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Octreotide-Targeted Liposomes Loaded with CPT-11 Enhanced Cytotoxicity for the Treatment of Medullary Thyroid Carcinoma

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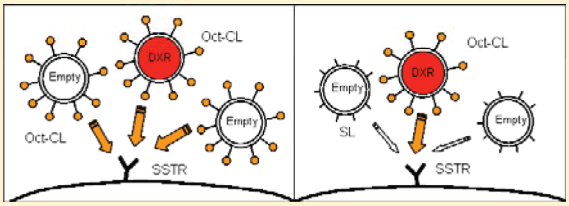
Supporting Information

ABSTRACT: Medullary thyroid carcinoma (MTC) is a rare endocrine tumor that frequently metastasizes, but treatment with irinotecan (CPT-11) is limited because of side effects. MTC is known to overexpress the somatostatin receptor subtype 2 (SSTR2). Octreotide (Oct) is a somatostatin analogue that has a high binding affinity for SSTR and can be used as a tumor-targeting ligand. We prepared Oct-targeted liposomes loaded with CPT-11 using Oct-poly (ethylene glycol) (PEG)-lipid and evaluated Oct-mediated association and cytotoxicity of the liposomes with an MTC cell line TT. The association of higher concentrations of modified Oct-targeted liposomes with TT cells was significantly higher than PEGylated liposomes and was significantly inhibited by empty Oct-targeted liposomes but not by free Oct. With exposure for 96 h, the cytotoxicity of Oct-targeted liposomal CPT-11 (IC50: 1.05 ± 0.47 μM) was higher than free CPT-11 (IC50: 3.76 ± 0.61 μM) or PEGylated liposomal CPT-11 (IC50: 3.05 ± 0.28 μM). In addition, empty Oct-targeted liposomes showed significantly higher cytotoxicity than empty nontargeted liposomes at a concentration where free Oct did not show cytotoxicity, suggesting that Oct as a ligand showed cytotoxicity. Moreover, Oct-targeted liposomal CPT-11 led to significantly higher antitumor activity and prolonged the survival time compared with nontargeted liposomal and free CPT-11 at a one-third dose and lower administration times with free CPT-11. These findings indicated that Oct-targeted liposomes loaded with CPT-11 may offer considerable potential for MTC chemotherapy because cytotoxicity of both CPT-11 and Oct was enhanced by effective cellular uptake *via* SSTR2.



KEYWORDS: octreotide, CPT-11, medullary thyroid carcinoma, somatostatin receptor, targeting liposome, antitumor activity

1. INTRODUCTION

Medullary thyroid carcinoma (MTC) is a rare endocrine tumor comprising a malignant neoplasm of calcitonin-secreting C cells of the thyroid and represents approximately 3−5% of all thyroid cancers.[1] MTC occurs in a sporadic form in about 75% of cases, and the remaining 25% are three familial forms: multiple endocrine neoplasia type IIA (MEN 2A), multiple endocrine neoplasia type IIB (MEN2B), and familial MTC not associated with MEN (FMTC).[2] The only effective treatment for MTC is surgical removal of the neoplastic tissue with central lymph node dissection.[3] In the case of total thyroidectomy, however, life-long hormone replacement therapy is necessary for the patient. Most chemotherapy or radiotherapy for MTC has inconclusive results. The lack of effective systemic therapy for MTC shows the importance of developing new approaches for the treatment of MTC.

Recently, therapeutic approaches for MTC using irinotecan (CPT-11) have been reported.[4,5] CPT-11 is a water-soluble derivative of camptothecin and is converted to SN-38, the active form of CPT-11, by carboxyl esterase.[6,7] CPT-11 inhibits the resealing of single-strand DNA breaks mediated by topoisomerase I by stabilizing cleavable complexes and is a cell-cycle-specific drug.[8−10] Based on this, a long period of exposure to CPT-11 mediates a reduction in tumor cells, but treatment with CPT-11 is limited because of its short half-life and serious side effects, such as bone-marrow suppression.[11] Success in the treatment of MTC with CPT-11 requires selective delivery to tumor tissues and limited distribution to normal tissues.

For this purpose, liposomal CPT-11 could be advantageous. Liposomal drugs reduce renal clearance and, when formulated for long circulation, may increase accumulation of the drug in a tumor by a passive targeting process based on the enhanced permeability and retention (EPR) effect in leaky tumor tissue. To further enhance the therapeutic effects of liposomal drugs in tumors, a variety of targeting ligands have been investigated. Somatostatin receptors (SSTRs) belong to a seven-transmembrane domain G-protein coupled receptor family (SSTR1, SSTR2, SSTR3, SSTR4, and SSTR5)[12] and can serve as a functional tumor-specific receptor. MTC tumors express all the SSTR subtypes, and the MTC cell line TT overexpresses SSTR1, SSTR2 and SSTR5.[13] SSTR2 expression was significantly higher than the other SSTR subtypes in TT cells and was the most

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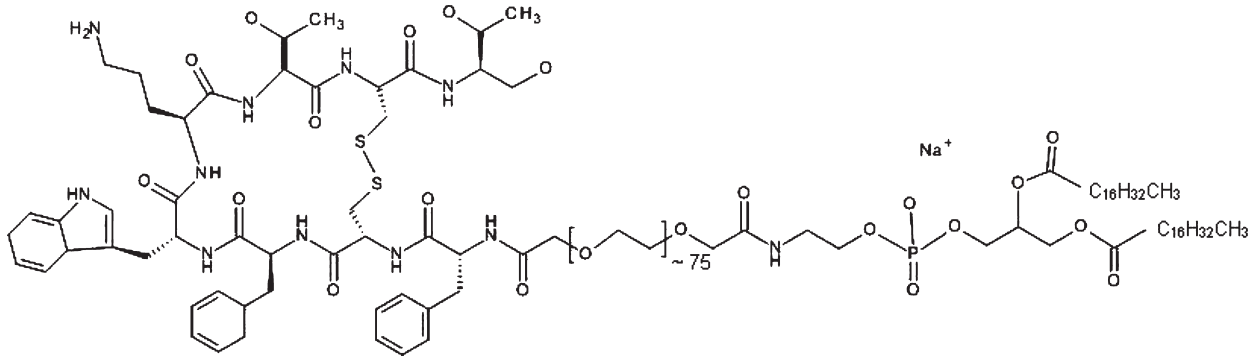

Figure 1. Chemical structure of Oct-PEG_{3400}-DSPE (MW = ~5200).

frequently detected subtype in human MTC.[13−15] Octreotide (Oct) has high binding affinity to all SSTR subtypes,[16] especially SSTR2 (affinity 0.56 nM).[12] Oct is an octapeptide analogue of natural somatostatin with markedly increased metabolic stability,[17] resulting in an increased plasma-half-life of >1 h in rats[17] and humans.[18] Oct has been marketed as a diagnostic agent.[19−21] It was reported that Oct-modified particles loaded with anticancer drugs (cantharidin and dihydrotanshinone I) were efficacious in breast cancer and gastric cancer, respectively.[22,23] To the best of our knowledge, this report is the first to explore the use of Oct-targeted liposomes for the treatment of MTC.

