Bioconjugate Chemistry

Article

pubs.acs.org/bc

Folate-Appended β-Cyclodextrin as a Promising Tumor Targeting Carrier for Antitumor Drugs *in Vitro* and *in Vivo*

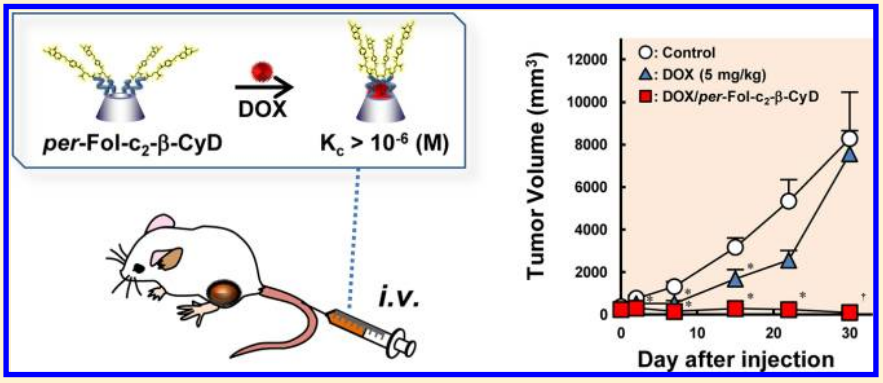
Ayaka Okamatsu,[†] Keiichi Motoyama,[†] Risako Onodera,[†] Taishi Higashi,[†] Takahiro Koshigoe,[‡] Yasutaka Shimada,[§] Kenjiro Hattori,[‡,§] Tomoko Takeuchi,[‡] and Hidetoshi Arima*[,†]

[†]Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Chuo-ku, Kumamoto 862-0973, Japan

[‡]Faculty of Engineering, Tokyo Polytechnic University, 1583 Iiyama, Atsugi 243-0297, Japan

[§]R&D Lab, NanoDex Inc., 705-1 Shimoimaizumi, Ebina 243-0435, Japan

Supporting Information



ABSTRACT: A large number of antitumor drug delivery carriers based on passive targeting and/or active targeting have been developed. However, encapsulation of antitumor drugs into these drug carriers is often complicated, and antitumor activities of these targeting systems are not satisfactory. In the present study, we first prepared *heptakis*-6-folic acid (FA)-appended β-cyclodextrin (β-CyD) possessing two caproic acids between FA and a β-CyD molecule as a spacer (Fol-c_2-β-CyD) and evaluated the potential as a novel tumor targeting carrier for antitumor drugs through a complexation. Fol-c_2-β-CyD formed an inclusion complex with doxorubicin (DOX) at a 1:1 molar ratio with a markedly high stability constant (>10^6 M^{-1}). Cellular uptake of DOX was increased by the addition of Fol-c_2-β-CyD in KB cells, a folate receptor-α (FR-α)-positive cell line. Additionally, Fol-c_2-β-CyD increased *in vitro* antitumor activities of antitumor drugs such as DOX, vinblastine (VBL), and paclitaxel (PTX) in KB cells, but not in A549 cells, a FR-α-negative cell line. The complex of DOX with Fol-c_2-β-CyD markedly increased antitumor activity of DOX, not only after intratumoral administration but also after intravenous administration to mice subcutaneously inoculated Colon-26 cells, a FR-α-positive cell line. These findings suggest that Fol-c_2-β-CyD could be useful as a promising antitumor drug carrier.

INTRODUCTION

Although many antitumor drugs are used in the pharmaceutical field, they have significant adverse effects because of nonspecific delivery and/or drug resistance in cancer cells. Drug delivery system (DDS) such as targeting is one of the most promising approaches to improve adverse effects of antitumor drugs. Targeting techniques are classified into passive targeting and active targeting, and the former is based on the enhanced permeability and retention (EPR) effect.[1−3] The representative product possessing passive targeting ability is Doxil, which is polyethylene glycol (PEG)-modified liposome containing doxorubicin (DOX). Doxil can avoid capture from the reticuloendothelial system (RES) because of the introduction of PEG with liposomes.[4−6] Although passive targeting is useful in cancer chemotherapy, it is difficult to completely eliminate the adverse effects of antitumor drugs.[4] Therefore, the development of drug carriers possessing both passive and active targeting ability to tumor tissues and cells is strongly expected to enhance the therapeutic effects and to reduce any adverse effects.

To achieve active targeting drug delivery, a chemical modification of drug carriers with tumor targeting ligands such as sugar,[7] transferrin,[8,9] antibody,[10] peptides,[6,11,12] and folic acid (FA)[13,14] is utilized. Of these ligands, FA is one of the most widely used because of the following advantages:[15,16] (1) folate receptor-α (FR-α) is highly expressed in many human tumor cells, including malignancies of the ovary, kidney, breast, myeloid cells, brain, and lung, (2) FA can be recognized by FR-α with high binding constants (K_d ∼ 10^{10} M^{-1}), (3) high compatibility with a variety of organic and aqueous solvent, (4) low immunogenicity, (5) low molecular weight (MW 441.4),

Received: January 8, 2013

   dx.doi.org/10.1021/bc400015r | *Bioconjugate Chem.* XXXX, XXX, XXX−XXX

and (6) low cost. Hence, a large number of folate-appended drug carriers, e.g., liposomes,[13,17−19] dendrimers,[20] polysaccharides,[21,22] and micelles,[23] have been developed. However, encapsulation of antitumor drugs into these drug carriers is often complicated, and antitumor activities of these targeting systems are not satisfactory. Recently, folate-conjugated vinca alkaloids, EC145, have emerged as a candidate for clinical development.[24] In this conjugate, however, only one FA was modified in one drug molecule, highly possibly resulting in lower binding to FR-α than FA. Thus, folate-appended drug carriers which enable easiest drug encapsulation and more effective drug delivery are required.

Cyclodextrins (CyDs) were first isolated in 1891 as degradation products of starch, and α-, β-, and γ-CyDs are the most common natural CyDs, consisting of six, seven, and eight glucose units, respectively. CyDs are cyclic oligosaccharides forming inclusion complexes with a wide range of hydrophobic molecules, and are utilized as drug delivery carriers.[25−29]

Recently, some folate-appending CyDs as tumor-targeting drug delivery carriers have been reported. Salmaso et al.[30,31] demonstrated that the conjugate of FA with β-CyD through a PEG spacer promotes the uptake of rhodamine-B into KB cells, FR-α-overexpressing human epidermal carcinoma, but not into MCF7 cells, non-FR-α expressing human lung carcinoma cell line. Also, Zhang et al.[32] prepared the FA-modified β-CyD with a PEG spacer through a click chemistry strategy. This conjugate forms nanoparticle by entrapping 5-fluorouracil (5-FU) in aqueous solution, and the resulting nanoparticle shows selective uptake through FR-α-mediated endocytosis in the FR-α-overexpressing cells. However, there are few studies which achieved antitumor drug delivery *in vivo* using FA-conjugated CyDs. Generally, inclusion complexes of drugs with CyDs rapidly dissociate to free CyDs and free drugs after parenteral administration due to their weak interaction.[33] Besides, multivalent interaction between carriers and targeting receptors promotes the recognition of ligands by the receptors.[34,35] Thus, to achieve *in vivo* antitumor drug delivery using FA-conjugated CyDs, the optimal carrier design, which allows strong interaction between CyDs and drugs, as well as multivalent interaction between carriers and FR-α, is considered. Hattori et al.[36−40] prepared *heptakis*-sugar-branched CyDs with spacers between sugars and CyDs. The wide-open sugar antenna of these conjugates attracts and interns the drugs in aqueous solution (sea anemone effect), and these complexes show markedly high stability constants ($K_c \sim 10^9$ M^{-1}). Moreover, these conjugates also bind strongly with rat liver cells due to multivalent interaction between the sugar moieties of conjugates and the asialoglycoprotein receptor expressing on hepatocytes ($\sim 10^{10}$ M^{-1}).

On the basis of these backgrounds, we first prepared *heptakis*-6-FA-appended β-CyD possessing two caproic acids as a spacer between FA and β-CyD molecules (Fol-c_2-β-CyD) in this study. Then, the potential of this conjugate as an antitumor drug carrier was evaluated *in vitro* and *in vivo*.

EXPERIMENTAL PROCEDURES

Materials. β-CyD was donated by Nihon Shokuhin Kako (Tokyo, Japan). FA was purchased from Nacalai Tesque (Kyoto, Japan). RPMI-1640 culture medium (FA-free) was purchased from GIBCO (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Nissui Pharmaceuticals (Tokyo, Japan) and Nichirei (Tokyo, Japan), respectively. Tetramethyl rhodamine isothiocyanate (TRITC) was obtained from Funakoshi (Tokyo, Japan). DOX hydrochloride was donated by Mercian (Tokyo, Japan). All other chemicals and solvents were of analytical reagent grade, and deionized double-distilled water was used throughout the study.

