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Research Article

In Vitro and *In Vivo* Co-delivery of siRNA and Doxorubicin by Folate-PEG-Appended Dendrimer/Glucuronylglucosyl-β-Cyclodextrin Conjugate

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Abstract. We have previously reported the utility of folate-polyethylene glycol-appended dendrimer conjugate with glucuronylglucosyl-β-cyclodextrin (Fol-PEG-GUG-β-CDE) (generation 3) as a tumor-selective carrier for siRNA against polo-like kinase 1 (siPLK1) *in vitro*. In the present study, we evaluated the potential of Fol-PEG-GUG-β-CDE as a carrier for the low-molecular antitumor drug doxorubicin (DOX). Further, to fabricate advanced antitumor agents, we have prepared a ternary complex of Fol-PEG-GUG-β-CDE/DOX/siPLK1 and evaluated its antitumor activity both *in vitro* and *in vivo*. Fol-PEG-GUG-β-CDE released DOX in an acidic pH and enhanced the cellular accumulation and cytotoxic activity of DOX in folate receptor-α (FR-α)-overexpressing KB cells. Importantly, the Fol-PEG-GUG-β-CDE/DOX/siPLK1 ternary complex exhibited higher cytotoxic activity than a binary complex of Fol-PEG-GUG-β-CDE with DOX or siPLK1 in KB cells. In addition, the cytotoxic activity of the ternary complex was reduced by the addition of folic acid, a competitor against FR-α. Furthermore, the ternary complex showed a significant antitumor activity after intravenous administration to the tumor-bearing mice. These results suggest that Fol-PEG-GUG-β-CDE has the potential of a tumor-selective co-delivery carrier for DOX and siPLK1.

KEY WORDS: PAMAM dendrimer; doxorubicin; siRNA; folate; tumor-selective drug delivery.

INTRODUCTION

Cancer is one of the most lethal diseases resulting in a global mortality rate of around 13%. The incidence of new cases of cancer is expected to rise up to 21.7 million resulting in a mortality of 13 million by the year 2030 (1). To treat cancer successfully, low-molecular weight drugs, peptide, proteins, antibodies, genes, small interfering RNAs (siRNAs), etc. have been tried. Low-molecular weight antitumor drugs have been extensively used for cancer chemotherapy. The antibodies have demonstrated good efficacy in different tumors, and hence, are recently developed aggressively.

Of these different antitumor agents, siRNAs have attracted great interests in recent years. siRNA induces RNA interference (RNAi) and reduces the target gene expression (2). Therefore, siRNAs are expected to be promising antitumor agents in the field of cancer therapy. For example, siRNA against polo-like kinase 1 (siPLK1) is a representative antitumor siRNA, as PLK1 is essential for regulating cell division and maintaining genome stability in mitosis, spindle assembly, and response to DNA damage (3). Naked siRNA, however, shows low blood retention, low stability in the blood or endosomes, low tumor selectivity, and low cellular uptake. Therefore, the development of tumor-selective siRNA carriers is extremely important.

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Cyclodextrins (CyDs), cyclic oligosaccharides, have been acknowledged to form inclusion complexes with hydrophobic guest molecules. CyDs are widely used to improve solubility, stability, taste, bioavailability, etc. of drugs (4). In recent years, CyD-based drug, gene and oligonucleotide carriers have been developed as advanced functional materials (5–10). CyD-based materials are very useful because of (1) their supramolecular properties rendering new functions, (2) facile preparation through self-assembly properties of CyDs, (3) safety profile of CyDs, (4) low cost, etc.

We have previously developed folate-polyethylene glycol (PEG)-appended polyamidoamine (PAMAM) dendrimer (generation 3; G3) conjugates with glucuronylglucosyl-β-CyD (GUG-β-CyD) as tumor-selective siRNA carriers and termed them as Fol-PEG-GUG-β-CDEs. GUG-β-CyD moiety in Fol-PEG-GUG-β-CDE has an essential role in the endosomal escaping ability of the siRNA complex, as it interacts with the endosomal membranes, resulting in endosome disruption (11). Among the Fol-PEG-GUG-β-CDEs having various degrees of folate substitutions (DSF), Fol-PEG-GUG-β-CDEs (DSF 6.7) have significantly enhanced cellular uptake and the RNAi effect of siRNA in the folate receptor-α (FR-α)-overexpressing cancer cells, such as KB cells and 786-0-luc cells, unlike the other Fol-PEG-GUG-β-CDEs (DSF 3.9 and 7.3) (12). Although Fol-PEG-GUG-β-CDEs have the potential of tumor-selective siRNA carriers, less is known about their utility as carriers for low-molecular weight antitumor drugs.

Doxorubicin (DOX) is a potent antitumor drug which is extensively used for the treatment of a large number of cancers in human. DOX, however, shows low tumor selectivity and causes serious cardiotoxicity by lipid peroxidation and generation of free radicals (13). In this context, to improve the tumor selectivity and safety of DOX, dendrimer-based drug carriers have been developed (14–16). Choi *et al.* have developed folate-modified PAMAM dendrimer (G5.5) conjugate with DOX for targeted delivery of DOX to the FR-α-expressing cancer cells (16). In addition, Han *et al.* have developed a PEG-peptide-modified PAMAM dendrimer (G5) loading DOX and demonstrated its potential as a tumor-selective antitumor agent (17). Thus, dendrimers can be useful to fabricate the carriers for DOX.

