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ORIGINAL ARTICLE

Design and evaluation of folate-appended methyl-β-cyclodextrin as a new antitumor agent

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Abstract Methyl-β-cyclodextrin (M-β-CyD) is widely used as a raft disrupting agent through extraction of cholesterol from lipid rafts which are highly expressed in cell membranes of tumor cells, but it does not have tumor cell-selective action. Meanwhile, the widespread use of folic acid (FA) as a tumor-targeting ligand has been known, because folate receptor (FR) overexpresses in various kinds of epithelial tumor cells. In the present study, in order to obtain more tumor cell-selectivity and antitumor activity of M-β-CyD, we designed folate-appended M-β-CyD (FA-M-β-CyD), and evaluated its physicochemical properties and antitumor activity. The ^{1}H-NMR study demonstrated that FA-M-β-CyD having average degree of substitution of FA (DSF) of 1.0 was prepared. In addition, FA-M-β-CyD (DSF 1.0) was found to be amorphous in a solid state and surface-active. Importantly, FA-M-β-CyD (DSF 1.0) had potent cytotoxicity, compared to M-β-CyD in KB cells, but not in A549 cells. These results suggest that FA-M-β-CyD (DSF 1.0) has the potential as a novel antitumor agent.

Keywords Folic acid · Methyl-β-cyclodextrin · Tumor targeting · Antitumor drug

Introduction

Drug delivery system (DDS) aims at maximizing the therapeutic effects through the regulation of pharmacokinetics of drugs, and comprises (1) controlled release of the drugs, (2) absorption enhancement of drugs, and (3) drug targeting. Drug targeting is one of the important techniques in the DDS field, and classified into a passive targeting and an active targeting. The representative product with passive targeting is Doxil™, which is polyethylene glycol-modified liposomes containing doxorubicin as an anticancer drug, avoiding the capture from reticuloendothelial system (RES) [1]. Although the passive targeting is necessary for the cancer chemotherapy, it is completely difficult to eliminate the adverse effects of anticancer drugs. Therefore, the development of drug carriers having an active targeting-ability is strongly expected to enhance the therapeutic effects and to reduce the adverse effects. To give an active targeting-ability to drug carrier, chemical modification by tumor targeting ligands is known, e.g., antibody [2], sugar [3], folic acid (FA) [4, 5], transferrin [6, 7], epidermal growth factor [8], and Arg-Gly-Asp-Ala-Pro-Arg-Pro-Gly peptide [9]. Of these ligands, FA is widely used because of its several advantages [10, 11], i.e., (1) folate receptor (FR) is upregulated in many human tumor cells, including malignancies of the ovary, brain, kidney, breast, myeloid cells and lung, (2) FA has a potent binding affinity to FR ($K_d \sim 10^{-10}$ M), (3) low immunogenicity, (4) low molecular weight (Mw 441.4), (5) high compatibility with a variety of organic and aqueous solvent, and (6) low cost.

Cyclodextrins (CyDs) and their hydrophilic derivatives form inclusion complexes with hydrophobic molecules. CyDs can improve the solubility, dissolution rate and bioavailability of the drugs, and so the widespread use of CyDs is well known in the pharmaceutical field [12, 13]. CyDs have been reported to interact with cell membrane constituents such as cholesterol and phospholipids, resulting in the induction of hemolysis of human and rabbit red blood cells at high concentrations of CyDs [14–16]. Additionally, methyl-β-cyclodextrin (M-β-CyD) is acknowledged to disrupt the structures of lipid rafts and caveolae [17, 18], which

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are lipid microdomains formed by lateral assemblies of cholesterol and sphingolipids in the cell membrane, through extraction of cholesterol from the microdomains [19]. Furthermore, we demonstrated that 2,6-di-*O*-methyl-*β*-cyclodextrin (DM-*β*-CyD) induced apoptosis through the inhibition of PI3K-Akt-Bad pathway, leading to cholesterol depletion from lipid rafts in NR8383 cells, a rat alveolar macrophage cell line [20]. Recently, Grosse et al. reported that intraperitoneal administration of M-*β*-CyD had antitumor activity in human tumor-xenografted athymic nude mice [21]. However, the cytotoxicity of M-*β*-CyD has low tumor cell-selectivity. Therefore, in the present study, to obtain more potent tumor cell-selectivity and antitumor activity of M-*β*-CyD, we newly prepared folate-appended M-*β*-CyD (FA-M-*β*-CyD), and evaluated its physicochemical properties and antitumor activity.

