articles

molecular pharmaceutics

Higher Liposomal Membrane Fluidity Enhances the in Vitro Antitumor Activity of Folate-Targeted Liposomal Mitoxantrone

Kumi Kawano, Eri Onose, Yoshiyuki Hattori, and Yoshie Maitani*

Institute of Medicinal Chemistry, Hoshi University, Ebara 2-4-41, Shinagawa-ku, Tokyo 142-8501, Japan

Received June 23, 2008; Revised Manuscript Received November 5, 2008; Accepted November 17, 2008

Abstract: The efficacy of folate-targeted liposomal drug delivery has not been fully achieved in part because of the slow release of the encapsulated drugs following uptake of the liposomes by target cells. Since liposomal mitoxantrone (MXN) composed of lipids with high fluidity was reported to achieve strong anticancer effects in vivo, we hypothesized that folate-targeted liposomal MXN uptake via folate receptor (FR)-mediated endocytosis could effectively release drugs into the endosomal compartment. Folate-targeted liposomal MXN was prepared using two lipids with different fluidities. MXN was released slowly from all types of liposome into PBS, indicating that the cellular uptake of MXN was considered to be in the liposomal form. Folate-targeted liposomes with high fluidity exhibited lower cellular uptake of loaded FITC-labeled dextran into FR (+) KB cells, but, when MXN was loaded, higher cytotoxicity than liposomes with lower fluidity. On the other hand, the cellular uptake of non-folate liposomes was not affected by the membrane fluidity, but higher cytotoxicity was observed in liposomal MXN with high fluidity, which suggested a higher rate of release of the drug from the liposomes. High levels of cytotoxic activity were achieved with folate-targeted liposomal MXN though the cellular uptake rate was restricted by selecting liposomes with higher lipid membrane fluidity. This finding provides a new insight into folate-targeted carrier drug delivery.

Keywords: Liposome; folate targeting; mitoxantrone; membrane fluidity

1. Introduction

Liposome formulations have been developed to enhance the therapeutic activity of anticancer agents. To increase the therapeutic effect on tumors, various targeting ligands have been investigated as tumor targeted drug carriers. Folate receptor (FR), a 38 kDa glycosyl−phosphatidylinositol−anchored glycoprotein, is overexpressed in many human cancer cells, including malignancies of the ovary, mammary gland, lung, kidney, and throat, but are expressed minimally in normal tissues.[1,2] Therefore, FR can serve as a functional tumor-specific receptor. Folic acid, a high-affinity ligand for FR, and its conjugate retain its receptor binding and endocytosis properties with FR positive cancer cells; FR-targeted liposome carriers have shown both selective binding and uptake. Liposomes conjugated to the folate ligand via a polyethylene glycol (PEG) spacer have been used to deliver chemotherapeutic agents.[3,4] It has already been shown that the length of the PEG linker chain and the amount of folate ligand are important for liposomes to be internalized fol-

* Corresponding author. Mailing address: Institute of Medicinal Chemistry, Hoshi University, Ebara 2-4-41, Shinagawa, Tokyo, 142-8501 Japan. Tel and fax: +81-3-5498-5048. E-mail: yoshie@hoshi.ac.jp.

(1) Toffoli, G.; Cernigoi, C.; Russo, A.; Gallo, A.; Bagnoli, M.; Boiocchi, M. Overexpression of folate binding protein in ovarian cancers. *Int. J. Cancer* **1997**, *74* (2), 193–198.

(2) Weitman, S. D.; Lark, R. H.; Coney, L. R.; Fort, D. W.; Frasca, V.; Zurawski, V. R., Jr.; Kamen, B. A. Distribution of the folate receptor GP38 in normal and malignant cell lines and tissues. *Cancer Res.* **1992**, *52* (12), 3396–3401.

(3) Gabizon, A.; Horowitz, A. T.; Goren, D.; Tzemach, D.; Shmeeda, H.; Zalipsky, S. In vivo fate of folate-targeted polyethylene-glycol liposomes in tumor-bearing mice. *Clin. Cancer Res.* **2003**, *9* (17), 6551–6559.

10.1021/mp800069c CCC: $40.75
Published on Web 12/15/2008

lowing recognition by FR[5] and uptake into cancer cells.[6] Longer PEG spacers (MW 5000) and lower concentrations of folate ligand were more effective for FR-targeting in vitro.[7]

Unfortunately, the efficacy of folate-targeted liposomal drug delivery has not achieved its full potential, in part because of the slow release of the encapsulated drugs following uptake of the liposomes by target cells. In order to overcome this obstacle, pH-sensitive liposomes have been developed.[8] However, there are few reports to our knowledge on the effect of lipid composition of folate-targeted liposomes on FR-targeting in regard to liposomal membrane fluidity. Focusing on lipid composition in drug release, it has been reported that liposomal mitoxantrone (MXN) composed of lipids with higher fluidity showed higher efficacy in vivo,[9] and MXN release from liposomes became the dominant factor contributing to therapeutic activity in vivo.[10,11] The lipid composition of liposomes might affect the cellular association and release of MXN into the cytoplasm differently. The possible explanations for this have not yet been clarified in vitro. Furthermore, from the in vivo results with MXN,[12] it was predicted that the mixing of lipids with shorter alkyl tails (<C18) in liposomes during the cellular uptake process within the endosome will promote endosomal membrane fluidity and enhance drug release. Therefore, in this study it was hypothesized that folate-targeted liposomal MXN composed of lipids with high fluidity could achieve stronger anticancer effects by higher uptake by FR-mediated endocytosis and then rapid release of the drug from the endosomal compartment.