In the present study, we have first evaluated the utility of Fol-PEG-GUG-β-CDE as a tumor-selective DOX carrier *in vitro*. Further, to fabricate the advanced antitumor agents, we have prepared a ternary complex of Fol-PEG-GUG-β-CDE/DOX/siPLK1 and evaluated its antitumor activity both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials

Fol-PEG-GUG-β-CDE (DSF 6.7) was prepared with PAMAM dendrimer (G3) and PEG (M.W. 2170) following a previously described method (12,18,19). RPMI-1640 (FA-containing) and RPMI-1640 (FA-free) media were purchased from Nissui Pharmaceuticals (Tokyo, Japan) and GIBCO (Tokyo, Japan), respectively. Fetal bovine serum (FBS) and DOX were obtained from Nichirei (Tokyo, Japan) and LC laboratories (MA, USA), respectively. The sequences of siGL2 and siPLK1 were as follows: siGL2: sense, dTdTGCAUGCGCCUUAUGAAGCU; antisense, dTdTAGCUUCAUAAGGCGCAUGC, siPLK1: sense, dTdTUUAUAAAUUCCUCCCCACUAGA; and antisense, dTdTUAUUUAAGGAGGGGUGAUCUUU. The other chemicals and solvents were of analytical reagent grade.

Preparation of the Fol-PEG-GUG-β-CDE/DOX Complex

Fol-PEG-GUG-β-CDE and DOX were dissolved in water in a molar ratio of 1:3 or 1:6, and stirred for 24 h in the dark at a room temperature. The free DOX was removed using Amicon® ultra centrifugal filters (MWCO 10,000, 7500*g*, 15 min) (Fig. 1). The amount of DOX in the filtrate was determined by UV spectrophotometry at λ_{max} 481 nm, and the amount of DOX loaded in the complex was calculated indirectly. The encapsulation efficiency (EE %) and the loading capacity (LC %) were calculated using the following equations:

EE (%) = [(drug added − free drug) / drug added] × 100, and.

LC (%) = (drug entrapped / total weight) × 100.

Release of DOX

Fol-PEG-GUG-β-CDE/DOX complex or DOX alone (as 3.5 mg DOX) was dialyzed (MWCO 3500) with 50 mL of PBS (pH 7.4) or citrate buffer (pH 5.5) at 37°C. At appropriate intervals, 1 mL of the medium was taken out and analyzed by UV-vis spectrophotometer at 481 nm. The volume of the release medium was maintained constantly by adding 1 mL of the buffer after withdrawing the sample. The released DOX was represented as percentage against initial amount of DOX (3.5 mg).

ζ-Potential and Particle Size

Fol-PEG-GUG-β-CDE/DOX/siRNA complex was prepared in mannitol solution (5% *w*/*v*) at a molar ratio of 1:3 (carrier/DOX) and a charge ratio of 50 (carrier/siRNA). The particle sizes and ζ-potential of the ternary complex were determined using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, U.K.).

Cell Culture

KB cells which are HeLa cell (human cervical cancer cells) subtype and generated by contamination were obtained from the Institute of Development, Aging, and Cancer, Tohoku University. KB cells were grown in RPMI-1640 (FA-containing) culture medium containing penicillin (1×10^5 mU/mL) and streptomycin (0.1 mg/mL) supplemented with 10% FBS at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air (20). Hepatocytes which are immortalized cell line of human hepatocytes were also grown in RPMI-1640 (FA-containing) culture medium containing penicillin (1×10^5 mU/mL) and streptomycin (0.1 mg/mL) supplemented with 10% FBS at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

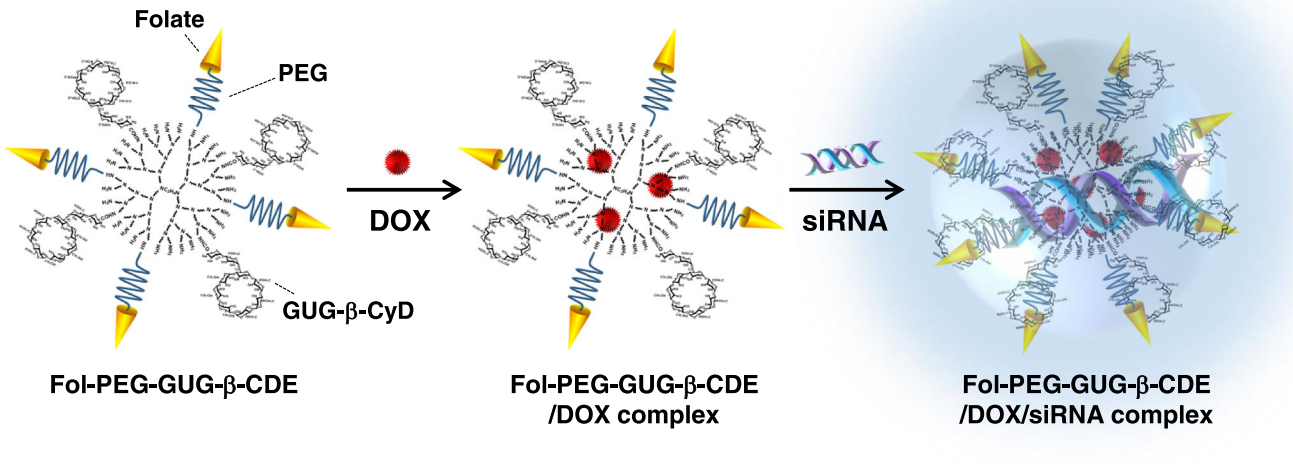

Fig. 1. Pathway of preparation of the Fol-PEG-GUG-β-CDE/DOX binary complex and the Fol-PEG-GUG-β-CDE/DOX/siRNA ternary complex

Cellular Uptake of DOX

KB cells and hepatocytes (1×10^5/35 mm) were incubated with DOX or Fol-PEG-GUG-β-CDE/DOX complex solution (200 μL, 5 μM DOX) on a glass bottom dish for 1, 2, 4, and 6 h at 37°C, and then washed with RPMI-1640 medium (FA-free) twice. The cells were observed under a fluorescence microscope (Biorevo BZ-9000, Keyence, Osaka, Japan), and the fluorescence intensity was evaluated using BZ-II analyzer software (Keyence, Osaka, Japan).