Materials and methods

Materials

β-CyD was donated by Nihon Shokuhin Kako (Tokyo, Japan). M-*β*-CyD with average degree of substitution (DS) of methyl group of 12.2 was obtained from Tokyo Kasei (Tokyo, Japan). DM-*β*-CyD was purchased from Wako Pure Chemical Industries (Osaka, Japan). FA, *p*-toluenesulfonyl chloride and ammonia water (25%) were purchased from Nakalai Tesque (Kyoto, Japan). 2-Chloro-2,6-dimethoxy-1,3,5-triazin was purchased from Kanto Chemicals (Tokyo, Japan). All other chemicals and solvents were of analytical reagent grade, and deionized double-distilled water was used throughout the study.

Apparatus

Nuclear magnetic resonance (NMR) spectra were taken on a JEOL JNM-R 500 instrument (Tokyo, Japan), operating at 500 MHz for protons at 25 °C. The concentration of the sample was 1.5 mg/750 μL in deuterated oxide (D_2O), and the chemical shifts were given as parts per million (ppm) downfield from that of tetramethylsilane (TMS). Fast atom bombardment mass (FAB-MS) spectra were measured in a negative mode at 25 °C by a JEOL JMS-DX 303 mass spectrometer (Tokyo, Japan). Powder X-ray diffraction patterns were measured with Rigaku Rint-2500 diffractometer (Tokyo, Japan) under the following conditions: Ni-filtered Cu-Kα radiation (1.542 Å), 40 kV, 40 mA, divergent slit of 1.74 mm (1°), scanning slit of 0.94 mm (1°), a receiving slit of 0.15 mm, and goniometer angular increment of 1°/min. Differential thermal analysis (DTA) was carried out using Rigaku Thermo Plus 2 (DTA8120, Tokyo, Japan) with a heating rate of 20 °C/min. Surface tension was measured at 25 °C by a DuNouy surface tensionmeter (Shimadzu, Kyoto, Japan).

Preparation of FA-M-*β*-CyD

Preparation of Tosylated M-β-CyD (Ts-M-β-CyD)

To synthesize FA-M-*β*-CyD, firstly Ts-M-*β*-CyD was prepared. Briefly, M-*β*-CyD (15 g, 1.1×10^{-2} mol) was dissolved in 0.4 M sodium hydroxide solution (NaOH, 300 mL), then *p*-toluenesulfonyl chloride (13 g, 6.9×10^{-2} mol) was added under stirring on ice for 6 h. After filtration, the solution was neutralized by 1 N hydroxyl chloride (HCl). The neutralized solution was concentrated by a rotary evaporator (EYELA N-1000S, Tokyo Rikakikai, Tokyo, Japan) and dried under reduced pressure at 40 °C, and then Ts-M-*β*-CyD was obtained. The reaction was monitored by thin-layer chromatography (TLC: silica gel Merck F_{254} (Darmstadt, Germany)); indicator *p*-anisaldehyde; ethyl acetate:2-propanol:ammonia water:water = 7:7:5:4 (v/v/v/v); FAB-MS (m/z): $[M]^-$ 1488; yield 21.5 g.

Preparation of Amino-M-β-CyD (NH_2-M-β-CyD)

Next, we prepared NH_2-M-*β*-CyD. Briefly, Ts-M-*β*-CyD (7 g, 4.7×10^{-3} mol) was dissolved in 25% ammonia water (120 mL) and the mixture was stirred for 24 h at 40 °C. The reactant was concentrated by a rotary evaporator (EYELA N-1000S, Tokyo Rikakikai, Tokyo, Japan) and dried under reduced pressure at 40 °C, and then NH_2-M-*β*-CyD was obtained. The reaction was monitored by TLC (silica gel Merck F_{254}); 1-buthanol:ethanol:water = 5:4:3 (v/v/v); indicator *p*-anisaldehyde and ninhydrin; FAB-MS (m/z): $[M]^-$ 1314.