Here, we hypothesized that Oct-targeted liposomal CPT-11 could enable selective delivery of CPT-11 and increase the therapeutic efficacy of CPT-11 for MTC. Previously, we succeeded in preparing liposomes with a high loading efficacy for CPT-11.[24] In this study, we prepared Oct-targeted liposomes loaded with CPT-11 and evaluated the Oct-mediated association and therapeutic potential of Oct-targeted liposomes with TT cells and TT tumor xenografts in mice, for the comparison of free and liposomal CPT-11.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Materials. CPT-11 was a kind gift from Yakult Co., Ltd. (Tokyo, Japan). Oct-poly(ethylene glycol)$_{3400}$-distearoylphosphatidylethanolamine (Oct-PEG_{3400}-DSPE)[25] was purchased from KNC Laboratories. Co., Ltd. (Kobe, Japan) (Figure 1). Oct was purchased from Acris Antibodies GmbH (Herford, Germany). Distearoylphosphatidylcholine (DSPC) and methoxy-PEG_{2000}-DSPE (PEG_{2000}-DSPE) were purchased from the NOF Corp. (Tokyo, Japan). Cholesterol, doxorubicin (DXR) hydrochloride, and Ham's F-12 medium were purchased form Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Phytic acid (IP-6) solution was obtained from Nacalai Tesque Inc. (Kyoto, Japan). Fetal bovine serum (FBS) was purchased from Invitrogen Corp. (Carlsbad, CA, USA). Nonessential amino acids were purchased from MP Biomedicals (Cleveland, OH, USA). Other reagents were of analytical or HPLC grade.

2.1.2. TT Cell Line Culture. The TT cell was obtained from the European Collection of Cell Cultures (ECACC, Wiltshire, UK). This cell line was routinely maintained in Ham's F-12 medium supplemented with 10% heat-inactivated FBS at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Preparation of Oct-Targeted Liposomes Loaded with Drug

2.2.1. Preparation of Liposomal CPT-11 and DXR. Liposomes containing IP-6 (IP-6 liposomes) were formulated following previously described methods.[24] Briefly, DSPC and cholesterol at a molar ratio of 55:45 (80 mg/32 mg) were dissolved in ethanol. Ethanol was removed by rotary evaporation to a smaller volume, and 80 mM IP-6 solution adjusted to pH 6.5 using triethanolamine was added immediately, followed by sonication to decrease the size to approximately 150 nm. Then, the extra liposomal IP-6 suspension was exchanged for HBS buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) by gel filtration chromatography using a Sephadex G50 column. The concentration of phospholipid (DSPC) of IP-6 liposomes was determined using a phospholipid C Test Wako (Wako Pure Chemical Industries, Ltd.), and then total lipid was calculated using DSPC and cholesterol at a molar ratio of 55:45. IP-6 liposomes were loaded with DXR or CPT-11 by incubation with CPT-11 (drug:total lipid = 0.6:1, w/w) at 60 °C for 60 min or with DXR (drug:total lipid =0.2:1, w/w) at 60 °C for 25 min, and then quenched in ice for 5 min. Unincorporated CPT-11 or DXR was removed using a Sephadex G-50 column eluted with saline as the mobile phase.

2.2.2. Modification of Liposomal Drug with Oct. Three types of liposomes were prepared: conventional non-PEGylated and nontargeted liposomes (CL), Oct-targeted liposomes (Oct-CL), and PEGylated, sterically stabilized liposomes (SL). Oct-CL was prepared by incubation of CL with an aqueous dispersion of 0.25−1.6 mol % Oct-PEG-DSPE with total lipids at 60 °C for 20 min by the postinsertion technique as reported previously.[26] Above 1.6 mol %, Oct-PEG-DSPE precipitates because of its insolubility in water. SL was prepared by incubation of CL with aqueous dispersion of 1.6 mol % PEG-DSPE in total lipids. CL modified with x mol % Oct-PEG-DSPE of total lipids are henceforth abbreviated as xOct-CL. For example, 0.25Oct-CL indicates liposomes with 0.25 mol % Oct-PEG-DSPE in total lipids (Table 1). Empty liposomes were prepared using the same protocol but without loading drug.

The mean diameter and zeta-potential of the resulting liposomes were determined using a ELS-Z2 (Otsuka, Electronics CO., Ltd. Osaka, Japan) at 25 ± 1 °C after diluting the liposome suspension with water. Drug concentration in the liposomes was determined after disruption of liposomes using 1% of Triton; DXR was determined using a UV-1700 PharmaSpec (Shimadzu, Kyoto, Japan) at 480 nm[26] and CPT-11 using a fluorometer (excitation wavelength 355 nm and emission wavelength 535 nm, Wallac ARVO SX1420 multilabel counter, PerkinElmer Japan, Yokohama, Japan).[24] No drug leaked out from the liposomes during the Oct-PEG-DSPE insertion procedure. The final Oct concentration after modification of the liposomes was determined using an Oct-EIA kit (Peninsula Laboratories, LLC, San Carlos, CA, USA) after disruption of the liposomes by dilution

Table 1. Oct-PEG Component (mol %) for Each Liposome Formulation

formulation	Oct-PEG$_{3400}$-DSPE	PEG$_{2000}$-DSPE
CL	0	0
0.25Oct-CL	0.25	0
0.8Oct-CL	0.8	0
1.0Oct-CL	1.0	0
1.2Oct-CL	1.2	0
1.4Oct-CL	1.4	0
1.6Oct-CL	1.6	0
SL	0	1.6

1:1,000 in 1% Triton. About 84%, 85%, and 70% of Oct ligand was inserted into 0.25Oct-CL, 0.8Oct-CL, and 1.6Oct-CL, respectively.