Synthesis of *heptakis*-6-Cl-β-CyD (Cl-β-CyD, 1). β-CyD (3.00 g) dehydrated with ethanol was dissolved in dry DMF (21 mL) containing CaH_2 and methylsulfonyl chloride (2.9 mL) under a stream of nitrogen gas, and then the solution was stirred for 4.5 h at 75 °C. After cooling the solution for 30 min, ethanol (5 mL) was added and stirred for 30 min to stop the reaction. Next, 10 mL of sodium methanolate (28% methanol solution) was added to the reactant, and concentrated using a rotary evaporator (EYELA N-1000S, Tokyo Rikakikai, Tokyo, Japan). The sample was filtered and washed with methanol and H_2O. After drying the precipitate under reduced pressure for 16 h at 40 °C, Cl-β-CyD was obtained. The reaction was monitored by TLC (silica gel F_{254}, Merck, Whitehouse Station, NJ). Eluent: 1-buthanol/ethanol/water = 5:4:3 (v/v/v). Indicator: *p*-anisaldehyde. MALDI-TOF MS $[M+H]^+$ m/z 1266.

Synthesis of *heptakis*-6-N_3-β-CyD (N_3-β-CyD, 2). Cl-β-CyD (1.26 g) was dissolved in dimethylacetamide (DMAc)/H_2O (60:8 v/v), and sodium azide (1.42 g) was added to the solution. The mixture was stirred for 24 h at 110 °C. After cooling the solution for 1 h, large volume of H_2O was added to obtain precipitate. After filtration, the sample was dried under reduced pressure for 13 h at 40 °C, and N_3-β-CyD was obtained. The reaction was monitored by TLC (silica gel F_{254}, Merck, Whitehouse Station, NJ). Eluent: 1-buthanol/ethanol/water = 5:4:3 (v/v/v). Indicator: *p*-anisaldehyde. MALDI-TOF MS $[M+H]^+$ m/z 1308.

Synthesis of *heptakis*-6-NH_2-β-CyD (NH_2-β-CyD, 3). N_3-β-CyD (1.00 g) and triphenylphosphine (3.20 g) were dissolved in DMAc (73 mL) and stirred for 1 h at room temperature. Aqueous ammonia (25%, 73 mL) was added in the mixture, and stirred for 22.5 h at room temperature. The reactant was precipitated with H_2O (730 mL) and dissolved in DMAc. And then, the reactant was precipitated and washed with acetone. After drying under reduced pressure for 20 h at 40 °C, NH_2-β-CyD was obtained. The reaction was monitored by TLC (silica gel F_{254}, Merck, Whitehouse Station, NJ). Eluent: 1-buthanol/ethanol/water = 5:4:3 (v/v/v). Indicator: *p*-anisaldehyde and ninhydrin. MALDI-TOF MS $[M+H]^+$ m/z 1129.

Synthesis of Boc-cap$_1$-OH. 6-Aminohexanoic acid (65.60 g) was dissolved in dioxane/H_2O (500:250 v/v), and triethylamine (84 mL) and di-*tert*-butyl dicarbonate (131.00 g) were added under stirring for 30 min on ice. Then, the mixture was stirred for 36 h at room temperature. The reactant was concentrated using a rotary evaporator, and sodium hydrogen carbonate (84.02 g) and H_2O (500 mL) were added to the reactant. After adjusting pH at 8.0, the sample was transferred into separatory funnel, and then washed six times with acetic ether (200 mL). Next, citric acid (150 g) was added to the sample and the pH of the sample was adjusted at 3.9. The sample was extracted four times with acetic ether (200 mL). Sodium sulfate anhydrate (200 g) was added to the sample, and stirred for 11 h at room temperature. After filtration, the sample was washed with acetic ether. *n*-Hexane (50 mL) was added to the sample, and concentrated by a rotary evaporator. This operation was repeated ten times. Acetic ether

dx.doi.org/10.1021/bc400015r | *Bioconjugate Chem.* XXXX, XXX, XXX−XXX

(50 mL) and petroleum ether (30 mL) were added, and precipitates were extracted by standing the solution for 12 h in the freezer. Finally, the sample was dried under reduced pressure for 73 h at room temperature and Boc-cap$_1$-OH was obtained. The reaction was monitored by TLC (silica gel F_{254}, Merck, Whitehouse Station, NJ). Eluent: 1-buthanol/ethanol/water = 5:4:3 (v/v/v). Indicator: ninhydrin. MALDI-TOF MS $[M+Na]^+$ m/z 254.

Synthesis of 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium Chloride (DMT-MM). *N*-Methylmorpholine (NMM, 14.8 mL) and 2-chloro-2,6-dimethoxy-1,3,5-triazin (25.29 g) were dissolved in tetrahydrofuran (THF, 450 mL), and the mixture was vigorously stirred for 1 h at room temperature. The white precipitate was washed three times with THF (100 mL). After drying under reduced pressure, DMT-MM was obtained. MALDI-TOF MS $[M-Cl]^+$ m/z 241.

Synthesis of *heptakis*-6-Boc-cap$_1$-β-CyD (Boc-c$_1$-β-CyD, 4). Boc-cap$_1$-OH (6.52 g), NH$_2$-β-CyD (4.32 g) and DMT-MM (8.48 g) were dissolved in methanol/H_2O (35:35 v/v), and stirring for 42 h at room temperature. The reactant was precipitated by H_2O (1200 mL). After filtration, the sample was washed with H_2O and dissolved in methanol. After condensation and lyophilization, Boc-c$_1$-β-CyD was obtained. The reaction was monitored by TLC (silica gel F_{254}, Merck, Whitehouse Station, NJ). Eluent: 1-buthanol/ethanol/water = 5:4:3 (v/v/v). Indicator: *p*-anisaldehyde and ninhydrin. MALDI-TOF MS $[M+Na]^+$ m/z 2644.

Synthesis of *heptakis*-6-NH$_2$-cap$_1$-β-CyD (NH$_2$-c$_1$-β-CyD, 5). Boc-c$_1$-β-CyD (8.03 g) was dissolved in 4 M HCl/dioxane (36 mL) and the mixture was stirred for 2 h on ice. After condensation, the reactant was lyophilized, and then NH$_2$-c$_1$-β-CyD was obtained. The reaction was monitored by TLC (silica gel F_{254}, Merck). Eluent: 1-buthanol/ethanol/water = 5:4:3 (v/v/v). Indicator: *p*-anisaldehyde and ninhydrin. MALDI-TOF MS $[M+H]^+$ m/z 1920.

Synthesis of *heptakis*-6-Boc-cap$_2$-β-CyD (Boc-c$_2$-β-CyD, 6). NH$_2$-c$_1$-β-CyD (9.65 g) and Boc-cap$_1$-OH (24.30 g) were dissolved in methanol (210 mL), and NMM (11.1 mL) and DMT-MM (29.01 g) were added into the mixture. After agitation for 37 h at room temperature, the reactant was precipitated with H_2O (2 L). The crude product was filtered and washed five times with H_2O (100 mL). The reactant was dissolved in methanol and concentrated using a rotary evaporator. The sample was dried under reduced pressure for 73 h at 40 °C, and then Boc-c$_2$-β-CyD was obtained. The reaction was monitored by TLC (silica gel F_{254}, Merck, Whitehouse Station, NJ). Eluent: 1-buthanol/ethanol/water = 5:4:3 (v/v/v). Indicator: *p*-anisaldehyde and ninhydrin. MALDI-TOF MS $[M+Na]^+$ m/z 3435.

Synthesis of *heptakis*-6-NH$_2$-cap$_2$-β-CyD (NH$_2$-c$_2$-β-CyD, 7). Boc-c$_2$-β-CyD (21.86 g) was dissolved in 4 M HCl/dioxane (85 mL) and the mixture was stirred for 4 h on ice. After condensation, the reactant was lyophilized, and then NH$_2$-c$_2$-β-CyD was obtained. The reaction was monitored by TLC (silica gel F_{254}, Merck, Whitehouse Station, NJ). Eluent: 1-buthanol/ethanol/water = 5:4:3 (v/v/v). Indicator: *p*-anisaldehyde and ninhydrin. MALDI-TOF MS $[M+Na]^+$ m/z 2735.