Preparation of DMT-MM

4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) was prepared as a condensation reagent. Briefly, *N*-methylmorpholine (21.7 μL, 20 mmol) was added to tetrahydrofuran (THF, 60 mL) dissolved with 2-chloro-2,6-dimethoxy-1,3,5-triazin (4 g, 22 mmol), and the mixture was vigorously stirred for 30 min at room temperature. The white precipitant was obtained after washing with THF (30 mL) for 3 times. After drying under reduced pressure, DMT-MM was obtained. FAB-MS (m/z): $[M\text{-}Cl]^+$ 241; yield 6 g (97%).

Introduction of FA to NH_2-M-β-CyD

NH_2-M-*β*-CyD (7 g, 5.3×10^{-2} mol) and FA (14 g, 3.2×10^{-2} mol) were completely dissolved in dimethylsulfoxide

(DMSO, 800 mL) by stirring at 40 °C. After cooling down the solution to room temperature, DMT-MM (9 g, 3.2×10^{-2} mol) dissolved with 100 mL of methanol was added, and the mixture was stirred for 3 days at room temperature. Acetone (8 L) was added to the reactant to get rid of free FA by precipitation. After filtration by glass filter, the elute was concentrated by a rotary evaporator (EYELA N-1000S, Tokyo Rikakikai, Tokyo, Japan), and dialyzed using a dialysis membrane, Spectra/pore (MWCO = 1,000), in 0.1 M ammonia water for 48 h followed by the dialysis in water for 24 h. After dialysis, the sample was concentrated and freeze dried to obtain FA-M-β-CyD. The reaction was monitored by TLC (silica gel Merck F_{254}); 1-buthanol: ethanol: water: 25% ammonia water = 5:4:3:5 (v/v/v/v); indicator *p*-anisaldehyde and iodine; FAB-MS (m/z): $[M]^-$ 1738; yield 366.8 mg (5%); ^{1}H-NMR (500 MHz, D_2O): δ (ppm) 8.64–8.61 (H7, FA), 7.55–7.51 (H13/15, FA), 6.74–6.61 (H12/16, FA), 5.11–4.85 (H1, M-β-CyD), 4.59–4.44 (H9, H19, FA), and 2.34–1.91 (H22, H21, FA).

Cell culture

A549 cells, a human lung epithelium cell line, were cultured as reported previously [22, 23]. KB cells, a human carcinoma of the nasopharynx, were grown in a RPMI-1640 culture medium (FA-free) containing penicillin (1×10^5 mU/mL) and streptomycin (0.1 mg/mL) supplemented with 10% FCS at 37 °C in a humidified 5% CO_2 and 95% air atmosphere.

Cytotoxicity

Cytotoxicity was assayed by the WST-1 method (a Cell Counting Kit, Wako Pure Chemical Industries, Osaka, Japan), as reported previously [24, 25]. Briefly, KB and A549 cells were seeded at 2×10^4 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. Cells were washed twice with phosphate-buffered saline (PBS, pH 7.4), and then incubated for 2 h with 150 μL of PRMI culture medium (FA-free) containing 10 mM β-CyDs or Tween 20 in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. After washing twice with PBS to remove β-CyDs, 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 630 nm was measured with a microplate reader (Bio-Rad Model 550, Tokyo, Japan).

Data analysis

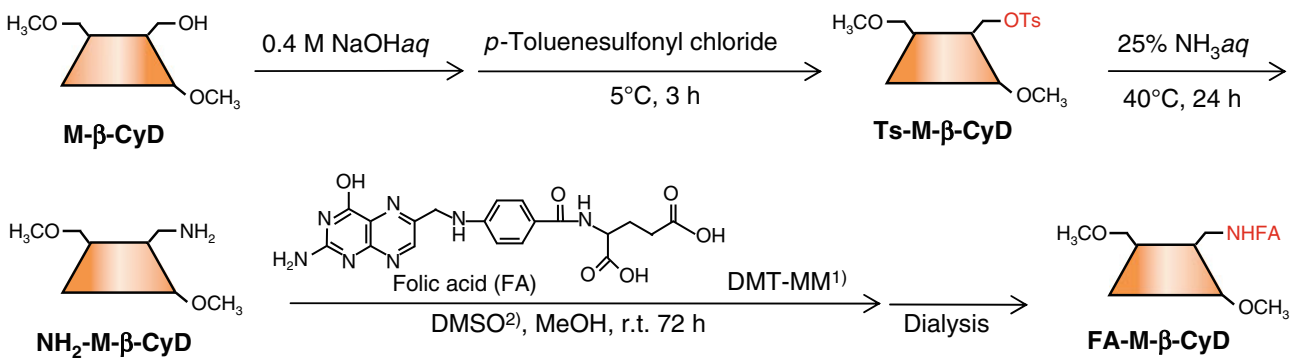
Data are given as the mean ± SEM. Statistical significance of mean coefficients for the studies was performed by analysis of variance followed by Scheffe's test. *p*-Values for significance were set at 0.05.

