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Role of Formulation Composition in Folate Receptor-Targeted Liposomal Doxorubicin Delivery to Acute Myelogenous Leukemia Cells

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Abstract: Targeted drug delivery has the potential to improve the efficacy of a therapeutic agent while reducing its side effects. The folate receptor type β (FR-β) is a cell surface marker selectively expressed in the leukemic cells of approximately 70% of acute myeloid leukemia (AML) patients. Upregulation of FR-β may also be selectively induced in AML cells by treatment with *all-trans*-retinoic acid (ATRA). In this study, the role of formulation composition in FR-targeted liposomal doxorubicin (DOX) delivery to AML cells was investigated. Liposomal formulations with a variable percentage of folate-polyethylene glycol distearoyl phosphatidylethanolamine (f-PEG-DSPE) were synthesized and evaluated for FR-β-targeted DOX delivery in MV4-11 AML cells in vitro and for their pharmacokinetic properties in vivo. The formulation containing 0.5 mol % f-PEG-DSPE exhibited the highest efficiency of cellular uptake and in vitro cytotoxicity, as well as a long systemic circulation time in mice. In MV4-11 cells, the binding and cytotoxicity of FR-targeted liposomal DOX based on this formulation was also enhanced by ATRA-induced FR-β upregulation.

Keywords: Folate receptor; liposomes; doxorubicin; *all-trans*-retinoic acid

1. Introduction

Acute myelogenous leukemia (AML) is one of the most common types of adult leukemia. Standard cytarabine- and anthracycline-based chemotherapy results in approximately 70% complete remission (CR) and 30–40% long-term disease-free survival (DFS).[1] In older patients, the outcome is more dismal with only 40% achieving CR and 10% achieving long-term DFS. In these patients, relapsed disease is frequently refractory to chemotherapy due to multidrug resistance (MDR).[2,3] Liposomal delivery of anthracycline drugs has been shown to overcome drug efflux in drug resistant AML cells,[4–6] athough this remains a controversial issue. Further, liposomal drug delivery could be selectively

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targeted to malignant tissues.[7] Therefore, development of tissue-targeted liposomal vehicles for optimal drug delivery to AML cells holds promise for improving the treatment of AML.

Human folate receptor (FR) α and β are high-affinity folate binding proteins with a glycosyl phosphatidylinositol (GPI) anchor.[8,9] Because of their selective expression in solid tumors and in leukemias, these receptors have been investigated as cellular markers for targeted drug delivery.[10–12] Expression of FR-β in normal tissues is restricted to placenta and hematopoietic cells, where it is expressed in the myelomonocytic lineage with an increase in its level of expression during neutrophil maturation or monocyte/macrophage activation.[13,14] FR-β is also known to be expressed in chronic myelogenous leukemia (CML) and in AML cells.[15,16] Furthermore, FR-β expression can be specifically upregulated by *all-trans*-retinoic acid (ATRA) in FR-β (+) KG-1, a human acute myeloid leukemia cell line, and primary AML cells without inducing myelomonocytic differentiation or growth inhibition.[17] In murine models of ascitic leukemia, median survival was increased by FR-targeted liposomal doxorubicin (DOX) and was further enhanced by coadministration of ATRA.[18] These findings support the use of FR-β-targeted delivery in combination with ATRA-mediated receptor upregulation.

In this study, we evaluated the effect of lipid composition on FR-β-selective liposomal DOX delivery to AML cells in vitro and on the systemic circulation time of the liposomes in vivo. The objective of this study is to identify an optimal liposomal formulation for clinical development and to confirm the beneficial effect of ATRA treatment on FR-β-targeted drug delivery in the context of this formulation.

2. Materials and Methods

2.1. Reagents. *all-trans*-Retinoic acid (ATRA), folic acid, cholesterol (CHOL), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), sodium dodecyl sulfate–polyacrylamide (SDS), and doxorubicin hydrochloride (DOX) were purchased from Sigma-Aldrich (St. Louis, MO). Hydrogenated phosphatidylcholine from soybean (HSPC) and methoxy-polyethylene glycol (MW = 2000) distearoyl phosphatidylethanolamine (PEG-DSPE) were purchased from Avanti Polar Lipids (Alabaster, AL). Folate polyethylene glycol (MW = 3350) distearoyl phosphatidylethanolamine (f-PEG-DSPE) was synthesized as described previously.[10]

2.2. Cell Culture. MV4-11 AML cells were grown continuously in folate-free RPMI1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) and 2 mM L-glutamine at 37 °C in a 5% CO_2/95% air-humidified atmosphere. The FBS contained its normal complement of endogenous folates that enabled the cells to sustain growth, and the serum-supplemented media contained a level of folate that is in the normal range of that of human plasma.