Synthesis of *heptakis*-6-Fol-cap$_2$-β-CyD (Fol-c$_2$-β-CyD, 8). NH$_2$-c$_2$-β-CyD (53.3 mg) and FA (276.9 mg) were dissolved in DMSO (20 mL) at 90 °C. After cooling, NMM (66 μL), DMT-MM (177.7 mg), and methanol (10 mL) were added to the mixture, and then the mixture was stirred for 45 h at 35 °C. The reactant was precipitated with acetone, and washed five times with acetone (5 mL). Finally, the sample was purified by Bio-Gel P4 (column, ϕ 4 cm × 69 cm; flow rate, 0.21 mL/min; sample size, 301.3 mg/3 mL 1 M NH_3 aq.), and then Fol-c$_2$-β-CyD was obtained. The reaction was monitored by TLC (silica gel F_{254}, Merck, Whitehouse Station, NJ). Eluent: 1-buthanol/ethanol/water/25% NH_3 aq = 5:4:3:5 (v/v/v/v). Indicator: *p*-anisaldehyde and ninhydrin. MALDI-TOF MS $[M+H]^+$ m/z 5674.

^{1}H NMR Spectrometry. ^{1}H NMR spectra were taken at 25 °C on a JEOL JNM-ECP500 (Tokyo, Japan), operating at 500 MHz, using a 5 mm sample tube. Deuterated water (D_2O) and DMSO (DMSO-d_6) were used as solvent. The samples were dissolved in D_2O or DMSO-d_6 at a concentration of 5 mg/0.6 mL. The signal of D_2O or DMSO-d_6 was used as an internal reference for ^{1}H NMR.

Surface Plasmon Resonance (SPR). The molecular interaction of DOX with Fol-c$_2$-β-CyD was examined using an optical biosensor "IAsys" based on SPR (Affinity Sensor, Cambridge, UK). The immobilization of DOX on the sensor cuvette was carried out by the reaction of a reactive linker molecule with the cuvette surface. After activation by washing with 8 M urea solution containing 10 mM $MnCl_2$, the interaction curves were measured at the concentration of Fol-c$_2$-β-CyD (0 to 1 μM) in 10 mM acetate buffer (pH 7.3) with 1 mM $CaCl_2$ and 100 mM NaCl at 25 °C. The association constant was obtained by measuring the change in the refractive index according to the usual procedure. The computational results were derived using a software FAST-fit equipped in the IAsys.

Fluorescence Spectrometry. DOX was dissolved in phosphate buffer (pH 7.3 or 6.8) in the presence of various concentrations of β-CyD, FA-appended β-CyD without a spacer (degree of substitution of FA, 5; FA-β-CyD), and Fol-c$_2$-β-CyD. Fluorescence spectra of the samples were measured at 25 °C using F-4500 fluorescence spectrometers (Hitachi, Tokyo, Japan).

Stoichiometry. The stoichiometry of the complex of DOX with Fol-c$_2$-β-CyD in phosphate buffer (pH 7.3) at 25 °C was determined by the continuous variation method,[41] analyzing the change in fluorescence intensity of DOX, where the total concentrations of DOX and Fol-c$_2$-β-CyD were kept constant at 50 μM.

Stability Constant. The stability constants of the complex of DOX with Fol-c$_2$-β-CyD in phosphate buffer (pH 7.3 or 6.8) were determined by the analysis of changes in fluorescence intensity of DOX at λ_{em} 554 nm (λ_{ex} 470 nm). The K_c values were obtained from the following Scott's equation,[42] assuming the 1:1 guest/host interaction

$$a \cdot b \cdot L/d = 1/(K_c \cdot \varepsilon_c) + b/\varepsilon_c$$

where a is the total concentration of DOX, b is the total concentration of Fol-c$_2$-β-CyD, L is length of cell, ε_c is the difference in fluorescence intensity for free and complexed DOX, and d is the change in fluorescence intensity of DOX by the addition of Fol-c$_2$-β-CyD.

Cell Culture. A549 cells, a human lung epithelium cell line (FR-α (−)), were cultured as reported previously.[43,44] KB cells, a human squamous carcinoma cell line (FR-α (+)), and Colon-26 cells, a mouse colon adenocarcinoma cell line (FR-α (+)), were grown in a RPMI-1640 culture medium (FA-free) containing penicillin (1 × 10^5 mU/mL) and streptomycin

dx.doi.org/10.1021/bc400015r | *Bioconjugate Chem.* XXXX, XXX, XXX−XXX

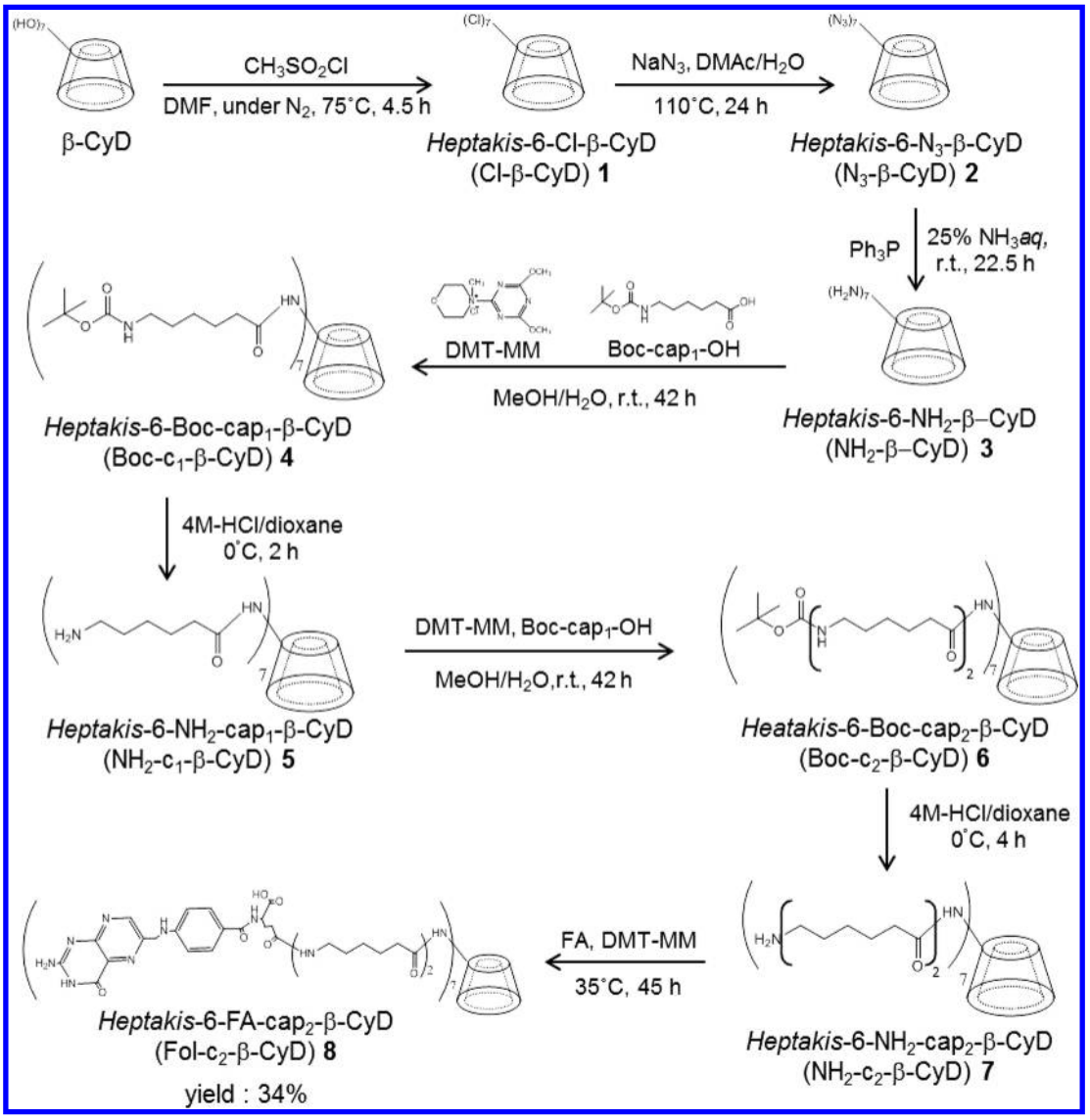


Figure 1. Preparation pathways of Fol-c_2-*β*-CyD.

(0.1 mg/mL) supplemented with 10% FBS at 37 °C in a humidified 5% CO_2 and 95% air atmosphere.