Results and discussion

Preparation of FA-M-β-CyD

In the present study, we attempted to prepare FA-appended M-β-CyD (FA-M-β-CyD) to obtain tumor cell-selective antitumor activity. Figure 1 shows the preparation pathway of FA-M-β-CyD. Ts-M-β-CyD was obtained by tosylation of hydroxyl group of M-β-CyD according to the Takahashi's method [26]. The FAB-MS spectrum of Ts-M-β-CyD showed parent peak at 1488 (m/z), suggesting that Ts-M-β-CyD having an average degree of substitution of tosyl groups (DST) of 1.2 was synthesized.

Next, we prepared NH_2-M-β-CyD by amination of tosyl group of Ts-M-β-CyD (DST 1.2) according to the Murakami's method with some minor modifications [27]. The endpoint of the reaction was judged from disappearance



1) 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholonium chloride, 2) Dimethyl sulfoxide.

Fig. 1 Preparation pathway of FA-M-β-CyD

and appearance of the spots on the TLC plate after treatment with UV light and ninhydrin, respectively. From the parent peak of NH_2-M-β-CyD in FAB-MS spectrum, the average degree of substitution of amino group (DSN) was determined to be 1.4.

Finally, FA was modified to the amino group of NH_2-M-β-CyD (DSN 1.4) by a condensation reaction using DMT-MM. Here, the results of TLC analysis showed that there was no intact form of FA in the reactant mixture after dialysis (data not shown). Next, ^{1}H-NMR spectrum was measured. Calculating from the integral values of the protons of benzene ring of FA and anomeric protons of glucose in M-β-CyD, the DSF value of FA-M-β-CyD was found to be 1.0 (Fig. 2). These results suggest that FA-M-β-CyD (DSF 1.0) was successfully synthesized. However, the yield of FA-M-β-CyD was still very low (ca. 5%). Thereby, further investigation should be required to obtain high yield of FA-M-β-CyD.

Physicochemical properties of FA-M-β-CyD

We performed the powder X-ray diffraction analysis of FA-M-β-CyD (DSF 1.0) (Fig. 3). Powder X-ray diffraction patterns of β-CyD, DM-β-CyD and FA gave many sharp peaks, suggesting that they are crystal forms in a solid state. On the other hand, X-ray diffraction patterns of FA-M-β-CyD (DSF 1.0) and M-β-CyD showed halo, indicating they are amorphous. In addition, the diffraction peaks derived from FA were completely disappeared in FA-M-β-CyD (DSF 1.0). These results suggest that FA was covalently bound to M-β-CyD, and there was no free FA in a solid state of FA-M-β-CyD (DSF 1.0). Next, we investigated DTA to reveal thermal property of FA-M-β-CyD (Fig. 4). Endothermic peak derived from FA at 130 °C was disappeared in FA-M-β-CyD (DSF 1.0), suggesting that FA was fully introduced to M-β-CyD. Taken together, these results indicate that FA-M-β-CyD was successfully prepared.