Although anthracyclines, such as MXN and doxorubicin (DXR), have a similar structure, they have significantly different octanol/buffer partition coefficient.[13] Liposomal DXR has been extensively investigated in this area, and consequently it was selected as a control. This study investigated the drug release, cellular association, and in vitro antitumor activity of folate-targeted liposomal MXN composed of two lipids with different fluidities compared with liposomal DXR in FR-positive KB cells, which is a human nasopharyngeal cell line.

2. Materials and Methods

2.1. Materials. Hydrogenated soybean phosphatidylcholine (HSPC) and dimyristoyl phosphatidylcholine (DMPC) were obtained, and amino-poly(ethyleneglycol)-distearylphosphatidylethanolamine (amino-PEG-DSPE, PEG mean molecular weight 5,000) was a kind gift from NOF Corp. (Tokyo, Japan). Egg phosphatidylcholine (EPC) was purchased from Q. P. Co., Ltd. (Tokyo, Japan). MXN hydrochloride was a kind gift from Wyeth K.K. (Tokyo, Japan). DXR hydrochloride and cholesterol (Ch) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). 1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Dextran-fluorescein isothiocyanate (D-FITC, molecular weight ~4,000) was purchased from Sigma Chemical Co. (St Louis, MO). Folate-PEG_{5000}-DSPE conjugate of folic acid was synthesized from amino-PEG-DSPE as reported previously.[5,14] Folate-deficient RPMI-1640 medium and fetal bovine serum (FBS) were obtained from Invitrogen Corp. (Carlsbad, CA). Other reagents used in this study were of reagent grade.

2.2. Preparation of Folate-Targeted Liposome. 2.2.1. Folate-Targeted Liposome Preparation. Liposomes were prepared from HSPC/Ch = 55/45 (mol/mol), DMPC/Ch = 55/45 (mol/mol), and EPC/Ch = 55/45 (mol/mol); HSPC-L, DMPC-L, and EPC-L, respectively, by a dry-film method as reported previously.[15] Briefly, all lipids were dissolved in chloroform, which was then removed by evaporation to leave a thin lipid film. The thin film was hydrated with citrate buffer (300 mM, adjusted to pH 4.0 with NaOH) and

(4) Pan, X. Q.; Wang, H.; Lee, R. J. Antitumor activity of folate receptor-targeted liposomal doxorubicin in a KB oral carcinoma murine xenograft model. *Pharm. Res.* **2003**, *20* (3), 417–422.
(5) Gabizon, A.; Horowitz, A. T.; Goren, D.; Tzemach, D.; Mandelbaum-Shavit, F.; Qazen, M. M.; Zalipsky, S. Targeting folate receptor with folate linked to extremities of poly(ethylene glycol)-grafted liposomes: in vitro studies. *Bioconjugate Chem.* **1999**, *10* (2), 289–298.
(6) Saul, J. M.; Annapragada, A.; Natarajan, J. V.; Bellamkonda, R. V. Controlled targeting of liposomal doxorubicin via the folate receptor in vitro. *J. Controlled Release* **2003**, *92* (1−2), 49–67.
(7) Kawano, K.; Maitani, Y. Effect of PEG spacer length and ligand density on folate receptor targeting of liposomal doxorubicin. *Drug Dev. Ind. Pharm.*, submitted.
(8) Turk, M. J.; Reddy, J. A.; Chmielewski, J. A.; Low, P. S. Characterization of a novel pH-sensitive peptide that enhances drug release from folate-targeted liposomes at endosomal pHs. *Biochim. Biophys. Acta* **2002**, *1559* (1), 56–68.
(9) Lim, H. J.; Masin, D.; McIntosh, N. L.; Madden, T. D.; Bally, M. B. Role of drug release and liposome-mediated drug delivery in governing the therapeutic activity of liposomal mitoxantrone used to treat human A431 and LS180 solid tumors. *J. Pharmacol. Exp. Ther.* **2000**, *292* (1), 337–345.
(10) Lim, H. J.; Masin, D.; Madden, T. D.; Bally, M. B. Influence of drug release characteristics on the therapeutic activity of liposomal mitoxantrone. *J. Pharmacol. Exp. Ther.* **1997**, *281* (1), 566–573.
(11) Wu, M.; Gunning, W.; Ratnam, M. Expression of folate receptor type alpha in relation to cell type, malignancy, and differentiation in ovary, uterus, and cervix. *Cancer Epidemiol. Biomarkers Prev.* **1999**, *8* (9), 775–782.
(12) Adlakha-Hutcheon, G.; Bally, M. B.; Shew, C. R.; Madden, T. D. Controlled destabilization of a liposomal drug delivery system enhances mitoxantrone antitumor activity. *Nat. Biotechnol.* **1999**, *17* (8), 775–779.
(13) Regev, R.; Yeheskely-Hayon, D.; Katzir, H.; Eytan, G. D. Transport of anthracyclines and mitoxantrone across membranes by a flip-flop mechanism. *Biochem. Pharmacol.* **2005**, *70* (1), 161–169.
(14) Shiokawa, T.; Hattori, Y.; Kawano, K.; Ohguchi, Y.; Kawakami, H.; Toma, K.; Maitani, Y. Effect of polyethylene glycol linker chain length of folate-linked microemulsions loading aclacinomycin A on targeting ability and antitumor effect in vitro and in vivo. *Clin. Cancer Res.* **2005**, *11* (5), 2018–2025.
(15) Horowitz, A. T.; Barenholz, Y.; Gabizon, A. A. In vitro cytotoxicity of liposome-encapsulated doxorubicin: dependence on liposome composition and drug release. *Biochim. Biophys. Acta* **1992**, *1109* (2), 203–209.