***In Vitro* Antitumor Activity.** *In vitro* antitumor activity was assayed by the WST-1 method (a Cell Counting Kit, Wako Pure Chemical Industries, Osaka, Japan), as reported previously.[45,46] Briefly, KB cells and A549 cells were seeded at 5×10^4 cells onto 96-well microplate (Iwaki, Tokyo, Japan) and incubated for 24 h in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. The cells were washed once with PBS (pH 7.4). In the case of KB cells system, 150 μL of RPMI-1640 culture medium (FA-free) containing 10 μM DOX in the absence and presence of Fol-c_2-*β*-CyD (molar ratio = 1:1) or Tween 20 was added to the cells and incubated for 24 h at 37 °C. In the case of A549 cells system, 150 μL of DMEM containing 1 μM DOX in the absence and presence of Fol-c_2-*β*-CyD (molar ratio = 1:1) or Tween 20 was added to the cells and incubated for 24 h at 37 °C. After washing once with PBS to remove the samples, 100 μL of fresh Hanks' balanced salt solution (HBSS, pH 7.4) and 10 μL of WST-1 reagent were added to the plates and incubated for 30 min at 37 °C. The absorbance at 450 nm against a reference wavelength of 620 nm was measured with a microplate reader (Bio-Rad model 550, Tokyo, Japan).

Cellar Uptake of Fol-c_2-*β*-CyD. KB cells and A549 cells were seeded at 4×10^5 cells onto 35 mm microplate (Iwaki, Tokyo, Japan), and incubated for 24 h in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. The cells were washed once with 1 mL of PBS, and then incubated for 1 h at 37 °C with 2 mL of RPMI-1640 culture medium (FA-free) in the KB cells system or DMEM in the A549 cells system in the presence of 10 μM TRITC-labeled Fol-c_2-*β*-CyD with or without FA (4 mM). Next, the cells were washed with 1 mL of PBS. Fluorescence intensity of TRITC was measured by FACSCalibur (BD Biosciences, Franklin Lakes, NJ).

Cellar Uptake of DOX. KB cells were seeded at 4×10^5 cells onto 35 mm microplate (Iwaki, Tokyo, Japan), and incubated for 24 h in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. The cells were washed once with 1 mL of PBS, and then incubated for 1 h at 37 °C with 1 mL of RPMI-1640 culture medium (FA-free) containing 10 μM DOX or DOX/Fol-c_2-*β*-CyD (molar ratio = 1:1). After washing with 1 mL of RPMI-1640 culture medium, the cells were observed by

dx.doi.org/10.1021/bc400015r | *Bioconjugate Chem.* XXXX, XXX, XXX–XXX

a fluorescence microscopy BZ-9000 (KEYENCE, Osaka, Japan).

***In Vivo* Antitumor Effect.** Four-week-old BALB/c male mice (ca. 20 g) were subcutaneously injected with the suspension containing Colon-26 carcinoma cells (2×10^5 cells/100 μL), FR-α-expressing cells. About 10 days later (diameter of tumor was ca. 8 mm), the mannitol solution (5%) including DOX (3 or 5 mg/kg) with or without Fol-c_2-β-CyD (25 mg/kg) was administered by single intratumoral or intravenous injection to Colon-26 cells (FR-α (+))-bearing mice. The tumor volume was determined by the equation (Volume = $LW^2/2$), where L is the longest dimension parallel to the skin surface and W is the dimension perpendicular to L and parallel to the surface. The body weight change of tumor-bearing mice was monitored for 30 days.

Data Analysis. Data are given as the mean ± SEM. Statistical significance of mean coefficients for the studies was performed by analysis of variance followed by Scheffe's test. p-Values for significance were set at 0.05.

RESULTS

Preparation of Fol-c_2-β-CyD. Figure 1 shows the preparation pathway of Fol-c_2-β-CyD. First, *per*-NH_2-β-CyD was obtained through chlorination, and azidation of primary hydroxyl groups of β-CyD. MALDI-TOF MS spectra of Cl-β-CyD, N_3-β-CyD, and NH_2-β-CyD indicated that they have seven chloro, azido, and amino groups, respectively (Figures S1−3). Next, two caproic acids were condensed with amino groups of NH_2-β-CyD by DMT-MM (Figures S4, S5). After deprotection (Figure S6), FA was modified to NH_2-c_2-β-CyD using DMT-MM, and Fol-c_2-β-CyD was obtained. Product yield of Fol-c_2-β-CyD was 34%, and no unreacted compounds were confirmed by TLC. According to the result of ^{1}H NMR spectrum, the degree of substitution of folate (DSF) was determined to be 7.0 from the integral values of the protons of benzene ring of folate and anomeric protons of glucose in β-CyD (Figure S7A). In addition, MALDI-TOF MS spectrum of Fol-c_2-β-CyD showed a parent peak at 5698 m/z, indicating the successful preparation of Fol-c_2-β-CyD (DSF 7.0) (Figure S7B).

Complex Formation between Fol-c_2-β-CyD and DOX. DOX is one of the most effective antitumor drugs. Hence, we selected DOX as an antitumor drug in this study, and the stoichiometry of a host−guest complex was determined by the continuous variation plot method (Figure 2).[41] The plots made by monitoring the fluorescence intensity change gave a maximum peak at 0.5, indicating that Fol-c_2-β-CyD forms an inclusion complex with DOX at a 1:1 molar ratio.

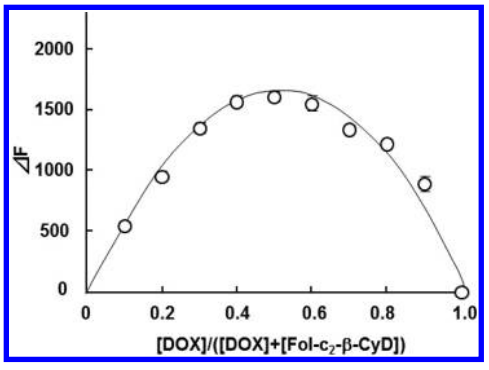


Figure 2. Continuous variation plots for DOX/Fol-c_2-β-CyD system monitored at 554 nm. The total concentration of DOX and Fol-c_2-β-CyD was 50 μM. Each points represent the mean ± SEM of 3 experiments.

Next, to determine the K_c of DOX/Fol-c_2-β-CyD complex at pH 7.3, fluorescence spectra of DOX in the absence and presence of Fol-c_2-β-CyD were measured (Figure 3A). Also, the

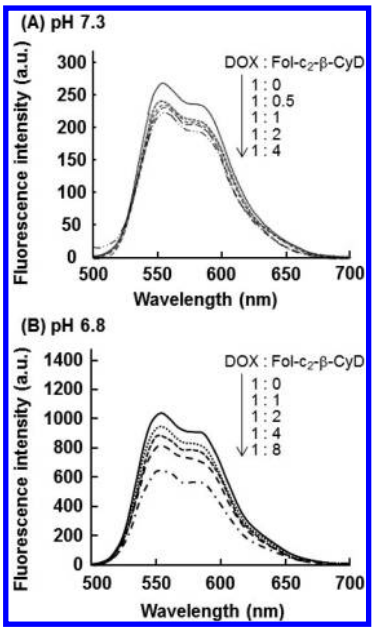


Figure 3. Effects of Fol-c_2-β-CyD on fluorescence spectrum of DOX in phosphate buffer (pH 7.3 or 6.8).

K_c value at pH 6.8 was determined (Figure 3B), since the pH value in FR-α-mediated endosomes, i.e., GPI-anchored-protein-enriched endosomal compartment (GEEC), is 6.0−6.8.[47] The fluorescence intensity of DOX decreased as the concentration of Fol-c_2-β-CyD increased, indicating a degree of interaction between both compounds. Strikingly, the K_c values of the complex determined using Scott's equation were 2.4×10^6 M^{-1} at pH 7.3 (Table 1). It should be noted that the K_c value of the

Table 1. Stability Constants (K_c) of DOX/β-CyDs Complexes

β-CyD system	method	pH	K_c (M^{-1})
Fol-$c_2$$\beta$-CyD	fluorescence spectrum	7.3	2.4×10^6
Fol-c_2-β-CyD	SPR	7.3	3.5×10^7
Fol-c_2-β-CyD	fluorescence spectrum	6.8	3.1×10^4
FA-β-CyD	fluorescence spectrum	7.3	4.9×10^5
β-CyD	fluorescence spectrum	7.3	2.2×10^2

complex was also determined to be 3.5×10^7 M^{-1}at pH 7.3 by SPR method (Table 1). Interestingly, the K_c values of the complexes of FA-β-CyD and β-CyD were 4.9×10^5 M^{-1} and 2.2×10^2 M^{-1}, respectively, indicating that the extremely high K_c value of the complex of Fol-c_2-β-CyD with DOX at pH 7.3 is reliable (Table 1). Meanwhile, the K_c value of DOX/Fol-c_2-β-CyD complex was 3.1×10^4 M^{-1} at pH 6.8 (Table 1). Hence, these results suggest that Fol-c_2-β-CyD forms the stable complex with DOX in bloodstream, and the complex could dissociate in endosomes after cellular uptake.