Cytotoxic Activity

KB cells (5×10^4 per well) were incubated for 4 h with serum-free medium (300 μL) containing DOX alone (0.5–5 μM), Fol-PEG-GUG-β-CDE/DOX complex (molar ratio of 1:3), or PEG-GUG-β-CDE/DOX/siPLK1 complex (100 nM siPLK1 at a charge ratio of 50, 5 μM DOX). After supplementing with fresh media containing 10% FBS, the cells were incubated at 37°C for 20 h (binary complex) or 44 h (ternary complex). The cell viability was assayed by the WST-1 method using a microplate reader (Multiskan FC, Thermo Scientific, Waltham, MA). To confirm the involvement of FR-α in the cytotoxic activity of the Fol-PEG-GUG-β-CDE/DOX/siPLK1 complex, KB cells [FR-α (+)] were pre-incubated with 4 mM folic acid for 1 h before the cytotoxic activity study.

Animal Studies

BALB/c *nu/nu* mice (male, 4-week-old) were inoculated with 100 μL of sterilized PBS containing KB cells (1.0×10^6) in the right flank under isoflurane anesthesia. After around 7 days, the mice that had developed a tumor of approximately 100 mm^3 volume were used for the subsequent study.

In Vivo Antitumor Activity

Five percent mannitol solution containing Fol-PEG-GUG-β-CDE/siPLK1 binary complex or Fol-PEG-GUG-β-CDE/DOX/siPLK1 ternary complex (50 μg of siPLK1 and 100 μg of DOX) was administered twice weekly into the mouse tail vein. The molar ratio of Fol-PEG-GUG-β-CDE/DOX was 1:3 and the charge ratio of Fol-PEG-GUG-β-CDE/siPLK1 was 50. The tumor volume and body weight were assessed regularly during the treatment period. The tumor volume was calculated according to the method of Corbett *et al.* (21) by measuring the major axis and minor axis of the tumor and using the following equation: Tumor volume = $L \times W^2/2$, where L = longest dimension of tumor and W = dimension perpendicular to L. The tumor weight was measured after intravenous administration for 30 days.

In Vivo RNAi Effect

The total RNA was isolated from 30 mg of the excised tumors using RNeasy® Mini Kit according to the manufacturer protocol. To determine the *PLK1* mRNA level, the real-time PCR was measured using following primers: 5′-GGC AAC CTT TTC CTG AAT GA-3′ and 5′-AAT GGA CCA CAC ATC CAC CT-3′.

In Vivo Safety Profile

To estimate the safety of the Fol-PEG-GUG-β-CDE complexes, biochemical investigations, such as blood urea nitrogen (BUN), serum creatinine (CRE), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) were performed (clinical chemistry analyzer, JCA-BM2250, JEOL, Tokyo, Japan) after intravenous administration to the mice bearing KB cells tumors.

Statistical Analysis

The data were represented as the mean ± S.E. Analysis of variance followed by Scheffe's test was performed. A p value of < 0.05 was considered as statistically significant.

RESULTS

Preparation of the Fol-PEG-GUG-β-CDE/DOX Complex

To prepare the Fol-PEG-GUG-β-CDE/DOX binary complex, Fol-PEG-GUG-β-CDE (G3, DSF 6.7) was selected as it showed a potent siRNA transfer activity in KB cells [FR-α (+)] (12). The Fol-PEG-GUG-β-CDE/DOX complex was prepared by mixing both the components at a molar ratio of 1:3 or 1:6 (Fig. 1). As shown in Table I, 90.7% and 47.5% of DOX were incorporated into the complex at a molar ratio of 1:3 and 1:6, respectively. LC (%) of DOX in the complex was almost the same with both the molar ratio. These results suggest that DOX was efficiently incorporated into the Fol-PEG-GUG-β-CDE at a molar ratio of 1:3. Hence, the complex prepared at a molar ratio of 1:3 was used for the subsequent studies.

Release of DOX from the Fol-PEG-GUG-β-CDE Complex

The releases of DOX from the binary complex with Fol-PEG-GUG-β-CDE at pH 5.5 and 7.4 were examined (Fig. 2). In the present experimental conditions, we used the dialysis membrane to evaluate release profiles of DOX. Therefore, ca. 2 h were required to diffuse free DOX, because few hours are generally needed to cause drug penetration through dialysis membrane. These data were corresponded with the data reported by Wang *et al.* (14). However, negligible difference in the release rate of free DOX was observed at pH 5.5 and 7.4. On the other hand, the complex released only 32% DOX after 2 h at pH 7.4. In contrast, approximately 85% of DOX was released at pH 5.5, suggesting that the Fol-PEG-GUG-β-CDE/DOX complex releases DOX in an acidic microenvironment.

Cellular Uptake of the Fol-PEG-GUG-β-CDE/DOX Complex

To evaluate cellular uptake of DOX in KB cells, the cells were observed under a fluorescence microscope after incubation with the Fol-PEG-GUG-β-CDE/DOX binary complex for 4 h (Fig. 3). The binary complex of Fol-PEG-GUG-β-CDE showed a markedly higher DOX accumulation than free DOX in KB cells. In addition, co-localization of DOX and Hoechst was observed in the binary complex, indicating the presence of DOX in nucleus of the cells. These results suggest that Fol-PEG-GUG-β-CDE increases cellular uptake of DOX in KB cells.