sonicated to decrease the size. The folate ligand was inserted into preformed liposomes; HSPC-L and DMPC-L, by incubation with an aqueous dispersion of folate-PEG_{5000}-DSPE (0.03 mol% of total lipid) at 60 °C for 1 h (F/HSPC-L and F/DMPC-L, respectively). The resulting mean diameter of the liposomes was about 130 nm with a narrow size distribution (polydispersity <0.3), as determined by a dynamic light scattering method (ELS-Z2, Otsuka Electronics Co., Ltd., Osaka, Japan) at 25 °C after diluting the liposome suspension with water.

2.2.2. Preparation of Folate-Targeted Liposomal MXN and DXR. Folate-targeted liposomes were actively loaded with MXN[9,10] and DXR[16] by a pH gradient method. After the external pH was adjusted to pH 7.4, folate-targeted liposomes were incubated with MXN (drug:lipid = 1:10, wt/wt) at 60 °C for 20 min, or incubated with DXR (drug:lipid = 1:5, wt/wt) at 60 °C for 25 min. The loading efficiencies of MXN and DXR were determined by separating the unencapsulated drug from the encapsulated drug on a Sephadex G-50 column with saline as a mobile phase. MXN and DXR concentrations were determined by measuring absorbance at 608 and 480 nm, respectively (UV-1700 Pharmaspec, Shimadzu Co., Kyoto, Japan).

2.2.3. Preparation of Folate-Targeted Liposome Encapsulating D-FITC. D-FITC encapsulating liposomes were prepared by hydration of lipids with 15 mM HEPES buffer (pH 7.0) containing 30 mg/mL (3%) D-FITC and sonication. The folate ligand was inserted into preformed liposomes as described in section 2.2.1, Folate-Targeted Liposome Preparation. The liposomes were then dialyzed against HEPES buffer without D-FITC at room temperature until the dialysis buffer was fluorescein free.[17]

2.3. In Vitro Drug Release. The release of MXN and DXR from liposomes in a dialysis tube was measured using seamless cellulose tube membranes (Viskase Sales Corp., IL) with a molecular weight cutoff of 12,000−14,000. The initial concentrations of MXN and DXR were 450 μg/mL and 500 μg/mL, respectively. The sample volume in the dialysis tube was 1 mL of liposomal MXN or DXR with 1 mL of FBS to examine the influence of protein, and the sink volumes were 100 and 200 mL of phosphate-buffered saline at pH 7.4 (PBS) for MXN and DXR, respectively, at 37 °C.[18] The MXN concentration was analyzed using an HPLC column for analysis.[19] Methylene blue was used as the internal standard (i.s.). The HPLC system was composed of an LC-20AT pump (Shimadzu Co.), SIL-20A autoinjector (Shimadzu Co.), SPD-M20A UV−visible detector (Shimadzu Co.), and a YMC-PACK ODS-AA-302, 150 × 4.6 mm i.d. column (YMC Co., Ltd., Kyoto, Japan). The isocratic mobile phase was 29:71 (v/v) acetonitrile:ammonium formate (160 mM) with hexanesulfonic acid (35 mM), adjusted to pH 2.7 with formic acid, running at a flow rate of 1.0 mL/min. The i.s. and MXN were detected by absorbance at 655 nm. The DXR concentration was analyzed using a fluorophotometer (Wallac 1420 ARVOsx multilabel counter, Perkin-Elmer Life Science, Tokyo, Japan) with excitation and emission wavelengths of 485 and 535 nm, respectively.

2.4. Fluorescence Anisotropy Measurements. The precise method of fluorescence anisotropy measurement was as reported previously.[20] The phospholipid bilayer was labeled with DPH by adding 10 μL of 10 mM freshly prepared DPH stock solution in tetrahydrofuran to 1000 μL of liposome suspension and then incubated at 37 °C for 2 h in the dark to complete the labeling. The fluorescence anisotropy of DPH in liposomes was measured with a fluorescence spectrophotometer (Hitachi F-450, Hitachi Co. Ltd., Tokyo, Japan) at 15−45 °C. The excitation and emission wavelengths used for DPH were 351 and 430 nm, respectively. The effect of temperature on the fluorescence anisotropy of DPH was measured at a heating rate of 1 °C /min. The steady-state fluorescent anisotropy was calculated using the following equation: $r = (I_{VV} - I_{VH})/(I_{VV} + 2I_{VH})$ where r is anisotropy and I_{VV} and I_{VH} are the intensity measured in directions parallel and perpendicular to the polarized excitation light, respectively.