Cellular Uptake. Cellular uptake of Fol-c_2-β-CyD fluorescence-labeled with TRITC was examined using both a fluorescence microscope and flow cytometry. Strong fluo-

dx.doi.org/10.1021/bc400015r | *Bioconjugate Chem.* XXXX, XXX, XXX−XXX

rescence of TRITC-Fol-c_2-β-CyD was observed after treatment for 1 h in KB cells, a FR-α-positive cell line (Figure S8). In contrast, the fluorescence of TRITC-β-CyD was negligible, suggesting that folate moieties in Fol-c_2-β-CyD molecule accelerate its uptake into KB cells. Furthermore, cellular uptake of TRITC-Fol-c_2-β-CyD in KB cells was significantly higher than that in A549 cells, a FR-α-negative cell line (Figure 4A),

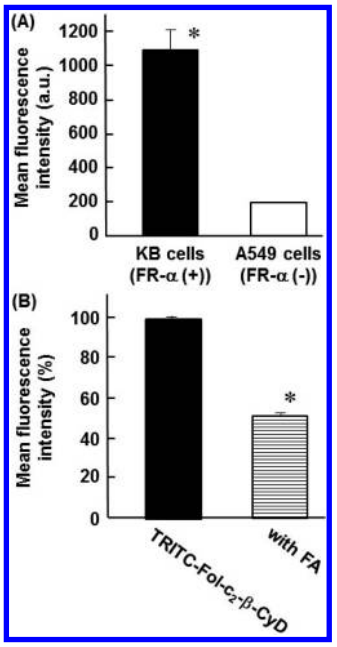


Figure 4. Cellular uptake of TRITC-Fol-c_2-β-CyD into KB (FR-α (+)) cells and A549 cells (FR-α (−)) (A), and the effects of FA on cellular uptake of TRITC-Fol-c_2-β-CyD into KB cells (B). The fluorescence intensity of TRITC in the cells was determined 1 h after incubation at 37 °C by a flow cytometer. The concentration of Fol-c_2-β-CyD was 10 μM. The concentration of FA was 4 mM. Each value represents the mean ± SEM of 3 experiments. *p < 0.05, compared with A549 cells or TRITC-Fol-c_2-β-CyD.

and was decreased by the addition of free FA as a competitor of FR-α (Figure 4B). The amount of DOX in KB cells also increased in the presence of Fol-c_2-β-CyD (Figures 5, S9). These results suggest that cellular uptake of Fol-c_2-β-CyD or its complex with DOX could be mediated by FR-α on KB cells.

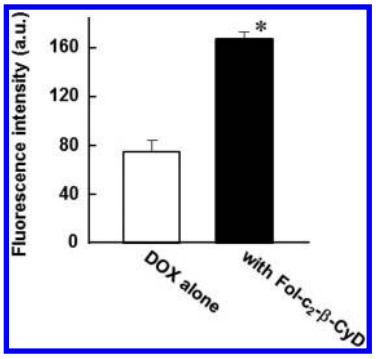


Figure 5. Cellular uptake of DOX into KB cells (FR-α (+)) in the absence and presence of Fol-c_2-β-CyD. The fluorescence intensity of DOX in the cells was determined 1 h after incubation at 37 °C by a fluorescence microscope. The concentration of Fol-c_2-β-CyD and DOX was 10 μM. Each value represents the mean ± SEM of 3−6 experiments. *p < 0.05, compared with DOX alone.

***In Vitro* Antitumor Activity.** The antitumor activity of DOX/Fol-c_2-β-CyD complex was evaluated in KB cells (Figure 6A) and A549 cells (Figure 6B). The DOX concentration was

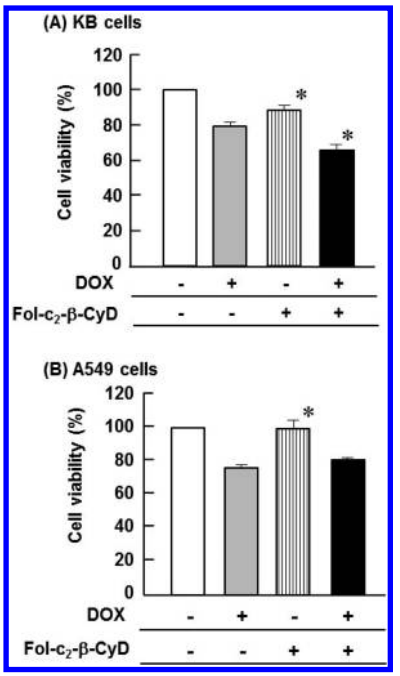


Figure 6. Cytotoxic activity of DOX/Fol-c_2-β-CyD complex in KB cells (FR-α (+)) (A) and A549 cells (FR-α (−)) (B) after treatment for 24 h. The molar ratio of DOX/Fol-c_2-β-CyD was 1:1. The concentrations of DOX in the KB cells and A549 cells systems were 10 μM and 1 μM, respectively. Each value represents the mean ± SEM of 3−18 experiments. *p < 0.05, compared with DOX alone.

set at 10 μM and 1 μM in the KB cells and A549 cells, respectively, because moderate antitumor activities of DOX were observed at the concentration in both cells. Fol-c_2-β-CyD increased antitumor activity of DOX in KB cells, but not in A549 cells. Importantly, these preferred effects of Fol-c_2-β-CyD were observed not only with DOX but also with other hydrophobic antitumor drugs such as vinblastine (VBL) and paclitaxel (PTX), whereas Fol-c_2-β-CyD did not increase antitumor activity of 5-FU, a hydrophilic antitumor drug (Figure S10). These results suggest that Fol-c_2-β-CyD could increase antitumor activities of hydrophobic antitumor drugs through markedly stable complexation, not hydrophilic drugs, in FR-α-positive cells.

***In Vivo* Antitumor Activity.** To investigate the antitumor activity of DOX/Fol-c_2-β-CyD complex *in vivo*, the solution containing the complex was administered intratumorally and intravenously to mice subcutaneously inoculated with Colon-26 cells, an FR-α-positive mouse colon carcinoma cell line. As shown in Figure 7A, an intratumoral administration of the DOX/Fol-c_2-β-CyD complex significantly inhibited the tumor growth, compared to that of control and DOX alone. Additionally, alterations in the body weight of the mice were almost the same among the all samples (Figure 7B). Thus, Fol-c_2-β-CyD could increase antitumor activity of DOX after intratumoral administration to tumor-bearing mice.

Next, antitumor activity of DOX/Fol-c_2-β-CyD complex after single intravenous administration to tumor-bearing mice was evaluated (Figure 7C). Intravenous injection of DOX did not inhibit the tumor growth under the present experimental

dx.doi.org/10.1021/bc400015r | *Bioconjugate Chem.* XXXX, XXX, XXX−XXX

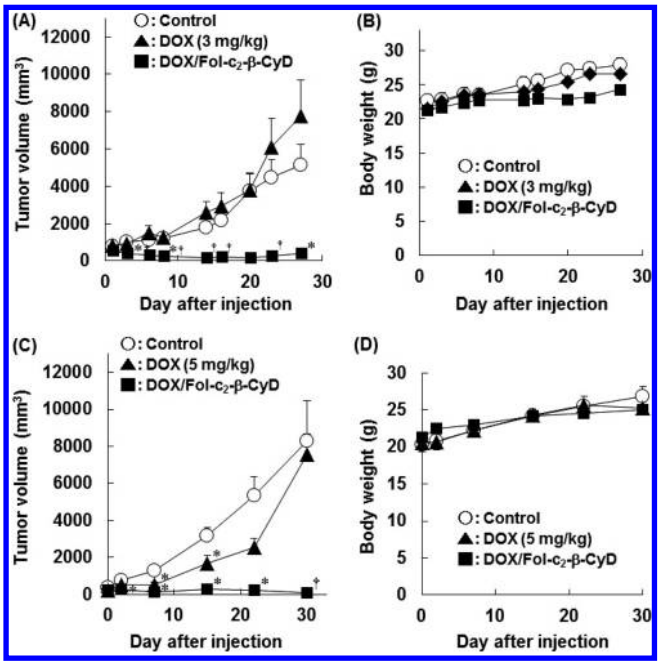


Figure 7. Tumor volume (A,C) and body weight (B,D) after intratumoral (A,B) and intravenous (C,D) administration of control (○), DOX (▲), and DOX/Fol-c_2-β-CyD complex (■) to Colon-26 cells (FR-α (+))-bearing mice. Each points represent the mean ± SEM of 3−10 experiments. $^*p < 0.05$, compared with control. $^\dagger p < 0.05$, compared with DOX alone.

conditions. In contrast, the DOX/Fol-c_2-β-CyD complex markedly inhibited the tumor growth after intravenous injection with no significant alteration in the body weight of the mice (Figure 7D). These results strongly suggest that Fol-c_2-β-CyD is useful for an antitumor drug carrier even after intravenous administration.