Table I. Encapsulation Efficiencies and Loading Content of DOX in Fol-PEG-GUG-β-CDE/DOX Complexes

Molar ratio	DSF	EE (%)	LC (%)
1:3	6.7	90.7 ± 1.0	5.4 ± 0.1
1:6	6.7	47.5 ± 1.8	5.5 ± 0.2

Each value represents the mean ± S.E. of 3–4 experiments
DSF degrees of folate substitutions, *EE* encapsulation efficiency, *LC* loading capacity

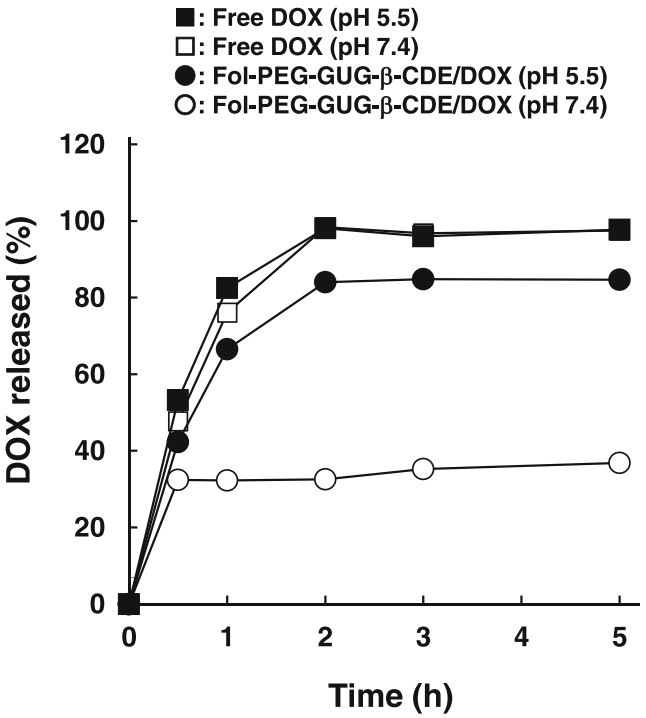

Fig. 2. Release profiles of DOX from the Fol-PEG-GUG-β-CDE/DOX complex at pH 5.5 or 7.4

Supplementary Fig. 1 shows time dependency of the cellular uptake of DOX or Fol-PEG-GUG-β-CDE/DOX complex in KB cells [FR-α (+)] and hepatocytes [FR-α (−)]. The cellular uptake of Fol-PEG-GUG-β-CDE/DOX complex was markedly higher than that of free DOX until 6 h in KB cells (Supplementary Fig. 1A). On the other hand, the cellular uptake of Fol-PEG-GUG-β-CDE/DOX complex in hepatocytes decreased compared with that in KB cells (Supplementary Fig. 1B). Moreover, the cellular uptake of free DOX was negligibly different between KB cells and hepatocytes. These results suggest that Fol-PEG-GUG-β-CDE/DOX complex was taken up by FR-α.

Cytotoxic Activity of the Fol-PEG-GUG-β-CDE/DOX Complex

Cytotoxic activity of the Fol-PEG-GUG-β-CDE/DOX complex in KB cells was further evaluated (Fig. 4). The Fol-PEG-GUG-β-CDE/DOX complex exhibited cytotoxic activity in a concentration-dependent manner. In addition, the complex showed a significantly higher cytotoxic activity than free DOX at a DOX concentration of 2 and 5 μM. Importantly, Fol-PEG-GUG-β-CDE alone showed a negligible cytotoxic activity by itself (data not shown). These results indicate that Fol-PEG-GUG-β-CDE enhances the cytotoxic activity of DOX in KB cells.

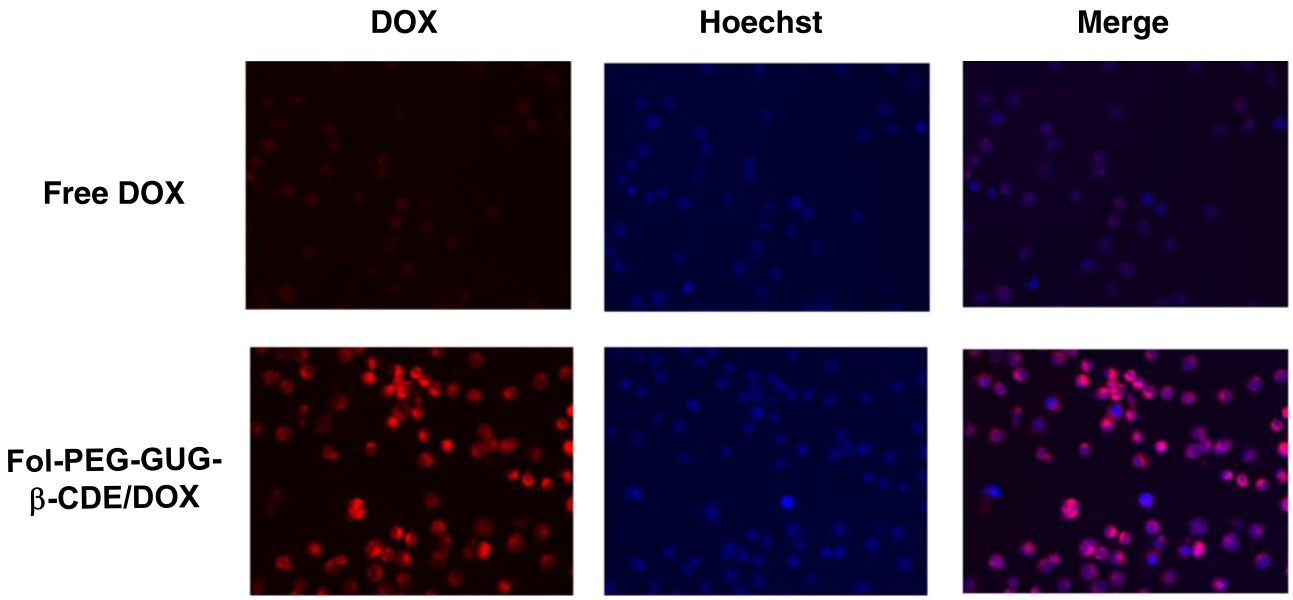

Fig. 3. Cellular uptake and intracellular distribution of the Fol-PEG-GUG-β-CDE/DOX complex in KB cells. The cells were incubated with free DOX or Fol-PEG-GUG-β-CDE/DOX complex (5 μM DOX) for 4 h and observed under a fluorescence microscope

Particle Size and ζ-Potential of the Fol-PEG-GUG-β-CDE/DOX/siRNA Complex

To fabricate the advanced antitumor agents, the ternary complex of PEG-GUG-β-CDE/DOX/siRNA was prepared by mixing the components at a molar ratio of 1:3 (carrier/DOX) and a charge ratio of 50 (carrier/siRNA) (Fig. 1). Subsequently, to evaluate the physicochemical properties of the complex, the particle sizes, polydispersity index (PDI), and ζ-potential values of the complex were estimated (Table II). The particle size of Fol-PEG-GUG-β-CDE/DOX/siRNA complex was approximately 92 nm with 0.4 of PDI at a charge ratio of 50. The ζ-potential value of the complex was approximately 32 mV, suggesting the formation of a positive nanoparticle.