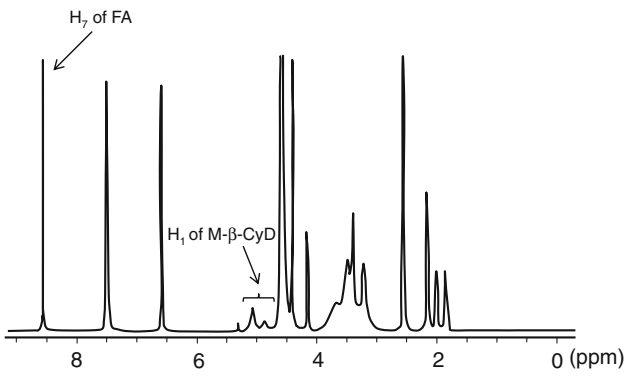


Fig. 2 ^{1}H-NMR spectrum of FA-M-β-CyD in D_2O

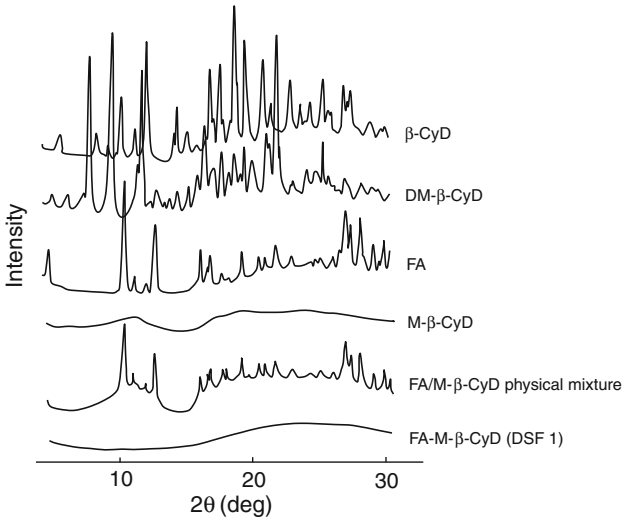


Fig. 3 Powder X-ray diffraction patterns of FA and β-CyDs

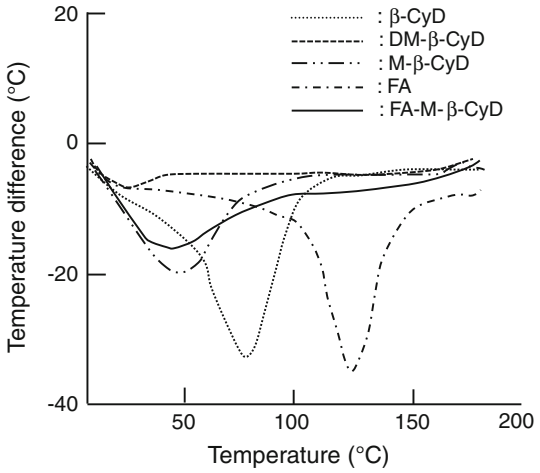


Fig. 4 DTA thermograms of FA and β-CyDs

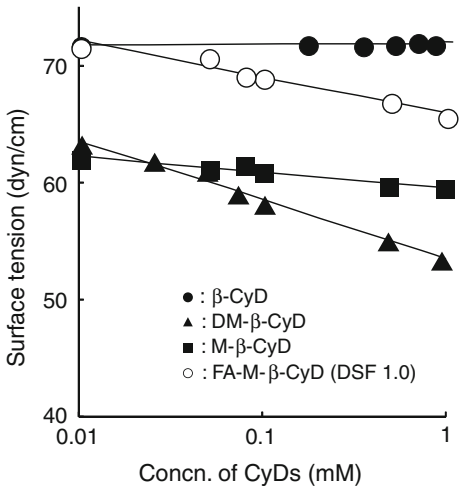


Fig. 5 Surface tension of β-CyDs solution in water at 25 °C

It is known that M-β-CyD is surface active, because M-β-CyD has hydroxyl group (hydrophilic group) and methyl group (hydrophobic group) in the molecule. In addition, M-β-CyD and DM-β-CyD are acknowledged to have a strong interaction with cell membranes including cholesterol due to its surface active and their inclusion ability. Therefore, we measured surface tension whether FA-M-β-CyD (DSF 1.0) is surface active or not (Fig. 5). β-CyD solution was confirmed to be surface-inactive.