2.3. Cytotoxicity Assay. TT cells were seeded onto 96-well plates at a density 10^4 cells/well for 72 h before addition of the drug. Culture medium was replaced with fresh medium containing various concentrations of liposomal CPT-11, free CPT-11 or empty liposomes. After 96 h incubation at 37 °C, the cells were washed with PBS three times and cultured with fresh medium for 48 h. Then, cell viability was determined using a WST-8 test (Dojindo Laboratories, Kumamoto, Japan). All measurement was carried out in quadruplicate. The 50% growth-inhibitory concentration (IC50) was calculated using the bootstrap method.[27]

2.4. Analysis of Cellular Uptake of Liposomes by Flow Cytometry. Cells were seeded onto 6-well plates at a density 10^4 cells/well for 72 h before addition of the drug. Cells were incubated with medium (2 mL/well) containing 0.25Oct-CL, 0.8Oct-CL, 1.0Oct-CL, 1.2Oct-CL, 1.4Oct-CL, 1.6Oct-CL, or SL at a concentration of 50 μg of DXR/ml for 1 or 2 h. In flow cytometry and confocal laser scanning microscopy studies, as described below, DXR was used instead of CPT-11 because CPT-11 is not excited at 488 nm. Subsequently, cells were washed three times with PBS (pH 7.4) to remove unbound liposomes, and the cellular uptake of liposomes was analyzed using a FACS Calibur flow cytometer (Becton Dickinson, CA, USA) equipped with a 488 nm argon ion laser and using CELL Quest software (Becton-Dickinson Immunocytometry System, CA, USA). A total of 10,000 events per sample were analyzed. The autofluorescence of cells was taken as a control. The cells were incubated without liposomes.

In competitive inhibition experiments, a 20-fold molar excess of free Oct (84 nmol/mL medium) was added to 1.6Oct-CL loaded with DXR (Figure 3). The medium in each well (2 mL) contained 50 μg of DXR/ml, 4.2 nmol of Oct originating from 1.6Oct-CL/ml, and 112 μg of phospholipid/ml. A two volume excess of empty 1.6Oct-CL or SL (258 μL) was added to 1.6Oct-CL loaded with DXR. Because all liposomes have a lipid concentration 194 μg of phospholipid/mL, the final concentration of phospholipid in medium was 582 μg/mL (Figure 4Bi,ii). This was nearly the maximum concentration because greater than 0.6 mg of phospholipid/mL induces cytotoxicity. The cells were incubated at 37 °C for 2 h.

2.5. Confocal Laser Scanning Microscopy. Cells were seeded onto 6-well plates at a density 10^4 cells/well for 72 h before addition of the drug. Cells were washed three times with PBS and then incubated with 1.6Oct-CL loaded with DXR in the presence or absence of a two volume excess of empty 1.6Oct-CL or SL for 2 h at 37 °C, as described above. After incubation, the cells were washed three times with PBS and fixed with 10% formaldehyde in PBS at room temperature for 15 min. Then, the cells were washed two times with PBS and coated with Aqua Poly/Mount (Polyscience, Warrington, PA, USA) to prevent fading and covered with coverslips. The fixed cells were observed using a Radiance 2100 confocal laser scanning microscope (Bio-Rad, Hercules, CA, USA) with an excitation wavelength at 488 nm and an emission wavelength at 560 nm utilizing a LP560 filter.

2.6. Cellular Distribution of Liposomal CPT-11 Observed by Fluorescence Microscopy. Cells were seeded onto 35 mm glass dishes at 10^4 cells/dish for 72 h before addition of the drug. Cells were treated with medium containing 1.6Oct-CL loaded with CPT-11 for 2 h at 37 °C. After incubation, the cells were fixed and coated as described above. Cells were examined using an inverted microscope, ECRIPS TS100 (Nikon, Tokyo, Japan) with an Epi-Fluorescence Attachment (Nikon) a utilizing a UV1A filter.

2.7. *In vitro* Drug Release. The release of the drug from liposomes into phosphate-buffered saline (PBS, pH 7.4) was monitored by a dialysis method. Dialysis was carried out at 37 °C under sink conditions using seamless cellulose tube membranes Spectra/Por CE (MWCO 2000, Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA). The initial concentration of CPT-11 was 500 μg/mL. The sample volume in the dialysis bag was 1 mL, and the sink solution was 200 mL. After various time intervals, aliquots were withdrawn and the CPT-11 concentrations were analyzed as described above.

2.8. Therapeutic Study. To generate TT tumor xenografts, 1×10^7 TT cells suspended in 100 μL of PBS containing 50% Matrigel (Collaborative Research, Bedford, MA, USA) were inoculated subcutaneously into the flank region of four female ICR nu/nu mice (6 weeks of age, purchased from Oriental Yeast Co., Ltd. Tokyo, Japan). The tumor volume was calculated using this formula: tumor volume = $0.5ab^2$, where a and b are the larger and smaller diameters, respectively. When the average volume of xenograft tumors reached about 100 mm^3, therapy was started (day 1). Antitumor activity was assessed by measuring tumor volume change after intravenous injection of CPT-11. Liposomal CPT-11 (10 mg/kg Oct-CL and 10 mg/kg CL) was administered on days 1 and 4, and 30 mg/kg free CPT-11 and saline were administered on days 1, 4, and 7. The data of free CPT-11-treated or saline-treated mice were referring to our previous data.[24] Tumor volume and body weights were measured for individual animals. The mean increase in life span (% ILS) was calculated using the formula $100 \times \{$(median day of death in treated tumor bearing mice) − (median day of death in control tumor-bearing mice)$\}$/(median day of death in control tumor-bearing mice). Animal experiments were performed with approval from the Industrial Animal Care and Use Committee at Hoshi University.

2.9. Statistical Analysis. Data are expressed as mean ± SD. The statistical significance of data was evaluated using Student's t test. $P < 0.05$ was considered as significant.

3. RESULTS

3.1. Characterization of Oct-Targeted Liposomes. Liposome size and zeta-potential of CL, Oct-CL and SL are listed in Table 2. The average diameter of prepared liposomes was approximately 134−154 nm with a narrow, monodisperse distribution (less than 0.2 polydispersity indexes). As the Oct concentration increased from 0 to 1.6 mol %, the zeta-potential of liposomes decreased. The Oct amount of each Oct-CL was

Table 2. Size and Zeta-Potential of Oct-Targeted Liposomes[a]

formulation	size (nm)	zeta-potential (mV)
CL	151.9 ± 5.5	−5.1 ± 0.1
0.25Oct-CL	141.3 ± 6.1	−11.2 ± 3.5
0.8Oct-CL	141.6 ± 9.4	−17.8 ± 6.9
1.0Oct-CL	153.9 ± 6.2	−18.1 ± 4.3
1.2Oct-CL	134.2 ± 5.4	−15.3 ± 1.9
1.4Oct-CL	147.6 ± 2.4	−17.3 ± 4.7
1.6Oct-CL	136.6 ± 3.2	−19.5 ± 1.3
SL	144.7 ± 1.6	−20.1 ± 1.6

[a] Mean ± SD (n = 3).