Cytotoxic Activity of the Fol-PEG-GUG-β-CDE/DOX/siPLK1 Complex

We have previously reported that Fol-PEG-GUG-β-CDE/siPLK1 complex shows significant RNAi effects manifesting around 50% cytotoxic activity in KB cells (12). Therefore, to improve the cytotoxic activity of the complex, the ternary complex of Fol-PEG-GUG-β-CDE/siPLK1/DOX was further evaluated (Fig. 5a). The Fol-PEG-GUG-β-CDE/siPLK1 binary complex showed around 50% of cytotoxic activity in KB cells, as reported previously. On the other hand, the Fol-PEG-GUG-β-CDE/siPLK1/DOX ternary complex showed around 94% of cytotoxic activity in KB cells, suggesting that the ternary complex possesses stronger cytotoxic activity than the binary complex in KB cells.

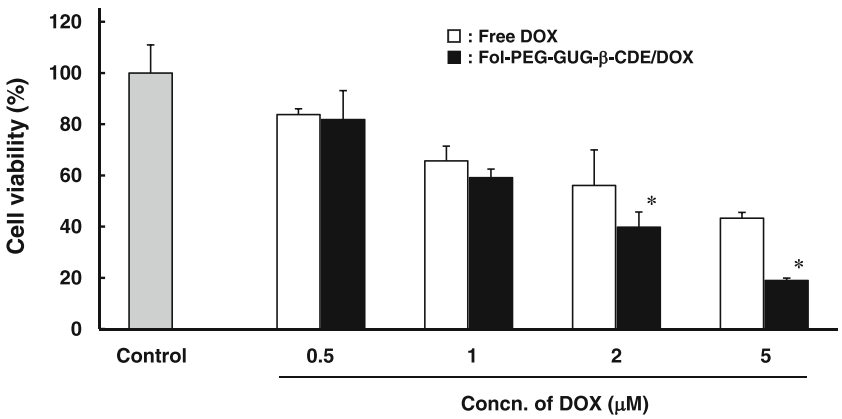

Fig. 4. Cytotoxic activity of the Fol-PEG-GUG-β-CDE/DOX complex in KB cells. The cells were incubated with free DOX or Fol-PEG-GUG-β-CDE/DOX complex for 4 h followed by supplementation with fresh media containing 10% FBS and incubation for 20 h. The cell viability was determined by the WST-1 method. Each value represents the mean ± S.E. of 3 experiments. $^{*}p < 0.05$ as compared with free DOX

Table II. Particle Size, Polydispersity Index, and ζ-potential of Fol-PEG-GUG-β-CDE/DOX/siRNA Complex

Molar ratio	Charge ratio	Particle size (nm)	PDI	ζ-potential (mV)
1:3	50	92.1 ± 3.9	0.42 ± 0.01	31.9 ± 0.6

Each value represents the mean ± S.E. of 3–4 experiments
PDI polydispersity index

To confirm the involvement of FR-α in the cytotoxic activity of the Fol-PEG-GUG-β-CDE/DOX/siPLK1 complex, the effects of folic acid, a competitor against FR-α on the cytotoxic activity, were examined. As shown in Fig. 5b, the cytotoxic activity of the ternary complex was significantly reduced by the addition of folic acid, indicating FR-α-overexpressing tumor cell-selectivity of the ternary complex.

In Vivo RNAi Effects and Antitumor Activity of the Fol-PEG-GUG-β-CDE/DOX/siPLK1 Complex

To evaluate *in vivo* RNAi effects of Fol-PEG-GUG-β-CDE/DOX/siPLK1 complex, the complex was intravenously injected twice a week to BALB/c *nu/nu* mice, and measured *PLK1* mRNA level in the tumor (Fig. 6). Fol-PEG-GUG-β-CDE/DOX/siPLK1 complex showed significant RNAi effects when compared with the siGL2 complex *in vivo*.

Next, to examine the *in vivo* antitumor activity of the Fol-PEG-GUG-β-CDE/DOX/siPLK1 complex, the complex solution was administered to KB cells-bearing BALB/c *nu/nu* mice twice a week, and the tumor size (Fig. 7a) or tumor weight (Fig. 7b) was monitored. As shown in Fig. 7a, the Fol-PEG-GUG-β-CDE/DOX/siPLK1 complex significantly suppressed the tumor growth compared with the Fol-PEG-GUG-β-CDE/siPLK1 binary complex. Moreover, tumor weight of KB cells-bearing BALB/c *nu/nu* mice was also decreased by the intravenous injection of Fol-PEG-GUG-β-CDE/DOX/siPLK1 complex for 30 days (Fig. 7b). Furthermore, a negligible change in body weight was observed after intravenous administration of the ternary complex compared with that of the control (5% mannitol solution), suggesting no severe adverse effects under the experimental conditions (Fig. 7c). These results suggest that the Fol-PEG-GUG-β-CDE/DOX/siRNA complex has potent antitumor activity *in vivo*.

In Vivo Safety Profile

To estimate the safety profiles of the Fol-PEG-GUG-β-CDE/DOX/siPLK1 complex, we have performed biochemical investigations after 24 h of intravenous administration of the complex to KB cells-bearing BALB/c *nu/nu* mice (Table III).