2.5. Cell Culture. KB cells were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Miyagi, Japan). The human lung adenocarcinoma A549 cell line was kindly provided by OncoTherapy Science, Inc. (Kanagawa, Japan). The cells were cultured in folate-deficient RPMI-1640 medium containing 10% heat-inactivated FBS and 50 μg/mL kanamycin sulfate in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.6. Determination of Cellar Uptake of D-FITC-Encapsulating Liposomes. KB cells were prepared by plating 3×10^5 cells/well in a 6-well culture plate 1 day before the assay. Cells were incubated with D-FITC-encapsulating liposomes containing 100 μg/mL lipids in 2 mL of folate-deficient RPMI-1640 medium containing 10% heat-inactivated FBS for 2 h at 37 °C. After incubation, the cells were washed two times with PBS at pH 7.4, and lysed with PBS containing 0.2% Triton X-100. Associated amounts of D-FITC were measured using a fluorophotometer (Wallac 1420 ARVOsx multilabel counter)

(16) Lee, R. J.; Low, P. S. Folate-mediated tumor cell targeting of liposome-entrapped doxorubicin in vitro. *Biochim. Biophys. Acta* **1995**, *1233* (2), 134–144.

(17) Shmeeda, H.; Mak, L.; Tzemach, D.; Astrahan, P.; Tarshish, M.; Gabizon, A. Intracellular uptake and intracavitary targeting of folate-conjugated liposomes in a mouse lymphoma model with up-regulated folate receptors. *Mol. Cancer Ther.* **2006**, *5* (4), 818–824.

(18) Johnston, M. J.; Semple, S. C.; Klimuk, S. K.; Edwards, K.; Eisenhardt, M. L.; Leng, E. C.; Karlsson, G.; Yanko, D.; Cullis, P. R. Therapeutically optimized rates of drug release can be achieved by varying the drug-to-lipid ratio in liposomal vincristine formulations. *Biochim. Biophys. Acta* **2006**, *1758* (1), 55–64.

(19) Johnson, J. L.; Ahmad, A.; Khan, S.; Wang, Y. F.; Abu-Qare, A. W.; Ayoub, J. E.; Zhang, A.; Ahmad, I. Improved liquid chromatographic method for mitoxantrone quantification in mouse plasma and tissues to study the pharmacokinetics of a liposome entrapped mitoxantrone formulation. *J. Chromatogr. B* **2004**, *799*, 149–155.

(20) Maitani, Y.; Katayama, S.; Kawano, K.; Hayama, A.; Toma, K. Artificial Lipids Stabilized Camptothecin Incorporated into Liposomes. *Biol. Pharm. Bull.* **2008**, *31* (5), 990–993.

with excitation and emission wavelengths of 485 and 535 nm, respectively.[21] The cellular uptake of D-FITC was quantified by the fluorescence of the cell lysate. The amount of cellular uptake was calculated using the following equation:

$$\text{dose}\ (\%) = \text{cellular uptake of D-FITC (mg)} / \text{applied D-FITC (mg)} \times 100 \quad (1)$$

2.7. Flow Cytometry Analysis. KB and A549 cells were prepared by plating 3×10^5 cells/well or 1.5×10^5 cells/well, respectively, in a 6-well culture plate 1 day before assaying. Cells were incubated with D-FITC-encapsulating liposomes containing 100 μg/mL lipids diluted in 2 mL of folate-deficient RPMI-1640 medium containing 10% heat-inactivated FBS for 2 h at 37 °C, with non-folate or folate-targeted liposomes. After incubation, the cells were washed with cold PBS two times, detached with 0.05% trypsin, and then suspended in PBS containing 0.1% bovine serum albumin and 1 mM EDTA. The suspended cells were directly introduced into a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 488 nm argon ion laser. Data for 10,000 fluorescent events were obtained by recording forward scatter and side scatter with 530/30 nm fluorescence. The autofluorescence of cells was taken as a control.

2.8. Cytotoxicity Study. KB cells were prepared by plating 1×10^4 cells in a 96-well culture plate 1 day before the experiment. Cells were then incubated with liposomes containing 0.02−200 μg/mL MXN or 0.01−100 μg/mL DXR diluted in folate-deficient RPMI-1640 medium containing 10% heat-inactivated FBS for 2 h at 37 °C. After incubation, the cells were washed with PBS and cultured in fresh medium containing 10% heat-inactivated FBS until 48 h. Cytotoxicity was determined using the WST-8 assay (Dojindo Laboratories, Kumamoto, Japan), based on enzymatic reduction of a tetrazolium salt, WST-8, to water-soluble formazan. The number of viable cells was then determined by absorbance measured at 450 nm using an automated plate reader (BioRad, CA).

