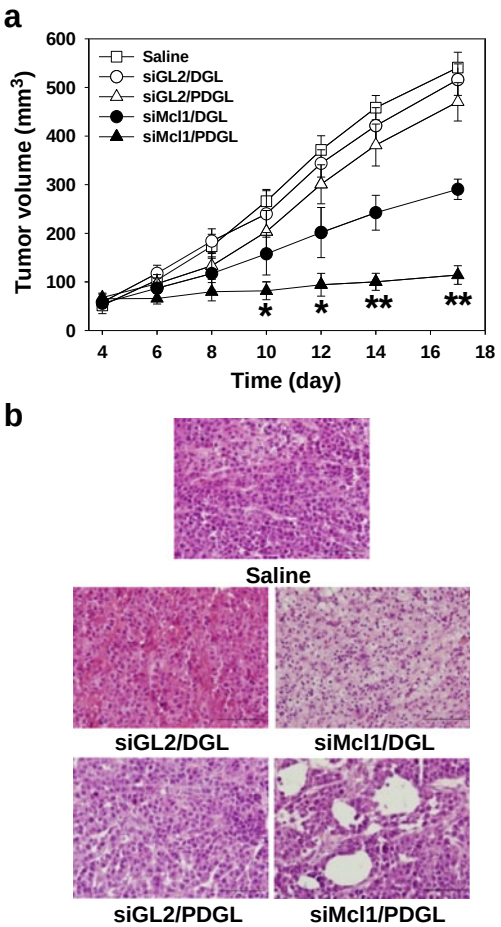

Fig. 6 *In vivo* anticancer effect of siMcl1 and MEK inhibitor co-delivered using PDGL. Xenografted mice ($n = 4$/group) were intratumorally injected with saline or lipoplexes of siGL2 or siMcl1 (0.7 mg/kg) every other day on five occasions beginning on day 4 after tumor inoculation. (**a**) Tumor volumes were periodically measured using calipers. (**b**) On day 17 after tumor inoculation, tumors were extracted and sectioned for hematoxylin and eosin staining. (*$p < 0.05$, **$p < 0.001$ *vs.* other groups; ANOVA and Student–Newman–Keuls).

encapsulation of PD0325901 did not alter the siRNA-delivery capability of DG-containing cationic liposomes.

Although siMcl1/PDGL complexes and siGL2/PDGL showed 25% of difference in mRNA reduction (Fig. 3a), we observed substantial difference of two groups in protein Mcl1 levels (Fig. 3b). Possible explanation for this discrepancy is the different time point of observation. We determined the mRNA levels of Mcl1 at 24 h after treatment, and protein levels of Mcl1 at 48 h after treatment. For fair comparison, mRNA measurement and western blot assay should be done at 48 h. However, at 48 h after treatment with siMcl1/PDGL, most cells were not viable, and it was technically difficult to extract mRNA possibly due to the degradation of cellular mRNA in apoptotic cells (26).

As compared to the *in vitro* reduction study (Fig. 3a), *in vivo* study showed higher reduction of Mcl1 mRNA levels (Fig. 5a). One possible explanation for the discrepancy between *in vitro* and *in vivo* mRNA reduction efficiencies is the time point of sample collection. For *in vitro* study, samples were taken at 24 h after treatment (Fig. 3). Meanwhile, for *in vivo* study, mRNA was extracted from tumor tissues 2 days after double injections of siRNA. Another possibility for the discrepancy might be attributed to the dose difference. For *in vitro* anticancer activity test, 0.02 μg of siRNA was used. Meanwhile, the dose of siRNA for *in vivo* injection was 0.7 mg/kg.

Unlike mRNA reduction data, the western blot data showed similar pattern between *in vitro* (Fig. 3b) and *in vivo* studies (Fig. 5b). Such a similarity between *in vitro* and *in vivo* data might be explained by the qualitative nature of western blot study, making it difficult to evaluate the exact silencing effect of target protein in each sample. For quantitative comparison of Mcl1 target protein silencing effects, other analytical methods such as enzyme-linked immunosorbent assays need to be done in the future.

We observed that co-delivery of siMcl1 and PD0325901 did not affect the expression of reciprocal target proteins. Specifically, our Western blot data showed that treatment of cells with siMcl1 did not affect the levels of pERK1/2, a downstream protein in the MEK pathway (11,15). Moreover, the similar levels of Mcl1 expression in siGL2 complexed to PDGL and siMcl1 complexed to PDGL indicate that the silencing of Mcl1 was not significantly affected by PD0325901. Our observations support the interpretation that the enhanced anticancer effects observed upon co-delivery are attributable to simultaneous inhibition of Mcl1 and MEK pathways in tumor cells.