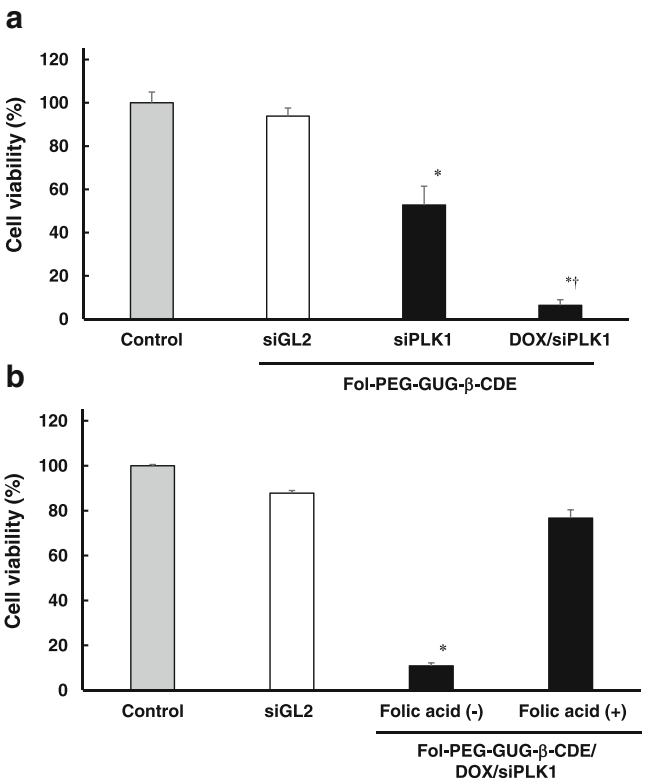

Fig. 5. **a** Cytotoxic activity of the Fol-PEG-GUG-β-CDE/DOX/siPLK1 complex in KB cells and **b** effects of folic acid on the cytotoxic activity of the Fol-PEG-GUG-β-CDE/DOX/siPLK1 complex in KB cells. The cells were transfected with the complexes (100 nM siRNA) for 4 h followed by supplementation with fresh media containing 10% FBS and incubation for 44 h. In the case of competition assay, the cells were pre-incubated with 4 mM FA for 1 h. Each value represents the mean ± S.E. of 3 experiments. $^{*}p < 0.05$ as compared with siGL2 and $^{\dagger}p < 0.05$ as to the siPLK1 complex

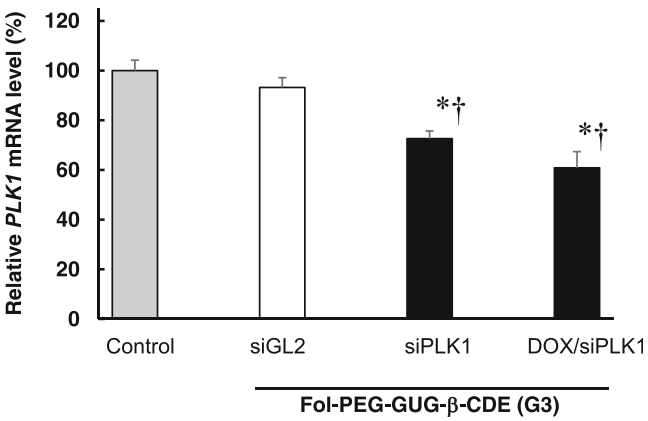

Fig. 6. *In vivo* RNAi effect of the Fol-PEG-GUG-β-CDE/DOX/siPLK1 complex after the intravenous administration in KB cells-bearing BALB/c *nu/nu* mice. Fol-PEG-GUG-β-CDE/DOX/siPLK1 complex (50 μg of siPLK1 and 100 μg of DOX) was intravenously administered to KB cells-bearing BALB/c *nu/nu* mice twice a week for 30 days. Each point represents the mean ± S.E. of 4–6 experiments. $^{*}p < 0.05$ as compared with control, $^{\dagger}p < 0.05$ as compared with siGL2 complex