2.9. Confocal Microscopy. After incubation with D-FITC-encapsulating liposome for 24 h or liposomal DXR containing 100 μg/mL lipids for 1, 2, 6 and 24 h as described in section 2.6, the medium was removed, and the cells were washed three times with PBS and fixed with 10% formaldehyde in PBS at 37 °C for 20 min. Then, the cells were coated with Aqua Poly/Mount (Polyscience, Warrington, PA) to prevent fading and covered with coverslips. The fixed cells were observed with a Radiance 2100 confocal laser scanning microscope (BioRad) with an excitation wavelength of 488 nm and an emission wavelength at 530 nm with a filter HQ515/30.

2.10. Statistical Analysis. Statistical comparisons were performed using Student's *t*-test. *P* values less than 0.05 were considered significant.

(21) Kawano, K.; Nakamura, K.; Hayashi, K.; Nagai, T.; Takayama, K.; Maitani, Y. Liver targeting liposomes containing beta-sitosterol glucoside with regard to penetration-enhancing effect on HepG2 cells. *Biol. Pharm. Bull.* **2002**, *25* (6), 766–770.

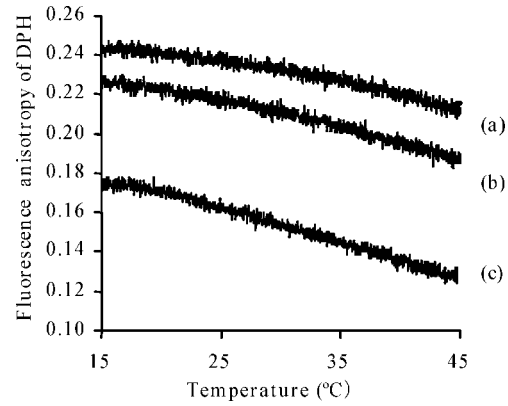

Figure 1. Temperature dependency of fluorescence anisotropy of DPH in liposomes. (a) HSPC-L, (b) DMPC-L, and (c) EPC-L.

3. Results and Discussion

3.1. Characterization of Liposomes. In this study, four types of liposome composed of either HSPC/Ch or DMPC/Ch were prepared with non-folate and folate-targeted liposomes. Folate-targeted liposomes were coated with 0.03 mol % folate-PEG_{5000}-DSPE because this modification showed the highest cellular uptake in KB cells (data not shown) as reported previously.[7] Both HSPC-L and DMPC-L were efficiently modified with folate-PEG_{5000}-DSPE of which insertion efficiency was 91.4% and 95.9%, respectively.

The use of the pH gradient method gave a high loading efficiency of MXN and DXR >95% at a drug-to-total lipid ratio of 1:10 (wt:wt) and of 1:5 (wt:wt), respectively. The average particle diameter of each liposome in water was approximately 130 nm. The loading efficiency of liposomal MXN and DXR did not change significantly for at least 1 month at 4 °C in the dark.

The phase-transition temperature (T_m) of the phospholipid component influences drug retention. In general, liposomal membranes are more permeable through high membrane fluidity when the incubation temperature is above the T_m. To investigate the membrane fluidity of liposomes, fluorescence anisotropy studies were performed using the fluorescent probe DPH, which distributes deeply into the hydrophobic interior of liposomes. Anisotropy increased in the order EPC-L < DMPC-L < HSPC-L (Figure 1), which reflected the decreasing trend in membrane fluidity. This finding that liposomes composed of Ch and lipids with low T_m value exhibited higher liposomal membrane fluidity corresponded well with the inherent T_m values of EPC, DMPC, and HSPC of −10 °C, 23 °C, and 50−60 °C, respectively.

3.2. Stability of Liposomal MXN or DXR. Stability of liposomal MXN or DXR formulations was evaluated from drug release. The release assay of liposome suspension with 50% FBS is based on dialysis against a large volume of PBS to mimic the condition of liposomes incubated with cells. Under these conditions, whereas <45% free-MXN release was observed over a 48 h incubation period at 37 °C, <2.5% MXN release was observed from all liposomal formulations

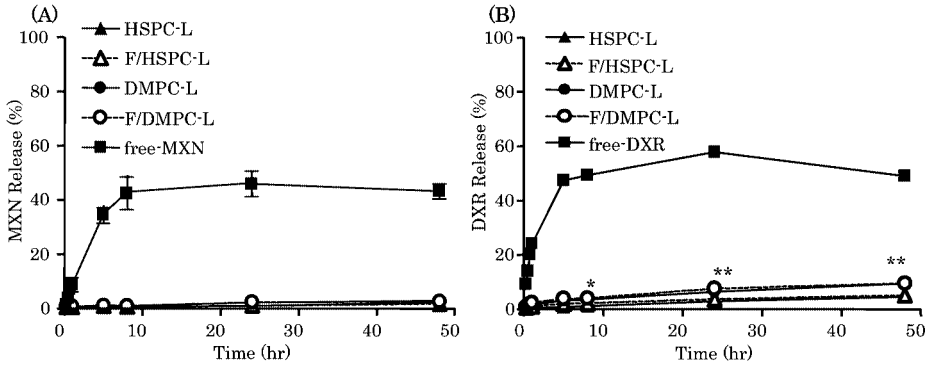

Figure 2. MXN (A) or DXR (B) release from liposomal MXN or DXR with FBS at 37 °C into PBS (pH 7.4). Each value represents mean ± SD ($n = 3$). *$P < 0.05$, **$P < 0.01$ for F/HSPC-L compared with F/DMPC-L and for HSPC-L compared with DMPC-L.