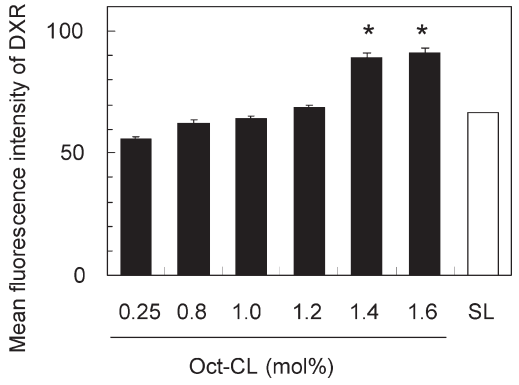

Figure 2. Effect of Oct surface density on cellular association of Oct-CL. DXR-loaded liposomes modified with various Oct-PEG-DSPE concentrations (mol %) were incubated with TT cells at 50 μg/mL DXR for 2 h at 37 °C. Each value represents the mean ± SD of three experiments. (*) Differences are statistically significant from SL at $P < 0.05$.

more than 70% of the theoretical values. The loading efficiencies of CPT-11 were approximately >82% in all liposomes except CL (data not shown). The average diameter and amount of CPT-11 loaded in all types of liposomes did not change for at least 1 month at 4 °C in the dark (data not shown).

3.2. Effects of Oct Surface Density of Oct-CL on Cellular Uptake. We examined the selectivity of Oct-CL for delivery into TT cells, which highly overexpress SSTR2, by flow cytometry. As shown in Figure 2, the mean fluorescence intensities of 0.8Oct-CL, 1.0Oct-CL, 1.2Oct-CL, 1.4Oct-CL and 1.6Oct-CL were approximately 1.1-fold, 1.2-fold, 1.2-fold, 1.7-fold and 1.7-fold greater than for SL, respectively, after a 2 h incubation. The cellular uptake of free DXR was ~3 times higher than 0.25Oct-CL (data not shown). When paying attention to the effects of the Oct surface density of liposomes on cellular association, a higher Oct surface density, more than 1.4 mol % of liposomes was more effectively associated with TT cells. The fluorescence intensities of 1.6Oct-CL after a 2 h incubation increased 2-fold more than after 1 h, whereas that of SL did not increase (data not shown). This finding indicated that the cellular association of Oct-CL increased in an incubation-time-dependent manner, but that of SL was not. From this result, 1.6Oct-CL was used in the following experiments as Oct-CL, and SL was used as a control for the 2 h incubation.

3.3. Competitive Inhibition Study. First, to investigate the cellular association of Oct-CL *via* SSTR, a competitive inhibition study was performed using free Oct as a competitive inhibitor. In the presence of a 20-fold excess of free Oct (84 nmol/mL

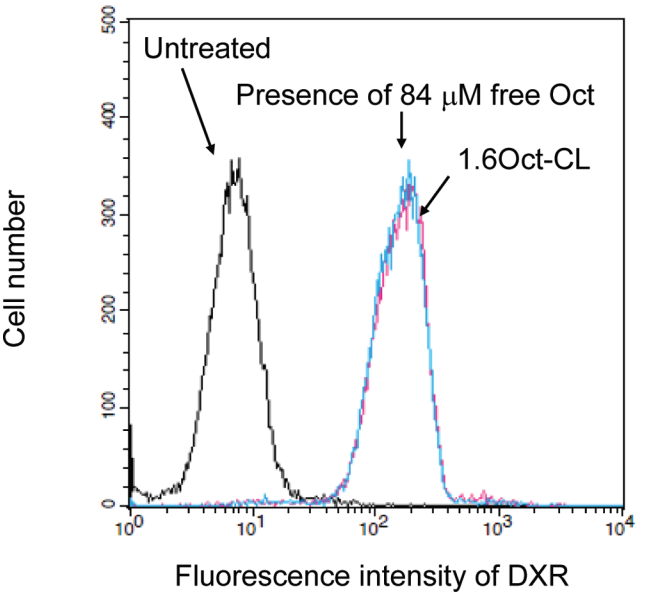

Figure 3. Association of Oct-CL loaded with DXR with TT cells in the presence or absence of free Oct by flow cytometry. 1.6Oct-CL loaded with DXR was incubated in the presence of a 20-fold excess of free Oct (84 μM) at 50 μg/mL DXR for 2 h at 37 °C. Untreated indicates autofluorescence of untreated cells.

medium) with a 2 h incubation, a competitive effect, a decrease of cellular uptake of Oct-CL, was not observed (Figure 3). Next, we tried to use Oct-CL without drug loading (empty Oct-CL) as a competitive inhibitor, because addition of drug-loaded liposomes has the possibility of increasing the cytotoxicity due to DXR. Figure 4A illustrates the scheme of the competitive cellular association of Oct-CL loaded with DXR with empty Oct-CL. The cellular uptake of Oct-CL loaded with DXR in the presence of two excess volumes of empty Oct-CL (i) was compared with that of empty SL (ii) by flow cytometry (Figure 4B) and confocal microscopy (Figure 5). As shown in Figure 4B, the mean fluorescence intensities of DXR loaded with Oct-CL in the presence of empty Oct-CL decreased by approximately half compared with in the presence of empty SL. This finding indicated that the cellular uptake of Oct-CL loaded with DXR was blocked significantly by empty Oct-CL compared with empty SL.

To confirm differences in the cellular uptake of Oct-CL loaded with DXR in the presence of empty Oct-CL or SL, intracellular localizations were observed by confocal microscopy (Figure 5). The presence of empty Oct-CL decreased the localization of DXR-loaded Oct-CL in the nucleus (red fluorescence of DXR) compared with that of empty SL, corresponding to the results of Figure 4Bi and Figure 4Bii, respectively.

3.4. Drug Release from Liposomal CPT-11. Before the investigation of cellular uptake of 1.6Oct-CL and SL, the release of drug from each liposome was examined. The profiles of CPT-11 release versus time are presented in Figure 6. Both 1.6Oct-CL and SL showed slow drug release, about 17% drug release for 24 h in PBS at 37 °C. There were no significant differences between 1.6Oct-CL and SL in drug release at each time point. This result suggested that 1.6 mol % Oct-modification did not affect drug release from liposomes.