■ DISCUSSION

In this study, we prepared folate-appended β-CyD with two caproic acids as a spacer, and evaluated antitumor activities of its complexes with DOX *in vitro* and *in vivo*. To our knowledge, this is the first report in which β-CyD derivatives show a systemic antitumor effect through not only its extremely stable complexation with DOX, but also FA-dependent tumor targeting after intravenous administration in tumor-bearing mice.

The complex of Fol-c_2-β-CyD and DOX showed the extremely high K_c value at pH 7.3 ($\sim 10^6$ M^{-1}) (Table 1). Hattori et al. reported that *heptakis*-sugar-branched CyDs with caproic acids as a spacer between sugars and CyDs can strongly interact with the hydrophobic drugs through "sea anemone effect", i.e., ligand−spacer moieties of the conjugates include and intern the guest drugs.[37] Therefore, Fol-c_2-β-CyD could also include and trap DOX through "sea anemone effect". In fact, β-CyD did not show the high K_c value with DOX ($\sim 10^2$ M^{-1}) like the Fol-c_2-β-CyD complex (Table 1). Importantly, it is reported that a K_c value of more than 10^5 M^{-1} is required to maintain a stable complex *in vivo*,[33,48] signifying the potential of Fol-c_2-β-CyD as a drug carrier *in vivo*. In addition, the K_c value of the complex of Fol-c_2-β-CyD with DOX decreased to 3.1×10^4 M^{-1} at pH 6.8, probably due to the ionization of amine group in a DOX molecule. Yang et al. reported that the pH value in the FR-α-mediated endosome is 6.0−6.8.[47] Taken together, the Fol-c_2-β-CyD/DOX complex stably exists in bloodstream, and then may dissociate, at least in part, in GEEC after cellular uptake through FR-α-mediated CLIC-GEEC endocytic pathway.

Cellular uptake of Fol-c_2-β-CyD in KB cells was higher than that in A549 cells (Figure 4A). Likewise, a significantly high amount of DOX was introduced into KB cells in the presence of Fol-c_2-β-CyD (Figure 5). Hattori et al. reported that *heptakis*-galactose-branched β-CyD shows a high binding constant with the asialoglycoprotein receptor, compared to *bis*-galactose-branched β-CyD due to their strong multivalent interaction.[37] Thus, Fol-c_2-β-CyD, possessing seven FA molecules in a β-CyD molecule, could show the multivalent interaction with FR-α. Meanwhile, a spacer between FA and a carrier is important for recognition by FR-α, because a spacer gives flexibility to the FA moiety.[49] Most recently, we revealed that cellular uptake of Fol-c_2-β-CyD was twice as high as that of Fol-c_1-β-CyD, having one caproic acid as a spacer between FA and a β-CyD molecule (data not shown). However, it is still unknown whether Fol-c_2-β-CyD has favorable length of spacer and the DSF for cellular uptake into FR-α-positive cells. Thereafter, we should prepare the various folate-appended β-CyDs having a different length spacer, and evaluate their binding ability to FR-α.

Fol-c_2-β-CyD may suppress efflux of DOX from P-glycoprotein (P-gp)-overexpressing cells through the complexation. In the present study, cellular uptake of DOX was actually increased twice by the addition of Fol-c_2-β-CyD in KB cells (Figure 5), and cellular efflux of DOX from KB cells was significantly impaired by the complexation with Fol-c_2-β-CyD (Figure S11). Riganti et al. reported that DOX-containing anionic liposomal nanoparticles was less extruded by P-gp than DOX, and inhibited the pump activity.[50] In addition, Roger et al. demonstrated that FA functionalized nanoparticles have the potential to enhance the oral absorption of drugs with poor oral bioavailability involving in overcoming P-gp-mediated drug efflux.[51] These lines of evidence make it tempting to speculate that Fol-c_2-β-CyD may decrease the amount of P-gp in plasma membrane and impairment of transport of P-gp. In fact, our previous data that 2,6-di-*O*-methyl-β-CyD (DM-β-CyD) decreased the P-gp level in Caco-2 cells and VBL-resistant Caco-2 cells[52] may support our speculation. Studies are currently underway to investigate the mechanism by which Fol-c_2-β-CyD suppresses P-gp-mediated efflux of DOX in P-gp-overexpressing cells.

Fol-c_2-β-CyD increased antitumor activity of DOX in KB cells, but did not do so in A549 cells (Figure 6). Similarly, antitumor activities of VBL and PXT were increased by the addition of Fol-c_2-β-CyD in KB cells (Figures S10A, B). Meanwhile, these effects were not observed with 5-FU (Figure S10C). DOX, VBL, and PTX are hydrophobic drugs, which can interact with CyDs. In contrast, 5-FU is a hydrophilic drug, and thereby the interaction of 5-FU and β-CyD is very weak.[53] Thus, a complexation ability of Fol-c_2-β-CyD with antitumor drugs such as DOX, VBL, PTX, and 5-FU plays an important role for the antitumor effects of their complexes. Upadhyay et al. reported that methyl-β-CyD enhances the susceptibility of human breast cancer cells to carboplatin and 5-FU at high concentration (5 mM).[54] However, the concentration of Fol-c_2-β-CyD was only 10 μM in the present study. Therefore, it is sure that Fol-c_2-β-CyD does not enhance the susceptibility of KB cells to 5-FU.

dx.doi.org/10.1021/bc400015r | *Bioconjugate Chem.* XXXX, XXX, XXX−XXX

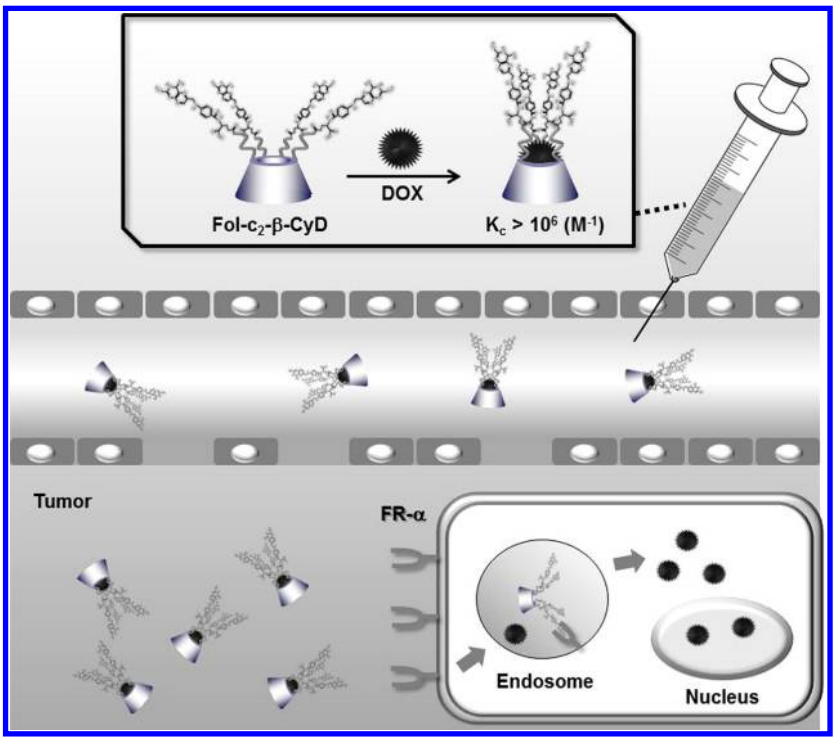


Figure 8. Proposed scheme for improved antitumor effect of DOX by Fol-c_2-β-CyD.

The complex of Fol-c_2-β-CyD with DOX showed markedly high antitumor activity after intratumoral and intravenous administration to tumor-bearing mice (Figure 7). It is noteworthy that this complex was administrated as a single dose, although the antitumor activity continued over 30 days. As shown in Figure S7B, the molecular weight of Fol-c_2-β-CyD is 5698, and is not sufficient for avoidance of glomerular filtration. In addition, Fol-c_2-β-CyD and DOX did not form any aggregates in phosphate buffer (data not shown). Soliman et al. demonstrated the presence of two folate-binding sites per albumin molecule and the equilibrium constant of $\sim 10^3$ M^{-1}.[55] Moreover, FA strongly binds to folate binding protein (FBP) in blood circulation.[56] Hence, the complex of Fol-c_2-β-CyD and DOX may bind to albumin and/or FBP, resulting in its long circulating half-life. Thereafter, pharmacokinetics of the complex of Fol-c_2-β-CyD and DOX after intravenous administration in mice should be clarified.