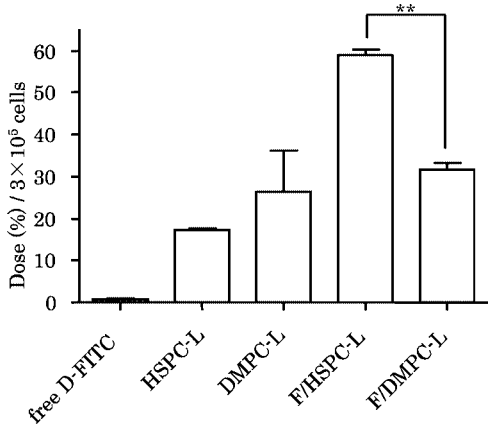

Figure 3. Cellular uptake of liposomes encapsulating D-FITC with KB cells 2 h at 37 °C after incubation by measuring the amount of D-FITC in the cells. Each value represents mean ± SE ($n = 3$). **$P < 0.01$ for F/HSPC-L compared with F/DMPC-L.

(Figure 2A). This result was consistent with MXN release studies in vitro that demonstrated no difference in drug release from liposomal formulations composed of distearyl phosphatidylcholine (DSPC) and DMPC[10] or that of dioleyl phosphatidylcholine and cardiolipin.[22] Different T_m values of the phospholipid species did not markedly affect MXN release. On the other hand, different DXR release rates were observed from different liposomal formulations. The release of DXR from both DMPC-L and F/DMPC-L exhibited a significantly more rapid release behavior than HSPC-L and F/HSPC-L, respectively, over 8 h ($P < 0.05$) (Figure 2B). This finding corresponded with the previous report that liposomes with higher T_m appeared to be more stable in PBS at 37 °C indicating T_m is directly proportional to stability.[23]

Concerning the difference in drug release from liposomes, MXN was released more slowly than DXR (Figure 2A,B). This finding adds to the report that liposomal MXN activity requires a modified lipid composition to promote release of the drug and improve efficiency.[10] The liposomal stability of MXN appears to be higher than that of DXR, using the current liposome formulations, supported by the report that more lipophilic MXN exhibited higher affinity toward liposome membranes compared to DXR.[13]

3.3. Cellular Uptake of Folate-Targeted Liposomes Encapsulating D-FITC. The cellular uptake of folate-targeted liposomes encapsulating D-FITC was investigated by incubation with FR (+) KB or FR (−) A549 cells both by measuring D-FITC in cells and by flow cytometry. Here D-FITC was used instead of MXN because MXN is not fluorescent and therefore its detection level was low. Moreover, since it is lipophilic, MXN alone from liposomal MXN could be transferred to cell membrane. D-FITC is fluorescent, is very hydrophilic and is unable to penetrate cells except via liposome endocytosis as shown in Figure 3. Liposomal DXR was leaky as shown in Figure 2B so that cellular uptake could not be investigated.

As for the influence of the lipid composition on cellular uptake 2 h after incubation, F/HSPC-L, which has lower lipid membrane fluidity, showed about 2-fold higher association than F/DMPC-L ($P < 0.01$). Non-folate liposomes were taken up similarly regardless of lipid composition.

Flow cytometry analysis showed a shift in the curve and a clear increase in cellular uptake of F/HSPC-L and F/DMPC-L after 2 h exposure to KB cells (Figure 4A). In addition, the mean fluorescence intensity of F/HSPC-L had 2.9-fold greater association than that of F/DMPC-L. In contrast, non-folate liposomes such as HSPC-L and DMPC-L shifted very little. The rank order of the potential of the liposomes to deliver encapsulated D-FITC into the cells was F/HSPC-L > F/DMPC-L > HSPC-L = DMPC-L. In contrast, the cellular uptake of liposomes into A549 cells was not observed (Figure 4B). The results indicate that folate-targeted liposomes were transported into cells by an FR-mediated endocytosis process. These findings were consistent with a previous report on the FR-mediated cellular uptake of folate-targeted liposomes and emulsions in KB cells.[5,14]

(22) Ugwu, S.; Zhang, A.; Parmar, M.; Miller, B.; Sardone, T.; Peikov, V.; Ahmad, I. Preparation, characterization, and stability of liposome-based formulations of mitoxantrone. *Drug Dev. Ind. Pharm.* **2005**, *31* (2), 223–229.

(23) Anderson, M.; Omri, A. The effect of different lipid components on the in vitro stability and release kinetics of liposome formulations. *Drug Delivery* **2004**, *11* (1), 33–39.