2.3. Liposome Preparation. Liposomes were prepared by the polycarbonate membrane extrusion method followed by pH gradient-driven remote loading of DOX, as described previously.[19] The lipid composition of nontargeted liposomes (L-DOX) was as follows: 54:40:6 HSPC/CHOL/PEG-DSPE (molar ratio). Compositions of FR-targeted liposomes (f-L-DOX) with varying percentages of f-PEG-DSPE are summarized in Table 1. For liposome preparation, lipid ingredients were dissolved in chloroform ($CHCl_3$) and dried

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Table 1. Formulations of L-DOX and f-L-DOX

	targeted formulations (f-L-DOX)					nontargeted formulations (L-DOX)
mol % of PEG-DSPE	5.8	5.5	5	4.5	4.0	6.0
mol % of f-PEG-DSPE	0.2	0.5	1.0	1.5	2.0	0.0
DOX loading efficiency (%)	97 ± 4	97 ± 4	96 ± 2	98 ± 3	96 ± 3	97 ± 3
particle size (nm)	118 ± 19	126 ± 8	130 ± 10	121 ± 14	115 ± 10	131 ± 15

into a thin film with rotary evaporation in a round bottom flask at 40 °C and then under vacuum. The lipid film was then hydrated with 0.25 M ammonium sulfate at 65 °C. The lipid suspension was extruded five times each through 0.2 and 0.1 μm pore size polycarbonate membranes on a nitrogen-driven Lipex lipid extruder (Northern Lipids Inc.). The liposome size distribution was determined by dynamic light scattering on a model 370 Nicomp Submicron Particle Sizer (NICOMP, Santa Barbara, CA). The resulting liposomes were then dialyzed (MWCO = 12000–14000) for 36 h at room temperature against phosphate-buffered saline (PBS, pH 7.4). Recovery of lipids and f-PEG-DSPE in the liposome fraction was assessed by size exclusion chromatography on a Sepharose CL-4B column. All lipids and f-PEG-DSPE were recovered in this fraction, indicating total incorporation of f-PEG-DSPE. Then the DOX solution and empty liposome suspension were mixed and incubated at 65 °C for 30 min with occasional mixing to enable pH gradient-driven remote loading. This is then followed by removal of free DOX by size exclusion chromatography on a Sepharose CL-4B column. Drug incorporation efficiencies for L-DOX and f-L-DOX were determined on the basis of the DOX concentration in the liposomal fractions, which was analyzed by UV absorption at 480 nm.

2.4. Cellular Uptake of Liposomal DOX Assessed by Fluorometry. Cellular uptake of various f-L-DOX formulations was assessed using MV4-11 AML cells. Cells were transferred in triplicate to 1.5 mL Eppendorf tubes at a density of 10^5 cells/tube in folate-free RPMI1640 medium with 10% FBS. The six f-L-DOX formulations containing 0–2 mol % f-PEG-DSPE (see Table 1) were mixed with the cells at a final DOX concentration of 50 μg/mL, and the Eppendorf tubes containing cells and DOX liposomes were then placed on a shaker at 37 °C for 2 h. Cells were then washed three times with PBS and lysed with 1% Triton X-100, and the fluorescence intensity of DOX was measured on a PerkinElmer LS-5B spectrofluorometer equipped with FTWinlab. The excitation and emission wavelengths were set at 500 and 550 nm, respectively, as described previously.[25] Cellular uptake of the liposomal DOX formulations was quantified by the fluorescence intensity. To determine the role of upregulation of FR-β expression, the same experiment was repeated using MV4-11 cells pre-exposed to 1 μM ATRA for 5 days.

2.5. Cellular Uptake of Liposomal DOX Assessed by Flow Cytometry. Cellular uptake of liposomal DOX was assessed by the fluorescence of DOX by flow cytometry. MV4-11 cells with or without the 5 day ATRA pretreatment were resuspended in PBS at a density of 1×10^6 cells/mL and divided into 1 mL aliquots in 1.5 mL microcentrifuge tubes. These were then incubated for 30 min at 4 °C with nontargeted or FR-targeted liposomes containing 0.5 mol % f-PEG-DSPE encapsulating DOX at a final DOX concentration of 50 μg/mL. In free folate competition studies, 1 mM folic acid was added to the incubation system. After the incubation, cells were washed three times with PBS and resuspended in 0.3 mL of PBS. For the flow cytometry assay, the samples were examined in a flow cytometer within 4 h using excitation and emission wavelengths of 488 and 575 nm, respectively. The results were expressed in mean fluorescence intensity. All the tests were performed in triplicate.