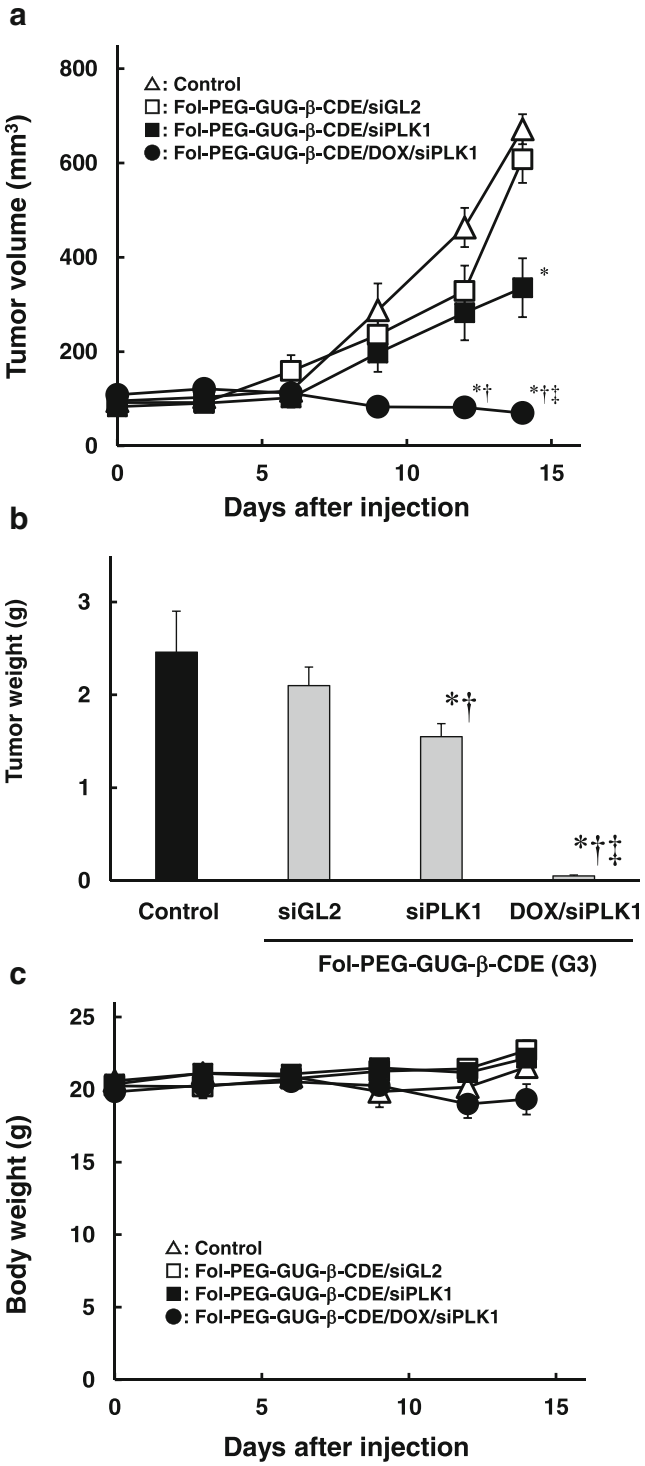

Fig. 7. *In vivo* antitumor activity of the Fol-PEG-GUG-β-CDE/DOX/siPLK1 complex. **a** Tumor size, **b** tumor weight, and **c** change in body weight of the mice after intravenous administration of the complex to KB cells-bearing BALB/c *nu/nu* mice. Fol-PEG-GUG-β-CDE/DOX/siPLK1 complex (50 μg of siPLK1 and 100 μg of DOX) was intravenously administrated to KB cells-bearing BALB/c *nu/nu* mice twice a week. Each point represents the mean ± S.E. of 4–6 experiments. $^{*}p < 0.05$ as compared with control, $^{\dagger}p < 0.05$ as compared with siGL2 complex, $^{\ddagger}p < 0.05$ as compared with siPLK1 complex

The CRE, BUN, AST, ALT, and LDH levels after administration of the Fol-PEG-GUG-β-CDE/DOX/siPLK1 complex were almost similar to those in the control (5% mannitol solution). These results suggest the safety of the Fol-PEG-GUG-β-CDE/DOX/siPLK1 complex *in vivo*.

DISCUSSION

In this study, we have prepared Fol-PEG-GUG-β-CDE/DOX binary complex and Fol-PEG-GUG-β-CDE/DOX/siRNA ternary complex, and evaluated their antitumor activities both *in vitro* and *in vivo*. During the preparation of the Fol-PEG-GUG-β-CDE/DOX complex, EE (%) of DOX was markedly decreased at a ratio of 1:6 (approximately 48%) compared to that at 1:3 (approximately 91%). In addition, Ke *et al.* previously reported that PAMAM dendrimer (G3) forms a complex with DOX at a molar ratio of 1:3.5 with 95% of EE (22). Therefore, one Fol-PEG-GUG-β-CDE molecule probably forms a complex with three DOX molecules.

As shown in Fig. 1, the Fol-PEG-GUG-β-CDE/DOX complex was successfully prepared by mixing both the components in water. Chandra *et al.* demonstrated that the interactions of DOX with oligo ethylene glycol-grafted PAMAM dendrimer are due to π–π stacking and/or hydrogen bond between DOX and the dendrimer (23). In addition, DOX forms an inclusion complex with β-CyD (24). Thus, these intermolecular interactions may be involved in the formation of the Fol-PEG-GUG-β-CDE/DOX complex. The stoichiometric characteristics of the Fol-PEG-GUG-β-CDE/DOX complex (1:3) and the PAMAM dendrimer (G3)/DOX complex (1:3.5) (22) are, however, almost the same, suggesting that GUG-β-CyD moiety in the Fol-PEG-GUG-β-CDE molecule may not be involved in the interaction between Fol-PEG-GUG-β-CDE and DOX, although GUG-β-CyD moiety in the Fol-PEG-GUG-β-CDE molecule is important for endosome escaping ability for siRNA delivery (11,25).

The release rate of DOX from the Fol-PEG-GUG-β-CDE/DOX complex at pH 5.5 was faster than that at pH 7.4 (Fig. 2). This may be due to the repulsion force between the positively charged DOX molecule and the protonated tertiary amino groups of dendrimer molecule at pH 5.5, resulting in accelerated release of DOX from the hydrophobic interior of dendrimer. Hereafter, we should clarify the release mechanism of DOX from the Fol-PEG-GUG-β-CDE/DOX complex.

It is pertinent to mention that the Fol-PEG-GUG-β-CDE/DOX complex exhibited significantly higher cytotoxic activity than free DOX in KB cells (Fig. 4). This was probably because of a higher cellular uptake of the Fol-PEG-GUG-β-CDE/DOX complex than that of free DOX (Fig. 3, Supplementary Fig. 1), and subsequent release of DOX from the complex at an acidic environment inside the endosome (Fig. 2). Herein, the complex showed a hydrodynamic size of 92 nm (Table II), which is a satisfactory diameter size for FR-α-mediated endocytosis (< 150 nm) (26). Therefore, efficient FR-α-mediated endocytosis of the complex may be responsible for its high cellular uptake. DOX, however, is generally taken up by a passive transport pathway aggressively. Hence,

Table III. Biochemical Results After Intravenous Administration of Fol-PEG-GUG-β-CDE/DOX/siRNA Complex in KB cells-bearing BALB/c *nu/nu* Mice

Sample	CRE (mg/dL)	BUN (mg/dL)	AST (U/L)	ALT (U/L)	LDH (U/L)
Control	0.2 ± 0.0	14.1 ± 0.5	54 ± 2	25.2 ± 1.5	435 ± 155
Fol-PEG-GUG-β-CDE/siRNA	0.2 ± 0.0	17.0 ± 1.1	77 ± 6	41.2 ± 4.6	505 ± 95
Fol-PEG-GUG-β-CDE/DOX/siRNA	0.2 ± 0.0	17.4 ± 0.8	60 ± 4	30.2 ± 2.5	461 ± 84

CRE creatinine, *BUN* blood urea nitrogen, *AST* alanine transaminase, *ALT* aspartate transaminase, *LDH* lactate dehydrogenase
Each value represents the mean ± S.E. of 4–6 experiments

some other mechanisms may be involved in a high cellular uptake of the complex. Hereafter, the effects complexation with Fol-PEG-GUG-β-CDE on DOX efflux by efflux pumps such as P-glycoprotein needs to be examined.