On the basis of the results obtained in this study, a proposed mechanism for improved antitumor effect of DOX by Fol-c_2-β-CyD is shown in Figure 8. Fol-c_2-β-CyD forms stable complexes with DOX and the other hydrophobic antitumor drugs in aqueous solution. After intravenous administration to tumor-bearing mice, the complex may accumulate in the tumor and may be incorporated in tumor cells through FR-α-mediated endocytosis. In addition, the complex may dissociate in endosome due to a decrease in the interaction between Fol-c_2-β-CyD and DOX. Finally, DOX may intercalate into DNA and inhibit the reaction of topoisomerase II, showing the antitumor activity (Figure 8). As described above, further investigations regarding the safety profile of the DOX complex with Fol-c_2-β-CyD as well as the detailed mechanism by which Fol-c_2-β-CyD enhanced antitumor activity of DOX are required.

CONCLUSION

In the present study, Fol-c_2-β-CyD was successfully prepared, and increased antitumor activities of antitumor drugs such as DOX, VBL, and PTX, not 5-FU *in vitro* and *in vivo*. In addition, Fol-c_2-β-CyD can very easily form complexes with antitumor drugs by simple mixing in aqueous solution. These results suggest that Fol-c_2-β-CyD could potentially be used as a promising antitumor drug carrier *in vitro* and *in vivo*, especially a systemic administration.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Telephone: +81-96-371-4160. Fax: +81-96-371-4420. E-mail: arimah@gpo.kumamoto-u.ac.jp.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This research was financially supported by the Sasagawa Scientific Research Grant from The Japan Science Society (R. Onodera, 2012).

ABBREVIATIONS

FA, folic acid; CyD, cyclodextrin; DDS, drug delivery system; EPR, enhanced permeability and retention; PEG, polyethylene glycol; DOX, doxorubicin; VBL, vinblastine; PTX, paclitaxel; 5-FU, 5-fluorouracil; RES, reticuloendothelial system; FR-α, folate receptor-α; NMM, N-methylmorpholine; DMT-MM, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride; DSF, degree of substitution of folate; SPR, surface

plasmon resonance; TRITC, tetramethyl rhodamine isothiocyanate; P-gp, P-glycoprotein; FBP, folate binding protein

REFERENCES

(1) Danhier, F., Feron, O., and Preat, V. (2010) To exploit the tumor microenvironment: Passive and active tumor targeting of nanocarriers for anti-cancer drug delivery. *J. Controlled Release 148*, 135−146.

(2) Matsumura, Y., and Maeda, H. (1986) A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. *Cancer Res. 46*, 6387−6392.

(3) Talekar, M., Kendall, J., Denny, W., and Garg, S. (2011) Targeting of nanoparticles in cancer: drug delivery and diagnostics. *Anticancer Drugs 22*, 949−962.

(4) Hong, R. L., Huang, C. J., Tseng, Y. L., Pang, V. F., Chen, S. T., Liu, J. J., and Chang, F. H. (1999) Direct comparison of liposomal doxorubicin with or without polyethylene glycol coating in C-26 tumor-bearing mice: is surface coating with polyethylene glycol beneficial? *Clin. Cancer Res. 5*, 3645−3652.

(5) Maruyama, K. (2011) Intracellular targeting delivery of liposomal drugs to solid tumors based on EPR effects. *Adv. Drug Delivery Rev. 63*, 161−169.

(6) Schiffelers, R. M., Koning, G. A., ten Hagen, T. L., Fens, M. H., Schraa, A. J., Janssen, A. P., Kok, R. J., Molema, G., and Storm, G. (2003) Anti-tumor efficacy of tumor vasculature-targeted liposomal doxorubicin. *J. Controlled Release 91*, 115−122.

(7) Roche, A. C., Fajac, I., Grosse, S., Frison, N., Rondanino, C., Mayer, R., and Monsigny, M. (2003) Glycofection: facilitated gene transfer by cationic glycopolymers. *Cell. Mol. Life Sci. 60*, 288−297.

(8) Hatakeyama, H., Akita, H., Maruyama, K., Suhara, T., and Harashima, H. (2004) Factors governing the *in vivo* tissue uptake of transferrin-coupled polyethylene glycol liposomes *in vivo*. *Int. J. Pharm. 281*, 25−33.

(9) Miyajima, Y., Nakamura, H., Kuwata, Y., Lee, J. D., Masunaga, S., Ono, K., and Maruyama, K. (2006) Transferrin-loaded nido-carborane liposomes: tumbetaor-targeting boron delivery system for neutron capture therapy. *Bioconjugate Chem. 17*, 1314−1320.

(10) Harata, M., Soda, Y., Tani, K., Ooi, J., Takizawa, T., Chen, M., Bai, Y., Izawa, K., Kobayashi, S., Tomonari, A., Nagamura, F., Takahashi, S., Uchimaru, K., Iseki, T., Tsuji, T., Takahashi, T. A., Sugita, K., Nakazawa, S., Tojo, A., Maruyama, K., and Asano, S. (2004) CD19-targeting liposomes containing imatinib efficiently kill Philadelphia chromosome-positive acute lymphoblastic leukemia cells. *Blood 104*, 1442−1449.

(11) Sugahara, K. N., Teesalu, T., Karmali, P. P., Kotamraju, V. R., Agemy, L., Girard, O. M., Hanahan, D., Mattrey, R. F., and Ruoslahti, E. (2009) Tissue-penetrating delivery of compounds and nanoparticles into tumors. *Cancer Cell 16*, 510−520.

(12) Sugahara, K. N., Teesalu, T., Karmali, P. P., Kotamraju, V. R., Agemy, L., Greenwald, D. R., and Ruoslahti, E. (2010) Coadministration of a tumor-penetrating peptide enhances the efficacy of cancer drugs. *Science 328*, 1031−1035.

(13) Chen, H., Ahn, R., Van den Bossche, J., Thompson, D. H., and O'Halloran, T. V. (2009) Folate-mediated intracellular drug delivery increases the anticancer efficacy of nanoparticulate formulation of arsenic trioxide. *Mol. Cancer Ther. 8*, 1955−1963.

(14) Lu, Y., and Low, P. S. (2002) Folate-mediated delivery of macromolecular anticancer therapeutic agents. *Adv. Drug Delivery Rev. 54*, 675−693.

(15) Leamon, C. P., and Low, P. S. (2001) Folate-mediated targeting: from diagnostics to drug and gene delivery. *Drug Discovery Today 6*, 44−51.

(16) Parker, N., Turk, M. J., Westrick, E., Lewis, J. D., Low, P. S., and Leamon, C. P. (2005) Folate receptor expression in carcinomas and normal tissues determined by a quantitative radioligand binding assay. *Anal. Biochem. 338*, 284−293.

(17) Gabizon, A., Tzemach, D., Gorin, J., Mak, L., Amitay, Y., Shmeeda, H., and Zalipsky, S. (2010) Improved therapeutic activity of folate-targeted liposomal doxorubicin in folate receptor-expressing tumor models. *Cancer Chemother. Pharmacol. 66*, 43−52.

(18) Riviere, K., Huang, Z., Jerger, K., Macaraeg, N., and Szoka, F. C., Jr. (2011) Antitumor effect of folate-targeted liposomal doxorubicin in KB tumor-bearing mice after intravenous administration. *J. Drug Targeting 19*, 14−24.

(19) Watanabe, K., Kaneko, M., and Maitani, Y. (2012) Functional coating of liposomes using a folate-polymer conjugate to target folate receptors. *Int. J. Nanomed. 7*, 3679−3688.

(20) Wang, Y., Cao, X., Guo, R., Shen, M., Zhang, M., Zhu, M., and Shi, X. (2011) Targeted delivery of doxorubicin into cancer cells using a folic acid-dendrimer conjugate. *Polym. Chem. 2*, 1754−1760.

(21) Sahu, S. K., Mallick, S. K., Santra, S., Maiti, T. K., Ghosh, S. K., and Pramanik, P. (2010) *In vitro* evaluation of folic acid modified carboxymethyl chitosan nanoparticles loaded with doxorubicin for targeted delivery. *J. Mater. Sci. Mater. Med. 21*, 1587−1597.

(22) Scomparin, A., Salmaso, S., Bersani, S., Satchi-Fainaro, R., and Caliceti, P. (2011) Novel folated and non-folated pullulan bioconjugates for anticancer drug delivery. *Eur. J. Pharm. Sci. 42*, 547−558.

(23) Kim, D., Lee, E. S., Oh, K. T., Gao, Z. G., and Bae, Y. H. (2008) Doxorubicin-loaded polymeric micelle overcomes multidrug resistance of cancer by double-targeting folate receptor and early endosomal pH. *Small 4*, 2043−2050.