2.6. Reverse Transcription Polymerase Chain Reaction (RT-PCR) Assay for FR-β mRNA. Total RNA from MV4-11 cells treated with vehicle control or ATRA was extracted using the RNeasy Mini Kit purchased from Qiagen (Chatsworth, CA) following the manufacturer's protocol. Real-time RT-PCR was used to measure endogenous mRNAs for FR-β as well as a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control in the same samples. The reverse transcription step was carried out using Taqman Reverse Transcript Reagents from Applied Biosystems (Foster City, CA) following the manufacturer's protocol. Essentially, 400 ng of total RNA was mixed with random hexamer primers (50 μM), RNase inhibitor (1 unit/μL), MultiScribe reverse transcriptase (5 units/μL), and deoxynucleoside triphosphate mix (2.5 mM each) in reverse transcriptase buffer. The 10 μL reaction mixture was first incubated at 25 °C for 10 min, then at 48 °C for 15 min, and finally at 95 °C for 5 min. The subsequent real-time PCR step for FR-β was carried out in the presence of 12.5 μL of PCR Mastermix (Applied Biosystems), 0.5 μL each of forward primer (CTGGCTCCTTGGCTGAGTTC) and reverse primer (GCCCAGCCTGGTTATCCA), and 0.5 μL of the TaqMan probe (6FAM-TCCTCCCAGACTACCTGCCCTCAGC-TAMERA). The primers and the TaqMan probe for the control GAPDH gene were purchased from Applied Biosystems. The PCR conditions were as follows: 2 min at 50 °C, then 10 min at 95 °C, followed by 40 cycles of 15 s each at 95 °C, and finally 1 min at 60 °C. Fluorescence data that were generated were monitored and recorded by the Gene Amp 5700 sequence detection system (Applied Biosystems). All samples were set up in triplicate and normalized to GAPDH values.

2.7. Western Blot Analysis. Cell membrane preparations were used in Western blots to detect FR-β. Samples were mixed with an equal volume of 2× sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer [62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.00125% bromophenol blue].

The samples were resolved on 12% SDS–PAGE gels and electrophoretically transferred to nitrocellulose filters. The blots were probed with a rabbit anti-human FR-β antiserum followed by goat anti-rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase. The bands were visualized by enhanced chemiluminescence.

2.8. In Vitro Cytotoxicity Study. MV4-11 cells in folate-free RPMI1640 medium with 10% FBS were seeded in 96-well plates at a density of 5×10^4 cells/well, 24 h prior to the addition of drug. The culture medium was then replaced with 200 μL of medium containing serial dilutions of DOX formulations in f-L-DOX, L-DOX, and DOX. Following a 2 h incubation at 37 °C, the cells were washed twice with PBS and cultured in fresh medium until untreated control wells reached >90% confluence. Then, 15 μL of MTT reagent (5 mg/mL) was added to each well, and the plates were incubated for an additional 4 h at 37 °C. Then, 50 μL of Triplex solution consisting of 10% SDS, 5% 2-propanol, and 0.012 N HCl was added to dissolve the formazan crystals. Cell viability was assessed by the absorbance at 570 nm, measured on a Bio-Rad microplate.

2.9. Pharmacokinetic Analysis. The effect of formulation on the plasma clearance kinetics of f-L-DOX was evaluated in female ICR mice (18–22 g, purchased from Harlan). The mice were placed on folate-free rodent diet for 1 week to produce a plasma folate concentration that is in the physiological range for humans. This was necessary because normal rodent diet contained a very high level of folate supplementation. The mice were organized into seven groups of five mice each, receiving free DOX and six liposomal formulations containing 0–2 mol % f-PEG-DSPE. Mice were treated with 5 mg of DOX/kg administered via lateral tail vein injection. Blood samples were collected in heparin-containing tubes at various time points (0.083, 0.5, 1, 4, 8, and 16 h). Plasma was isolated by centrifugation (10 min at 1500 *g*) and stored at −20 °C. DOX was extracted with 20% SDS and 2-propanol and quantified by fluorometric analysis, as described above. Using Winnonlin software, pharmacokinetic parameters were determined, including area under the curve (AUC), mean residence time (MRT), total body clearance (CL), and plasma half-life for the distribution and elimination phases.