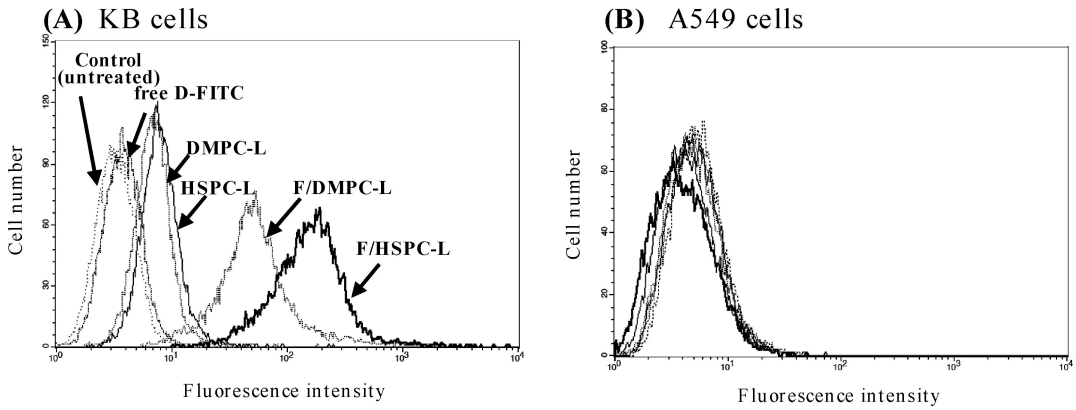

Figure 4. Cellular uptake of liposomes encapsulating D-FITC with KB (A) or A549 (B) cells by flow cytometry after 2 h incubation. Control indicates autofluorescence of untreated cells.

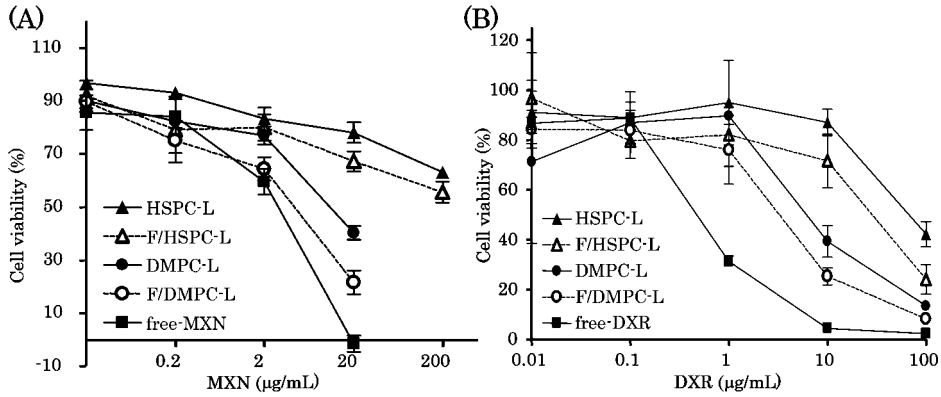

Figure 5. Cytotoxicity of liposomal MXN (A) or DXR (B) against KB cells. Cells were incubated with liposomes in folate-free medium for 2 h, then in fresh medium without the drug for 48 h at 37 °C. Each value represents mean ± SD ($n = 5$).

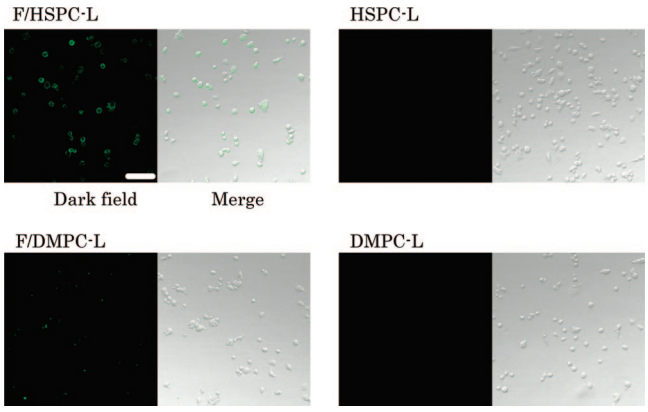

Figure 6. Confocal microscopic images of KB cells treated with liposomes encapsulating D-FITC after 24 h incubation. Scale bar denotes 100 μm (×200).

However, this is the first report that folate-targeted HSPC-L has a much higher uptake rate into FR (+) cells than DMPC-L. FR-mediated liposomes with low liposomal membrane fluidity were taken up more effectively into KB cells, suggesting that an anchor part with lower flexibility might restrict the movement of the hydrated, flexible PEG linker, resulting in high recognition efficiency of the folate ligand by FR.