3.5. Cellular Uptake Oct-CL Loaded with CPT-11. Next, we tried to observe the cellular distribution of liposomes loaded with CPT-11 by fluorescence microscopy (Figure 7). TT cells were

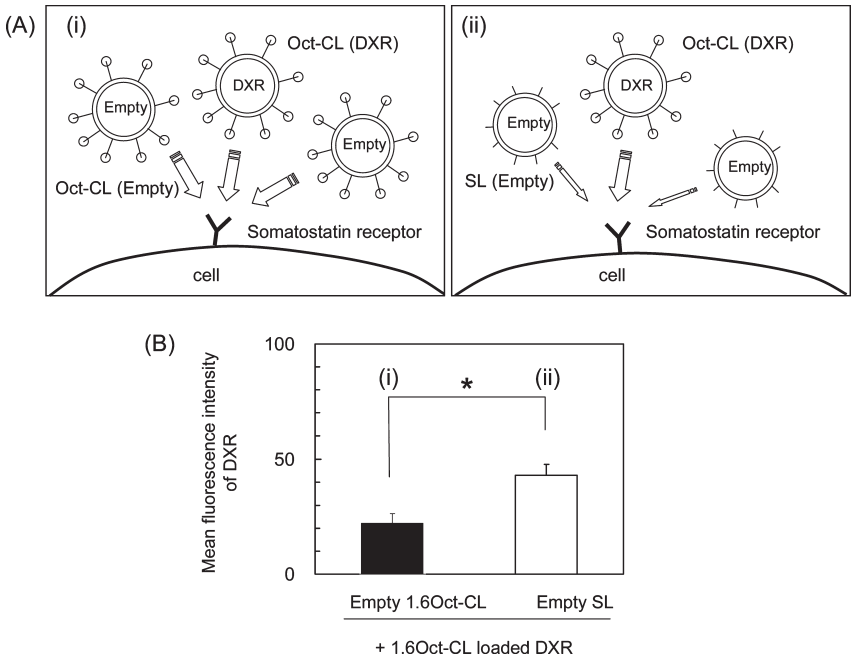

Figure 4. Scheme (A) and DXR fluorescence (B) of cellular association of 1.6Oct-CL loaded with DXR with TT cells in the presence of two excess volumes of empty 1.6Oct-CL (i) or empty SL (ii) for 2 h at 37 °C by flow cytometry. Each value is the mean ± SD for three experiments. (*) Differences are statistically significant at $P < 0.05$.

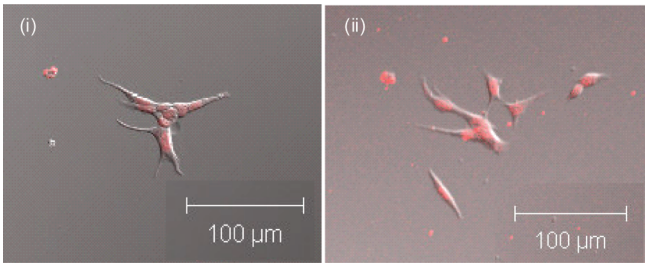

Figure 5. Effects of empty 1.6Oct-CL (i) or SL (ii) on the cellular association of 1.6Oct-CL loaded with DXR with TT cells determined by confocal microscopy. The experimental conditions were the same as for Figure 4B. Scale bar denotes 100 μm.

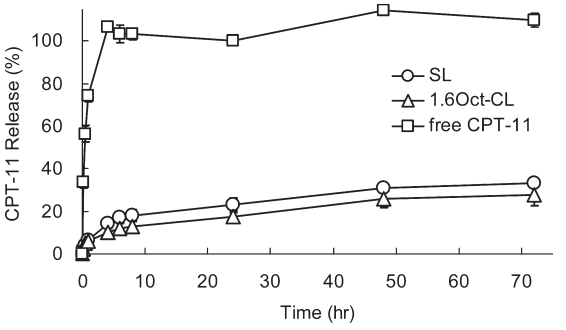

Figure 6. Percentage of CPT-11 released from 1.6Oct-CL and SL as a function time at 37 °C. CPT-11 concentrations were measured as described in Materials and Methods using PBS as a sink solution at pH 7.4. Each value represents the mean ± SD of three experiments.

incubated with Oct-CL loaded with CPT-11 for 2 h at 37 °C. Blue fluorescence due to CPT-11 (Figure 7A) was observed weakly in Oct-CL loaded with CPT-11 at the same location as TT cells (Figure 7B). This finding indicated that Oct-CL loaded with CPT-11 was taken up into the cells as well as Oct-CL loaded with DXR.

3.6. Effect on Cytotoxicity of Oct-Targeted Liposomes. To evaluate the cellular uptake of Oct-CL loaded with CPT-11, the cytotoxicity with TT cells was measured using a WST-8 assay. The doubling time of TT cells is 83 h; therefore, a 96 h incubation was set in this experiment. As shown in Table 3 and Figure S1 in the Supporting Information, free CPT-11, Oct-CL, and SL increased cytotoxicity in an incubation-time-dependent manner. Free CPT-11 showed higher cytotoxicity than liposomal CPT-11 with 48 h and 72 h incubations. After a 96 h incubation, the IC50 value of Oct-CL was the highest (1.05 ± 0.47 μM), whereas free CPT-11 (3.76 ± 0.61 μM) and SL (3.05 ± 0.28 μM) gave similar cytotoxicity results. Therefore cytotoxicity of Oct-CL loaded with CPT-11 may be due to cellular uptake of liposomal CPT-11. In addition, 8.4 μM Oct originating from empty 1.6Oct-CL significantly decreased TT cell viability to 60% after a 96 h incubation compared with empty SL (corresponds to the amount of PEG-DSPE of Oct-CL) (Figure 8). Cytotoxicity of liposomes modified with more than about 12 μM Oct was observed due to the lipids. On the other hand, cytotoxicity of free Oct was not observed independent of the Oct concentration (data not shown).

3.7. Antitumor Effect of Oct Modified Liposomes on TT Tumor Xenografts. To examine the effect of Oct modification of liposomes on cytotoxicity *in vivo*, the antitumor activity of Oct-targeted liposome and nontargeted CL loaded with CPT-11 was evaluated following two intravenous injections into TT tumor bearing mice (Figure 9). 1.6Oct-CL reduced the tumor size in mice after the final injections, and the reduced tumor size was maintained until day 23, whereas CL maintained tumor growth suppression only until day 10. 1.6Oct-CL suppressed tumor growth significantly compared with CL, free CPT-11, or saline.

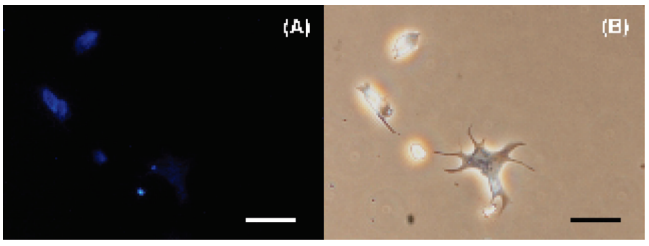

Figure 7. Cellular uptake of liposomal CPT-11 observed by fluorescence microscopy. TT cells were incubated with 1.6Oct-CL loaded with CPT-11 for 2 h at 37 °C. Blue fluorescence; location of CPT-11. Dark field (A), and bright field (B). Scale bar, 50 μm.