Our study revealed that tumor growth was significantly suppressed by co-delivery of siMcl1 and PD0325901 using PDGL. In this study, we tested whether it is possible to find the concentration of PD0325901 which exerts no significant antitumor activity, but significantly enhances therapeutic activity of siMcl1. The *in vitro* tumor cell survival responses after treatments with various concentrations of free PD0325901 (Fig. 4a) reveals that the survival of tumor cells began to decrease from 0.96 μg/ml. Indeed, the use of 0.72 μg/ml of PD0325901 did not show significant antitumor activity in free form, but increased the antitumor activity of siMcl1 in liposomal co-delivery (Fig. 4b). Notably, *in vivo* administration of siMcl1 or PD0325901 alone showed much lower suppression of tumor growth than did co-treatment with siMcl1 complexed to DGL or siGL2 complexed to PDGL. The mechanisms underlying the much greater anticancer activity of siMcl1 and PD0325901 co-delivered by PDGL remain to be studied. In general, combination therapy using different mechanisms could induce effective tumor suppression by interfering with different signal pathways involved in survival and escape from apoptosis. Thus, it is possible that simultaneously blocking Mcl1 and the Raf/MEK/ERK pathway might have promoted the death of cancer cells by sensitizing cells to apoptosis stimuli. Previously, a triblock copolymer-based micelleplex simultaneously delivering polo-like kinase 1-specific siRNA and paclitaxel was shown to induce a synergistic tumor-suppressive effect in MDA-MB-435 xenografted mice (27). Moreover, diblock copolymer nanoparticles delivering Bcl2 siRNA and doxorubicin into the same cancer cells were shown to yield synergistic anticancer effects (28). Another study reported that cellular co-delivery of siRNA targeting vascular endothelial growth factor and paclitaxel using cationic triblock copolymer micelles enhanced the efficacy of paclitaxel (29).

The PDGL-mediated co-delivery of siMcl1 might also reduce the effective dose of PD0325901. In our *in vivo* study, intratumoral administration of 0.72 mg/kg/day of PD0325901 combined with siMcl1 significantly suppressed tumor growth (by 79%) compared to saline-treated controls. This contrasts with a previous study in thyroid carcinoma xenografted mice that reported a 58% reduction in tumor mass after oral treatment with 20 to 25 mg/kg/day of PD0325901 (14). Since we used the intratumoral route for injection of PD0325901, the exact comparison between two routes is not possible. However, the oral bioavailability of PD0325901 may provide informative insights between two routes. In a recent study, the pharmacodynamic and toxicokinetic profiles of PD0325901 were compared in the rat following oral and intravenous administration with 10, 30, and 100 mg/kg doses (30). The study reported that the oral bioavailability of PD0325901 was as high as 56-109%. Moreover, the maximum-tolerated dose of PD0325901 was found to be 100 mg/kg for both oral and intravenous routes. In dogs, the oral bioavailability of PD0325901 was found to be higher than 90% (31). Given the high oral bioavailability of PD0325901, our intratumoral dose of 0.72 mg/kg may provide lower blood concentrations as compared to oral

doses of 20–25 mg/kg. Sun *et al.* recently described a similar anticancer-drug dose-saving effect in the context of co-delivered siRNA, reporting that co-delivery of a Plk1-specific siRNA and paclitaxel using self-assembled nanoparticles significantly reduced the effective dose of paclitaxel (27). Such dose-saving effects following co-delivery with siMcl1 might contribute to a reduction in the side effects of combined anticancer drugs in clinical applications.

CONCLUSION

PDGL effectively delivered siRNA into cells, and provided enhanced anticancer activity *in vitro* and *in vivo*. PDGL-mediated co-delivery of siMcl1 and a MEK inhibitor remarkably enhanced tumor growth inhibition compared to each treatment alone. The enhanced anticancer activity associated with PDGL-mediated co-delivery of siMcl1 and PD0325901 could reduce the doses of anticancer drugs required, and suggests that this combination may serve as a potential anticancer strategy. Importantly, DG-containing liposome formulations could be utilized for co-delivery of other effective combinations of siRNA and anticancer chemotherapeutics.

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