As shown in Fig. 5b, cytotoxic activity of Fol-PEG-GUG-β-CDE/DOX/siPLK1 complex significantly decreased in the presence of folic acid. Moreover, we previously reported that Fol-PEG-GUG-β-CDE shows higher siRNA transfer activity than GUG-β-CDE having no folate moiety in 786-0-luc cells [FR-α (+)] and KB cells [FR-α (+)] (12). In contrast, Fol-PEG-GUG-β-CDE showed lower siRNA transfer activity than GUG-β-CDE in A549 cells [FR-α (−)]. These results strongly suggest that cellular uptake of Fol-PEG-GUG-β-CDE/DOX complex is due to FR-α-mediated endocytosis.

To enhance the cytotoxic activity of the Fol-PEG-GUG-β-CDE/DOX complex, the ternary complex of Fol-PEG-GUG-β-CDE/DOX/siRNA was subsequently prepared (Fig. 1). As expected, the ternary complex showed a higher cytotoxic activity than the Fol-PEG-GUG-β-CDE/DOX binary complex in KB cells (Fig. 5a). This synergistically enhanced cytotoxic activity of DOX might be due to regulating the cell cycle of KB cells by PLK1 silencing which regulates a wide range of cell cycle proteins, causing an increase in the G2/M subpopulation. It is also known to increase the sensitivity of DOX (27–29).

Most importantly, the Fol-PEG-GUG-β-CDE/DOX/siPLK1 complex showed a potent antitumor activity *in vivo* (Fig. 7a, b). Fol-PEG-GUG-β-CDE possesses folate moiety and PEG chain in the molecules, anticipating targeting both DOX and siPLK1 passively and actively. Hereafter, the pharmacokinetics of DOX and siRNA after the administration of the ternary complex needs to be assessed. Meanwhile, the synergistic antitumor effects by co-delivery of various antitumor drugs and siRNAs have been demonstrated (30–33). The Fol-PEG-GUG-β-CDE/DOX/siPLK1 complex is also useful as a co-delivery system for low-molecular antitumor drugs and siRNA.

In recent years, a number of folate-dendrimer-based antitumor carriers have been reported. For instance, Quintana *et al.* prepared folate-modified dendrimers as tumor-selective carriers for low-molecular weight antitumor drugs (34). Singh *et al.* reported that folate-PEG/dendrimer conjugate shows high tumor selectivity compared with folate/dendrimer conjugate (35). Moreover, folate-CyD-based antitumor carriers have been also developed. Salmaso *et al.* developed folate-PEG/CyD conjugate as a tumor-selective antitumor drug carrier (36). We previously reported utility of multiple folate-modified CyDs as DOX carriers both *in vitro* and *in vivo* (37,38). In this context, Fol-PEG-GUG-β-CDE is useful as a drug carrier for both low-molecular weight drug and siRNA. In addition, Fol-PEG-GUG-β-CDE possesses CyD moiety and PEG moiety, resulting in its endosomal escaping ability and passive targeting ability. Moreover, Fol-PEG-GUG-β-CDE can form complex with DOX and siRNA by mixing in water, suggesting a facile preparation. Thus, we believe that Fol-PEG-GUG-β-CDE is useful as a tumor-selective carrier for low-molecular antitumor drug and siRNA.

While the results collected support that Fol-PEG-GUG-β-CDE/DOX/siRNA complex works in delivery and suppression of tumor growth, the detailed mechanism remains unclear. We believe that dendrimer in Fol-PEG-GUG-β-CDE works as a carrier for both siRNA and DOX, and that GUG-β-CyD is important for endosome escaping ability as reported previously (11,25).

CONCLUSION

In this study, we have prepared a Fol-PEG-GUG-β-CDE/DOX/siPLK1 ternary complex as an FR-α expressing tumor cell-selective co-delivery system for DOX and siRNA. The Fol-PEG-GUG-β-CDE/DOX binary complex exhibited a higher cellular uptake and a stronger cytotoxic activity than free DOX in KB cells. Moreover, the cytotoxic activity of the Fol-PEG-GUG-β-CDE/DOX/siPLK1 ternary complex was FR-α-mediated and superior to that of the binary complex. Furthermore, the Fol-PEG-GUG-β-CDE/DOX/siPLK1 ternary complex exhibited a potent antitumor activity *in vivo* without causing severe adverse effects. Taken together, these findings suggest that the Fol-PEG-GUG-β-CDE has the potential of a tumor-selective co-delivery carrier for low-molecular antitumor drugs and siRNA.

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AUTHOR CONTRIBUTIONS

AFAM, TH, KM, AO, RO, and HA had participated in the research design. AFAM and AO had conducted the experiments. AFAM, TH, KM, AO, and RO had performed the data analysis. AFAM and TH had drafted or contributed to the writing of the manuscript. TH, KM, KAK, HAS, AKH and HA had supervised the experiments.

COMPLIANCE WITH ETHICAL STANDARDS

All animal procedures were carried out in accordance with the approved guidelines and with the approval of the Ethics Committee for Animal Care and Use of Kumamoto University (approval no.: C29-162).

Conflict of Interest The authors declare that they have no conflict of interest.

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