3.4. Cytotoxicity Study. The cytotoxicity of liposomal MXN or DXR formulations to KB cells was evaluated by a WST-8 assay (Figure 5). Folate-targeted liposomes showed significantly higher cytotoxicity than non-folate-targeted liposomes, which corresponded to their cellular uptake rate. Empty-liposomes into which the drugs were not loaded did not show any toxicity to KB cells (data not shown). However, as for the influence of lipid composition on cytotoxicity, MXN-loaded DMPC-L with higher membrane fluidity showed higher toxicity than HSPC-L. The rank order of cytotoxicity to the cells was HSPC-L < F/HSPC-L < DMPC-L < F/DMPC-L < free-MXN in KB cells (Figure 5A). MXN concentrations leading to 50% cell death (IC_{50}) were determined from concentration-dependent cell viability curves. The IC_{50} values of each liposomal MXN formulation were HSPC-L = F/HSPC-L > 200 μg/mL, DMPC-L = 10.9 μg/mL, F/DMPC-L = 4.3 μg/mL, and free-MXN = 2.9 μg/mL. Surprisingly, this finding was in conflict with the results of the cellular uptake study. Since the release of MXN from all liposomes during incubation with cells was quite low regardless of folate-linkage, cytotoxicity was considered to reflect drug release from the liposomes in the endosomes into the cytoplasm. Therefore, this finding indicates that liposomal membranes with higher fluidity easily released MXN.

Similar to MXN, DXR-loaded DMPC-L with high fluidity showed higher cytotoxicity (Figure 5B), corresponding to the higher release of DXR (Figure 2B). This finding indicated that DXR released from DMPC-L into the extracellular medium mediated the cytotoxic effect. This finding corresponded with the previous report.[15] The IC_{50} values of each liposomal DXR formulation were HSPC-L = 66.8 μg/mL, F/HSPC-L = 28.3 μg/mL, DMPC-L = 3.9 μg/mL, F/DMPC-L = 3.3 μg/mL, and free-DXR = 0.47 μg/mL.

3.5. Cellular Uptake Observed by Confocal Microscopy. To investigate differences in cellular uptake and cytotoxicity of liposomal MXN, the intracellular localization of the various liposome formulations encapsulating D-FITC was observed by confocal microscopy (Figure 6). KB cells were exposed to liposomes for 24 h. Until 24 h, the liposomes were barely detectable because the fluorescence intensity of D-FITC was not particularly strong. The fluorescence of D-FITC in folate-targeted liposomes was observed clearly after 24 h incubation, whereas that of non-folate liposome was weak. In particular, the fluorescence from F/HSPC-L was significant in its extent, which corresponded with the results of cellular uptake and flow cytometry (Figures 3 and 4A).

To compare the cellular uptake of D-FITC with DXR, the intracellular localization of the various liposomal DXR formulations was observed (Figure 1s in the Supporting Information). After incubation for 1, 2, 6, and 24 h, the fluorescence of DXR in folate-targeted liposomes was observed more clearly than with non-folate liposomes. In contrast to D-FITC, DXR in both F/DMPC-L and DMPC-L were taken up into cells much more strongly than F/HSPC-L and HSPC-L. This finding reflected that DXR-loaded F/DMPC-L and DMPC-L were significantly more leaky than F/HSPC-L and HSPC-L (Figure 2B), which corresponded with the findings of the previous report.[24] Furthermore, although leakage of DXR from liposomes contributed to the cytotoxicity of liposomal DXR, the uptake via FR-mediated endocytosis also contributed, since there was no significant difference in release between F/DMPC-L and DMPC-L (Figure 2B), but the former showed higher toxicity than the latter (Figure 5B).

The cellular uptake of F/HSPC-L was higher than F/DMPC-L and non-folate liposomes, suggesting that folate was effectively recognized by FR when the anchor lipid was locked into the liposomes. On the contrary, the uptake of non-folate HSPC- and DMPC-L was similar, even if leakage and/or fusion of the liposomes with the plasma membrane occurred over endocytosis. From this study it can be concluded that, for FR-mediated uptake, liposomal membranes with lower fluidity were more effective. On the contrary, for the release of liposomal drugs from the endosome into the cytoplasm, liposomes with high fluidity were more effective irrespective of the existence of folate modifications. This is one of the reasons why the in vivo antitumor efficacy of liposomal MXN with higher fluidity liposomes was more effective, which was different from DXR. The choice of the appropriate lipid composition for FR-targeting liposomes is important to obtain a formulation that fits all the necessary criteria for each clinical application.

Conclusion

In this study, F/HSPC-L exhibited higher cellular uptake via FR-mediated endocytosis into FR (+) KB cells but lower cytotoxicity than F/DMPC-L. High cytotoxic activity of folate-targeted liposomal MXN was achieved by selecting lipids with higher lipid membrane fluidity though cellular uptake was restricted. This may be a novel finding for the design of liposomes in FR-targeting liposomes in the clinical setting. However, further in vivo evaluation of this new formulation is warranted.

Acknowledgment. This study was supported in part by the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by the Open Research Center Project.

Supporting Information Available: Figure 1s, confocal microscopic images of KB cells incubated with liposomal DXR for 1, 2, 6, and 24 h. This material is available free of charge via the Internet at http://pubs.acs.org.

MP800069C

(24) Kobayashi, T.; Ishida, T.; Okada, Y.; Ise, S.; Harashima, H.; Kiwada, H. Effect of transferrin receptor-targeted liposomal doxorubicin in P-glycoprotein-mediated drug resistant tumor cells. *Int. J. Pharm.* **2007**, *329* (1−2), 94–102.