(24) Leamon, C. P., Reddy, J. A., Vlahov, I. R., Westrick, E., Parker, N., Nicoson, J. S., and Vetzel, M. (2007) Comparative preclinical activity of the folate-targeted Vinca alkaloid conjugates EC140 and EC145. *Int. J. Cancer 121*, 1585−1592.

(25) Hirayama, F., and Uekama, K. (1999) Cyclodextrin-based controlled drug release system. *Adv. Drug Delivery Rev. 36*, 125−141.

(26) Li, J. J., Zhao, F., and Li, J. (2011) Supramolecular polymers based on cyclodextrins for drug and gene delivery. *Adv. Biochem. Eng. Biotechnol. 125*, 207−249.

(27) Szejtli, J. (1994) Medicinal applications of cyclodextrins. *Med. Res. Rev. 14*, 353−386.

(28) Szente, L., and Szejtli, J. (1999) Highly soluble cyclodextrin derivatives: chemistry, properties, and trends in development. *Adv. Drug Delivery Rev. 36*, 17−28.

(29) Uekama, K., Hirayama, F., and Irie, T. (1998) Cyclodextrin drug carrier systems. *Chem. Rev. 98*, 2045−2076.

(30) Salmaso, S., Semenzato, A., Caliceti, P., Hoebeke, J., Sonvico, F., Dubernet, C., and Couvreur, P. (2004) Specific antitumor targetable β-cyclodextrin-poly(ethylene glycol)-folic acid drug delivery bioconjugate. *Bioconjugate Chem. 15*, 997−1004.

(31) Caliceti, P., Salmaso, S., Semenzato, A., Carofiglio, T., Fornasier, R., Fermeglia, M., Ferrone, M., and Pricl, S. (2003) Synthesis and physicochemical characterization of folate-cyclodextrin bioconjugate for active drug delivery. *Bioconjugate Chem. 14*, 899−908.

(32) Zhang, H., Cai, Z., Sun, Y., Yu, F., Chen, Y., and Sun, B. (2012) Folate-conjugated β-cyclodextrin from click chemistry strategy and for tumor-targeted drug delivery. *J. Biomed. Mater. Res. A 100*, 2441−2449.

(33) Stella, V. J., and He, Q. (2008) Cyclodextrins. *Toxicol. Pathol. 36*, 30−42.

(34) Ooya, T., Eguchi, M., and Yui, N. (2003) Supramolecular design for multivalent interaction: maltose mobility along polyrotaxane enhanced binding with concanavalin A. *J. Am. Chem. Soc. 125*, 13016−13017.

(35) Ooya, T., Utsunomiya, H., Eguchi, M., and Yui, N. (2005) Rapid binding of concanavalin A and maltose-polyrotaxane conjugates due to mobile motion of α-cyclodextrins threaded onto a poly(ethylene glycol). *Bioconjugate Chem. 16*, 62−69.

(36) Abe, H., Kenmoku, A., Yamaguch, N., and Hattori, K. (2002) Structural effects of oligosaccharide-branched cyclodextrins on the dual recognition toward lectin and drug. *J. Incl. Phenom. Macrocycl. Chem. 44*, 39−47.

(37) Hattori, K., Kenmoku, A., Mizuguchi, T., Ikeda, D., Mizuno, M., and Inazu, T. (2006) Saccharide-branched Cyclodextrins as Targeting Drug Carriers. *J. Incl. Phenom. Macrocycl. Chem. 56*, 9−16.

(38) Oda, Y., Kobayashi, N., Yamanoi, T., Katsuraya, K., Takahashi, K., and Hattori, K. (2008) β-Cyclodextrin conjugates with glucose moieties designed as drug carriers: their syntheses, evaluations using concanavalin A and doxorubicin, and structural analyses by NMR spectroscopy. *Med. Chem. 4*, 244−255.
(39) Oda, Y., Yanagisawa, H., Maruyama, M., Hattori, K., and Yamanoi, T. (2008) Design, synthesis and evaluation of D-galactose-β-cyclodextrin conjugates as drug-carrying molecules. *Bioorg. Med. Chem. 16*, 8830−8840.
(40) Yamanoi, T., Yoshida, N., Oda, Y., Akaike, E., Tsutsumida, M., Kobayashi, N., Osumi, K., Yamamoto, K., Fujita, K., Takahashi, K., and Hattori, K. (2005) Synthesis of mono-glucose-branched cyclodextrins with a high inclusion ability for doxorubicin and their efficient glycosylation using Mucor hiemalis endo-β-N-acetylglucosaminidase. *Bioorg. Med. Chem. Lett. 15*, 1009−1013.
(41) Job, P. (1928) Formation and stability of inorganic complexes in solution. *Ann. Chim. 9*, 113−203.
(42) Scott, R. L. (1956) Some comments on the Benesi-Hildebrand equation. *Rec. Trav. Chim. 75*, 787−789.
(43) Arima, H., Chihara, Y., Arizono, M., Yamashita, S., Wada, K., Hirayama, F., and Uekama, K. (2006) Enhancement of gene transfer activity mediated by mannosylated dendrimer/α-cyclodextrin conjugate (generation 3, G3). *J. Controlled Release 116*, 64−74.
(44) Wada, K., Arima, H., Tsutsumi, T., Chihara, Y., Hattori, K., Hirayama, F., and Uekama, K. (2005) Improvement of gene delivery mediated by mannosylated dendrimer/α-cyclodextrin conjugates. *J. Controlled Release 104*, 397−413.
(45) Hamasaki, K., Kogure, K., and Ohwada, K. (1996) A biological method for the quantitative measurement of tetrodotoxin (TTX): tissue culture bioassay in combination with a water-soluble tetrazolium salt. *Toxicon 34*, 490−495.
(46) Ono, N., Arima, H., Hirayama, F., and Uekama, K. (2001) A moderate interaction of maltosyl-α-cyclodextrin with Caco-2 cells in comparison with the parent cyclodextrin. *Biol. Pharm. Bull. 24*, 395−402.
(47) Yang, J., Chen, H., Vlahov, I. R., Cheng, J. X., and Low, P. S. (2007) Characterization of the pH of folate receptor-containing endosomes and the rate of hydrolysis of internalized acid-labile folate-drug conjugates. *J. Pharmacol. Exp. Ther. 321*, 462−468.
(48) Stella, V. J., Rao, V. M., Zannou, E. A., and Zia, V. V. (1999) Mechanisms of drug release from cyclodextrin complexes. *Adv. Drug Delivery Rev. 36*, 3−16.
(49) Ghaghada, K. B., Saul, J., Natarajan, J. V., Bellamkonda, R. V., and Annapragada, A. V. (2005) Folate targeting of drug carriers: a mathematical model. *J. Controlled Release 104*, 113−128.
(50) Riganti, C., Voena, C., Kopecka, J., Corsetto, P. A., Montorfano, G., Enrico, E., Costamagna, C., Rizzo, A. M., Ghigo, D., and Bosia, A. (2011) Liposome-encapsulated doxorubicin reverses drug resistance by inhibiting P-glycoprotein in human cancer cells. *Mol. Pharm. 8*, 683−700.
(51) Roger, E., Kalscheuer, S., Kirtane, A., Guru, B. R., Grill, A. E., Whittum-Hudson, J., and Panyam, J. (2012) Folic acid functionalized nanoparticles for enhanced oral drug delivery. *Mol. Pharm. 9*, 2103−2110.
(52) Yunomae, K., Arima, H., Hirayama, F., and Uekama, K. (2003) Involvement of cholesterol in the inhibitory effect of dimethyl-β-cyclodextrin on P-glycoprotein and MRP2 function in Caco-2 cells. *FEBS Lett. 536*, 225−231.
(53) Wen, J. Q., and Cui, W. (1990) Studies on the composition and stability constant of inclusion complexes of β-cyclodextrin with fluorouracil and ftorafur by NMR. *Yao Xue Xue Bao 25*, 345−348.
(54) Upadhyay, A. K., Singh, S., Chhipa, R. R., Vijayakumar, M. V., Ajay, A. K., and Bhat, M. K. (2006) Methyl-β-cyclodextrin enhances the susceptibility of human breast cancer cells to carboplatin and 5-fluorouracil: involvement of Akt, NF-κB and Bcl-2. *Toxicol. Appl. Pharmacol. 216*, 177−185.
(55) Soliman, H. A., and Olesen, H. (1976) Folic acid binding by human plasma albumin. *Scand. J. Clin. Lab. Invest. 36*, 299−304.
(56) Antony, A. C. (1992) The biological chemistry of folate receptors. *Blood 79*, 2807−2820.