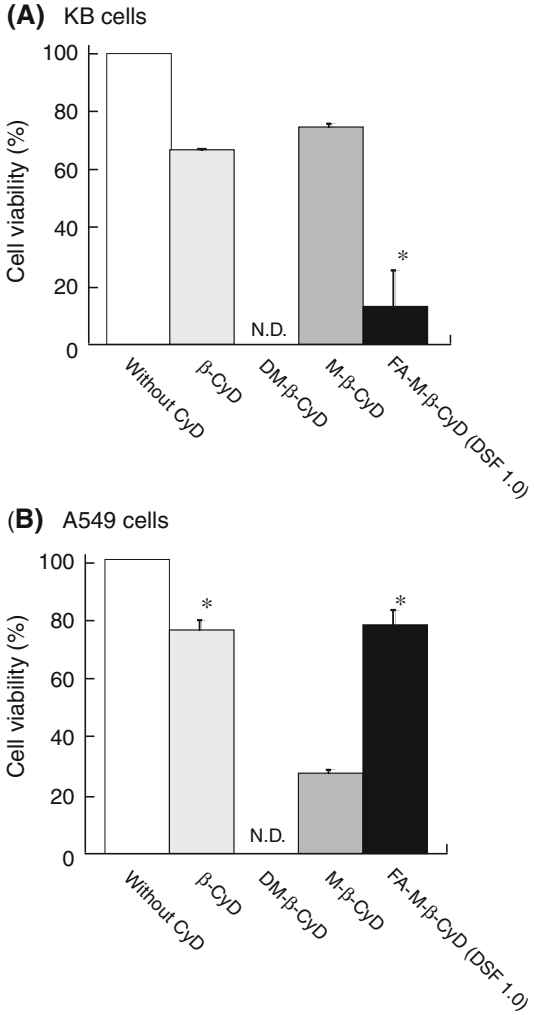


Fig. 6 Cytotoxic activity of β-CyDs in KB and A549 cells. KB and A549 cells (2×10^4 cells/96-well microplate) were incubated for 24 h in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Cells were washed twice with PBS (pH 7.4), and then incubated for 2 h with 150 μL of PRMI culture medium (Folic acid-free) containing 10 mM β-CyDs or Tween 20 in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. After washing twice with PBS to remove β-CyDs, 100 μL of fresh 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 630 nm was measured with a microplate reader. Each value represents the mean ± SEM of three experiments. *ND* not determined. *$p < 0.05$ versus without CyD

In addition, surface tension of FA-M-β-CyD (DSF 1.0) solution decreased in a concentration-dependent manner, suggesting that FA-M-β-CyD is surface-active. However, surface activity of FA-M-β-CyD (DSF 1.0) was relatively lower than that of M-β-CyD and DM-β-CyD. The low surface activity of FA-M-β-CyD (DSF 1.0) could be ascribed to the hydrophilicity of FA introduced in M-β-CyD. We previously reported that DM-β-CyD caused apoptosis in NR8383 cells through the extraction of cholesterol from lipid rafts due to its surface activity and inclusion ability [20]. In our preliminary study, we revealed that FA-M-β-CyD (DSF 1.0) induces hemolysis through release of cholesterol from cell membranes of erythrocytes (data not shown). Therefore, these results suggest that FA-M-β-CyD (DSF 1.0) has antitumor activity through not only the binding to FR but also the interaction with cell membranes of tumor cells.

To clarify the FR-selective antitumor activity of FA-M-β-CyD (DSF 1.0), we evaluated cytotoxicity of FA-M-β-CyD (DSF 1.0) in KB cells, FR-positive cells, and A549 cells, FR-negative cells (Fig. 6). Importantly, FA-M-β-CyD (DSF 1.0) had potent cytotoxicity, compared to M-β-CyD in KB cells, but not in A549 cells. Importantly, FA-M-β-CyD (DSF 1.0) had potent cytotoxicity, compared to M-β-CyD in KB cells, but not in A549 cells. Further investigations on cytotoxic activity of FA-M-β-CyD (DSF 1.0) in other cell lines are undergoing.

Conclusion

In the present study, we newly prepared FA-M-β-CyDs. In addition, FA-M-β-CyD (DSF 1.0) was found to be amorphous and surface active. Importantly, FA-M-β-CyD (DSF 1.0) had higher cytotoxic activity than M-β-CyD in KB cells. These results suggest that FA-M-β-CyD (DSF 1.0) has the potential as a novel antitumor agent.

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