Table 3. IC50 of Free CPT-11 and Liposomes Loaded with CPT-11 on TT Cells after Various Incubation Times[a]

	IC50 (μM)		
formulation	48 h	72 h	96 h
free CPT-11	7.43 ± 6.73	5.10 ± 1.85	3.76 ± 0.61
1.6Oct-CL	29.05 ± 19.40	8.72 ± 1.14	1.05 ± 0.47*
SL	22.50 ± 19.50	8.65 ± 2.26	3.05 ± 0.28

[a] Mean ± SD (n = 4). (*) Differences are statistically significant from SL at P < 0.05.

Body weight loss was not observed in any of the groups (data not shown). Median survival for mice treated with saline was 68 days, compared with 88 days for free CPT-11-treated, 103 days for CL-treated, and 217 days for 1.6Oct-CL-treated mice. Treatment with liposomal CPT-11 significantly increased survival time. Therefore, %ILS of 1.6Oct-CL treated group was significantly improved compared with that of the CL-treated and free CPT-11-treated groups. This finding indicated that Oct modification of liposomal CPT-11 enhanced antitumor effect *in vivo*.

4. DISCUSSION

In this study, we prepared Oct-CL loaded with CPT-11 and demonstrated that high Oct-surface-density significantly increased the cellular association of Oct-CL *via* SSTR and approximately 2-fold higher cytotoxicity when compared with free CPT-11 and PEGylated liposomes with TT cells using a 96 h exposure period. Recently, it was reported that 0.5 mol % Oct-modified liposomes loaded with anticancer drug were selectively taken by the cells and were effective for the treatment of SSTR-positive breast cancer and gastric cancer.[22,23] In the case of transferrin-targeted liposomes modified with transferrin-PEG-DSPE, the cellular uptake of liposomes was dependent on the concentration of transferrin-PEG-DSPE.[28] Therefore, we increased the concentration of Oct-PEG-DSPE in the liposomes and found that the cellular uptake of the Oct-CL increased more effectively with high Oct-surface-density, more than 1.4 mol % (Figure 2).

Two striking observations in this study were as follows. First, the cellular uptake of Oct-CL was significantly inhibited by empty Oct-CL. The competitive inhibition study of cellular uptake of ligand-modified liposomes was generally done using excess free ligands, not using ligand-liposomes such as Oct-CL. For example, it was reported that the cellular uptake of transferrin-targeted liposomes was inhibited by 20-fold excess transferrin.[28] In this study, 20-fold excess free Oct did not inhibit the cellular uptake of Oct-CL (Figure 3). In the competitive inhibition study *via*

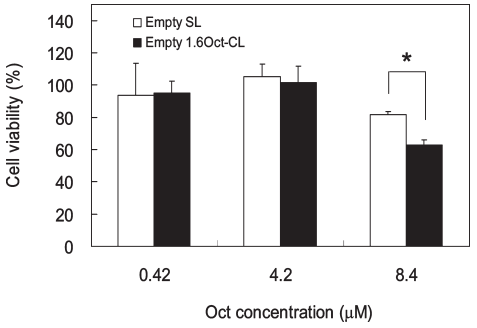

Figure 8. Effect of Oct concentrations on cell viability of Oct-CL compared with SL. TT cells were incubated with empty 1.6Oct-CL or empty SL for 96 h at 37 °C. (*) Differences are statistically significant at P < 0.05.

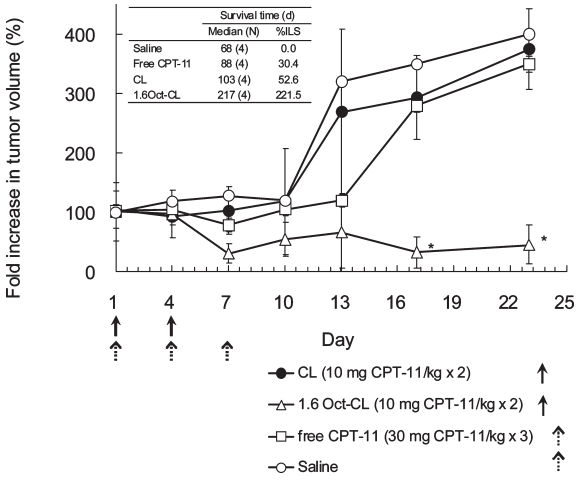

Figure 9. Effect of Oct-modification of liposomal CPT-11 on antitumor activity in mice bearing TT cells. Antitumor activity of Oct-CL loaded with CPT-11 was compared to that of CL or free drug. Liposomal CPT-11 (△, 10 mg/kg Oct-CL; ●, 10 mg/kg CL) was administrated on days 1 and 4, and free CPT-11 (□, 30 mg/kg) and saline (○) were administrated on days 1, 4, and 7. Arrows indicate the day of drug injections. Each value represents the mean ± SD (n = 4). (*) Differences are statistically significant from free CPT-11 at P < 0.05.

SSTR, 100-, 1,000- or 1,000,000-fold excess of Oct was used.[29−31] These experiments had a very high cost, and Oct has been reported to be used as an anticancer drug.[32,33] This information suggested that the use of such an excess Oct was not suitable for competition of the cellular uptake of Oct-CL because of increases in cytotoxicity. Gabizon et al.[34] reported that liposome binding is multivalent, in other words, several ligands contribute to cellular uptake, and the overall affinity for the target cell is the product of the individual affinities of the ligands participating in binding. It could be predicted that the affinity of free Oct and Oct-CL to SSTR are not the same. In this regard, we used empty Oct-CL and empty SL as a competitive inhibitor and as a control, respectively (Figure 4B). The cellular uptake of Oct-CL loaded with drug (Oct concentration 7.2 μM) was inhibited significantly by empty Oct-CL with 14.4 μM Oct, compared with empty SL. This finding indicated that Oct-CL associated *via* SSTR, and the affinity of Oct-CL to SSTR was substantially higher than that of free Oct.

Second, the cytotoxicity of Oct-CL loaded with CPT-11 incubated for 96 h was higher than that of free CPT-11 and SL loaded with CPT-11. The cytotoxicity of free CPT-11 increased in an incubation-time-dependent manner from 24 to 96 h in TT cells, as reported previously.[4] The long incubation times may lead to metabolization of CPT-11 to SN-38, the active form of CPT-11 in TT cells. As a result, the cytotoxicity of free CPT-11 after the 96 h incubation increased 6-fold compared with at 48 h, and that of Oct-CL increased ~25 times, resulting in the highest cytotoxicity among free CPT-11 and SL. These findings suggested that a long incubation time caused CPT-11 release from the inner liposomes, which was converted to the active form SN-38, and consequently increased the cytotoxicity. The receptor-mediated endocytosis mechanism of Oct-CL significantly facilitated cellular uptake and the cytotoxic potential of CPT-11 compared with SL.

The question remains why 1.6Oct-CL showed higher cytotoxicity than free CPT-11, which freely diffuses into cells. Empty 1.6Oct-CL as a control of 1.6Oct-CL (correspond to 4.2 μM of Oct-PEG-DSPE) at 96 h incubation in the cytotoxicity experiments was shown to have no effects (Figure 8). However, empty 1.6Oct-CL showed higher cytotoxicity than empty SL liposomes modified with PEG-lipid corresponded to Oct-PEG-DSPE (8.4 μM) of 1.6Oct-CL at 96 h incubation, at the concentration where free Oct did not show cytotoxicity, suggesting that Oct as a ligand showed cytotoxicity.

With regard to Oct activity, Oct was reported to produce an antiproliferative action in insulinoma cells and pituitary tumor cells.[12,35] These findings suggested that Oct may show an antiproliferative effect in TT cells. From this, Oct may lead empty Oct-CL to showing a stronger cytotoxicity than empty SL because Oct-targeted liposomes were taken up effectively *via* SSTR. However, further experiments are needed to clarify these points *in vitro*.

To examine the effect of Oct-modification of liposomal CPT-11 on cytotoxicity *in vivo*, the antitumor activity of 1.6Oct-CL loaded with CPT-11 was compared with that of free and liposomal CPT-11 in mice bearing TT cells (Figure 9). In an *in vitro* study, Oct increased the cellular association of liposomal CPT-11 (Figure 2), and empty 1.6Oct-CL decreased the viability of TT cells (Figure 8). Therefore, it was estimated that 1.6Oct-CL selectively associated with TT tumor xenografts, led to significantly higher antitumor activity, prolonged the survival time and improved % ILS, compared with CL and free CPT-11 at a one-third dose and lower administration times with free CPT-11. This finding suggested that Oct-modification of liposomal CPT-11 improved therapeutic efficacy for MTC.

5. CONCLUSION

In conclusion, the present study showed that higher concentrations of modified Oct-CL associated effectively with TT cells *via* the somatostatin receptor and had higher cytotoxicity than free CPT-11 or PEGylated liposome SL. These findings indicated that Oct-targeted liposomes loaded with CPT-11 may offer considerable potential for MTC chemotherapy because cytotoxicity of both CPT-11 and Oct was enhanced by effective cellular uptake *via* the somatostatin receptor.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Figure S1 depicting cytotoxicity of free CPT-11, SL loaded with CPT-11, and 1.6Oct-CL loaded with CPT-11 for TT cells incubated for 48, 72, or 96 h. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Vitale, G.; Caraglia, M.; Ciccarelli, A.; Lupoli, G.; Abbruzzese, A.; Tagliaferri, P.; Lupoli, G. Current Approaches and Perspectives in the Therapy of Medullary Thyroid Carcinoma. *Cancer* **2001**, *91*, 1797–1808.

(2) Cakir, M.; Grossman, A. B. Medullary Thyroid Cancer: Molecular Biology and Novel Molecular Therapies. *Neuroendocrinolgy* **2009**, *90*, 323–348.

(3) Giuffrida, D.; Gharib, H. Current diagnosis and management of medullary thyroid carcinoma. *Ann. Oncol.* **1998**, *9*, 695–701.

(4) Strock, C. J.; Park, J. I.; Rosen, D. M.; Ruggeri, B.; Denmeade, S. R.; Ball, D. W.; Nelkin, B. D. Activity of irinotecan and the tyrosine kinase inhibitor CEP-751 in medullary thyroid cancer. *J. Clin. Endocrinol. Metab.* **2006**, *91*, 79–84.

(5) Koga, K.; Hattori, Y.; Komori, M.; Narishima, R.; Yamasaki, M.; Hakoshima, M.; Fukui, T.; Maitani, Y. *Cancer Sci.* **2010**, *101*, 941–947.

(6) Kawato, Y.; Aonuma, M.; Hirota, Y.; Kuga, H.; Sato, K. Intracellular roles of SN-38, a metabolite of the camptothecin derivative CPT-11,in the antitumor effect of CPT-11. *Cancer Res.* **1991**, *51*, 4187–4191.

(7) Kawato, Y.; Furuta, T.; Aonuma, M.; Yasuoka, M.; Yokokura, T.; Matsumoto, K. Antitumor activity of a camptothecin derivative, CPT-11, against human tumor xenografts in nude mice. *Cancer Chemother. Pharmacol.* **1991**, *28*, 192–198.

(8) Hsiang, Y. H.; Wu, H. Y.; Liu, L. F. Topoisomerases: novel therapeutic targets in cancer chemotherapy. *Biochem. Pharmacol.* **1988**, *37*, 1801–1802.

(9) Hsiang, Y. H.; Liu, L. F. Identification of mammalian DNA topoisomerase I as an intracellular target of the anticancer drug camptothecin. *Cancer Res.* **1988**, *48*, 1722–1726.

(10) Hsiang, Y. H.; Lihou, M. G.; Liu, L. F. Arrest of Replication Forks by Drug-stabilized Topoisomerase I-DNA Cleavable Complexes as a Mechanism of Cell Killing by Camptothecin. *Cancer Res.* **1989**, *49*, 5077–5082.

(11) Ohno, R.; Okada, K.; Masaoka, T.; Kuramoto, A.; Arima, T.; Yoshida, Y.; Ariyoshi, H.; Ichimaru, M.; Sasaki, Y.; Oguro, M.; Ito, Y.; Morishima, Y.; Yokomaku, S.; Ota, K. An early phase II study of CPT-11: a new derivative of camptothecin, for the treatment of leukemia and lymphoma. *J. Clin. Oncol.* **1990**, *8*, 1907–1912.

(12) Grozinsky-Glasberg, S.; Shimon, I.; Korbonits, M.; Grossman, A. B. Somatostatin analogues in the control of neuroendocrine tumours: efficacy and mechanisms. *Endocr.-Relat. Cancer* **2008**, *15*, 701–720.

(13) Zatelli, M. C.; Tagliati, F.; Taylor, J. E.; Rossi, R.; Culler, M. D.; Uberti, E. C. Somatostatin Receptor Subtypes 2 and 5 Differentially Affect Proliferation *in Vitro* of the Human Medullary Thyroid Carcinoma Cell Line TT. *J. Clin. Endocrinol. Metab.* **2001**, *86*, 2161–2169.

(14) Zatelli, M. C.; Tagliati, F.; Taylor, J. E.; Piccin, D.; Culler, M. D.; Uberti, E. C. Somatostatin, but not somatostatin receptor subtypes 2 and 5 selective agonists, inhibits calcitonin secretion and gene expression in

the human medullary thyroid carcinoma cell line, TT. *Horm. Metab. Res.* **2002**, *5*, 229–233.

(15) Mato, E.; Matias-Guiu, X.; Chico, A.; Webb, S. M.; Cabezas, R.; Berná, L.; Leiva, A. Somatostatin and Somatostatin Receptor Subtype Gene Expression in Medullary Thyroid Carcinoma. *J. Clin. Endocrinol. Metab.* **1998**, *83*, 2417–2420.

(16) Froidevaux, S.; Eberle, A. N. Somatostatin analogues and radiopeptides in cancer therapy. *Biopolymers* **2002**, *66*, 161–183.

(17) Lemaire, M.; Azria, M.; Dannecker, R.; Marbach, P.; Schweitzer, A.; Maurer, G. Disposition of sandostatin, a new synthetic somatostatin analogue, in rats. *Drug Metab. Dispos.* **1998**, *17*, 699–703.

(18) Kutz, K.; Nüesch, E.; Rosenthaler, J. Pharmacokinetics of SMS 201−995 in healthy subjects. *Scand. J. Gastroenterol. Suppl.* **1986**, *119*, 65–72.

(19) Bakker, W. H.; Krenning, E. P.; Breeman, W. A.; Kooij, P. P.; Koper, J. C.; Jong, M.; Lameris, J. S.; Visser, T. J.; Lamberts, S. W. In vivo use of a radioiodinated somatostatin analogue: dynamics, metabolism, and binding to somatostatin receptor-positive tumors in man. *J. Nucl. Med.* **1991**, *32*, 1184–1189.

(20) Froidevaux, S.; Heppeler, A.; Eberle, A. N.; Meier, A. M.; Hausler, M.; Beglinger, C.; Behe, M.; Powell, P.; Macke, H. R. Preclinical comparison in AR4-2J tumor-bearing mice of four radiolabeled 1, 4, 7, 10-tetraazacyclododecane-1, 4, 7, 10-tetraacetic acid-somatostatin analogs for tumor diagnosis and internal radiotherapy. *Endocrinolgy* **2000**, *141*, 3304–3312.

(21) Smith-Jones, P. M.; Stolz, B.; Albert, R.; Ruser, G.; Briner, U.; Macke, H. R.; Bruns, C. Synthesis and characterisation of [90Y]-Bz-DTPA-oct: a yttrium-90-labelled octreotide analogue for radiotherapy of somatostatin receptor-positive tumors. *Nucl. Med. Biol.* **1998**, *25*, 1981–1988.

(22) Chang, C. C.; Liu, D. Z.; Lin, S. Y.; Liang, H. J.; Hou, W. C.; Huang, W. J.; Chang, C. H.; Ho, F. M.; Liang, Y. C. Liposome encapsulation reduces cantharidin toxicity. *Food Chem. Toxicol.* **2008**, *46*, 3116–3121.

(23) Chen, C. H.; Liu, D. Z.; Fang, H. W.; Liang, H. J.; Yang, T. S.; Lin, S. Y. Evaluation of multi-target and single-target liposomal drugs for the treatment of gastric cancer. *Biosci., Biotechnol., Biochem.* **2008**, *72*, 1586–1594.

(24) Hattori, Y.; Shi, L.; Ding, W.; Koga, K.; Kawano, K.; Hakoshima, M.; Maitani, Y. Novel irinotecan-loaded liposome using phytic acid with high therapeutic efficacy for colon tumors. *J. Controlled Release* **2009**, *136*, 30–37.

(25) Su, J. C.; Tseng, C. L.; Chang, T. G.; Yu, W. J.; Wu., S. K. A synthetic method for peptide-PEG-lipid conjugates: Application of Octreotide-PEG-DSPE synthesis. *Bioorg. Med. Chem. Let.* **2008**, *18*, 4593–4596.

(26) Yamada, A.; Taniguchi, Y.; Kawano, K.; Honda, T.; Hattori, Y.; Maitani, Y. Design of folate-linked liposomal doxorubicin to its antitumor effect in mice. *Clin. Cancer Res.* **2008**, *14*, 8161–8168.

(27) Arai, H.; Suzuki, T.; Kaseda, C.; Ohyama, K.; Takayama, K. Bootstrap Re-sampling Technique to Evaluate the Optimal Formulation of Theophylline Tablets Predicted by Non-linear Response Surface Method Incorporating Multivariate Spline Interpolation. *Chem. Pharm. Bull.* **2007**, *55*, 586–593.

(28) Ishida, O.; Maruyama, K.; Tanahashi, H.; Iwatsuru, M.; Sasaki, K.; Eriguchi, M.; Yanagie, H. Liposomes bearing polyethyleneglycol-coupled transferrin with intracellular targeting property to the solid tumors in vivo. *Pharm. Res.* **2001**, *18*, 1042–1048.

(29) Stroh, T.; Jackson, A. C.; Farra, C. D.; Schonbrunn, A.; Vincent, J. P.; Beaudet, A. Receptor-mediated internalization of somatostatin in rat cortical and hippocampal neurons. *Synapse* **2000**, *38*, 177–186.

(30) Huang, C. M.; Wu, Y. T.; Chen, S. T. Targeting delivery of paclitaxel into tumor cells via somatostatin receptor endocytosis. *Chem. Biol.* **2000**, *7*, 453–461.

(31) Barone, R.; Smissen, P. V. D.; Devuyst, O.; Beaujean, V.; Pauwels, S.; Courtoy, P. J.; Jamar, F. Endocytosis of the somatostatin analogue, octreotide, by the proximal tubule-derived opossum kidney (OK) cell line. *Kidney Int.* **2005**, *67*, 969–976.

(32) Modigliani, E.; Cohen, R.; Joannidis, S.; Siame-Mourot, C.; Guliana, J. M.; Charpentier, G.; Cassuto, D.; Pessayre, M. B.; Tabarin, A.; Roger, P. Results of long-term continuous subcutaneous octreotide administration in 14 patients with medullary thyroid carcinoma. *Clin. Endocrinol.* **1992**, *36*, 183–186.

(33) Lupoli, G.; Cascone, E.; Arlotta, F.; Vitale, G.; Celentano, L.; Salvatore, M.; Lombardi, G. Treatment of advanced medullary thyroid carcinoma with a combination of recombinant interferon alpha-2b and octreotide. *Cancer* **1996**, *78*, 1114–1118.

(34) Gabizon, A.; Shmeeda, H.; Horowitz, A. Z.; Zalipsky, S. Tumor cell targeting of liposome-entrapped drugs with phospholipid-anchored folic acid-PEG conjugates. *Adv. Drug Delivery Rev.* **2004**, *56*, 1177–1192.

(35) Theodoropoulou, M.; Zhang, J.; Laupheimer, S.; Paez-Pereda, M.; Erneux, C.; Florio, T.; Pagotto, U.; Stalla, G. K. Octreotide, a somatostatin analogue, mediates its antiproliferative action in pituitary tumor cells by altering phosphatidylinositol 3-kinase signaling and inducing Zac1 expression. *Cancer Res.* **2006**, *66*, 1576–1582.