3. Results

3.1. Synthesis and Characterization of f-L-DOX. f-L-DOX samples with varying mole percentage of f-PEG-DSPE were prepared by lipid film hydration, polycarbonate membrane extrusion, and remote loading, as described in Materials and Methods. f-PEG-DSPE was added to the initial mixture of lipids rather than by the alternative method of "postinsertion" into nontargeted liposomes, which has become a popular strategy for synthesis of immunoliposomes. Although this requires synthesis of multiple batches of liposomes with varying f-PEG-DSPE contents, premixing with lipids is likely to ensure total incorporation of the ligand into liposomes. In contrast, postinsertion of ligand into liposomes with significant PEGylation density may be quite inefficient. A major advantage of the postinsertion strategy, i.e., not having to carry out bioconjugation on liposomes, is absent in this particular scenario where f-PEG-DSPE is presynthesized. Furthermore, it is more convenient and practical to prepare liposomes by this method for large-scale synthesis of FR-targeted liposomes. DOX incorporation efficiency and particle size are given in Table 1. The results show that the DOX incorporation efficiency and particle size of liposomal formulations were relatively unaffected by the f-PEG-DSPE density.

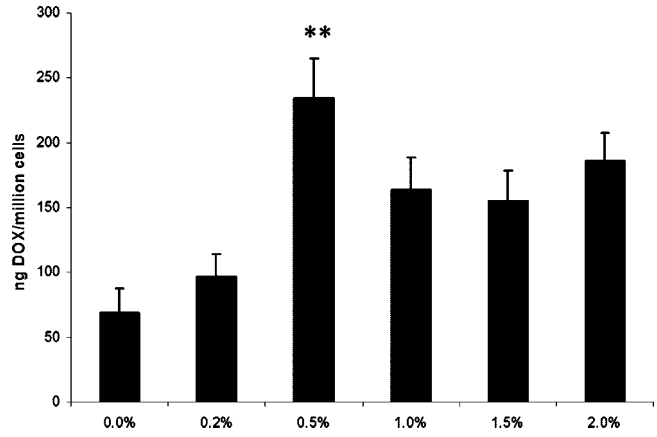

Figure 1. Uptake of f-L-DOX formulations with varying percentages of f-PEG-DSPE by MV4-11 cells. Cells were incubated for 2 h with 50 μg/mL L-DOX (0% f-PEG-DSPE) or f-L-DOX with the f-PEG-DSPE mole percentage ranging from 0.2 to 2. Cell-associated DOX was assessed on the basis of DOX fluorescence intensity by fluorometry. Values were means ± the standard deviation ($n = 3$). Statistical significance on differences between cells treated with 0.5 mol % f-PEG-DSPE and 0.2 mol % f-PEG-DSPE was measured by a Student's *t* test (two asterisks, $p = 0.003$).

3.2. Effect of Mole Percentage of f-PEG-DSPE on Cellular Uptake and Cytotoxicity of f-L-DOX. The cellular uptake of f-L-DOX with varying percentages of f-PEG-DSPE was determined by fluorometry, and the results are summarized in Figure 1. The liposomal formulation with 0.5% f-PEG-DSPE had higher binding and uptake efficiencies compared with those with 0 and 0.2% f-PEG-DSPE ($p < 0.05$). However, a further increase in the percentage of f-PEG-DSPE (i.e., up to 2%) did not result in a greater improvement in uptake efficiency. Under equal incubation conditions, cellular uptake of 50 μg/mL free DOX was 1.25 μg/million cells, which was much higher than that of the liposomes. However, given the very rapid systemic clearance rate of free DOX, liposomes, especially with FR targeting, are likely to exhibit a therapeutic advantage over free DOX in vivo.

Cytotoxicity of f-L-DOX with varying percentages of f-PEG-DSPE was determined in MV4-11 cells using a MTT assay (Table 2). f-L-DOX had a significantly lower IC_{50} than L-DOX. With an increase in the percentage of f-PEG-DSPE from 0.2 to 0.5%, the IC_{50} value decreased significantly. When the density of f-PEG-DSPE was further increased to 2%, no further significant changes were observed. On the

Table 2. Cytotoxicity of f-L-DOX with Various Percentages of f-PEG-DSPE[a]

	mol % of PEG2000-DSPE	mol % of f-PEG-DSPE	IC_{50} (μM)
targeted formulations	5.8	0.2	93 ± 8[b]
(f-L-DOX)	5.5	0.5	70 ± 5
	5.0	1.0	71 ± 7
	4.5	1.5	67 ± 6
	4.0	2.0	65 ± 5
nontargeted control (L-DOX)	6.0	0.0	337 ± 29
free DOX	N/A	N/A	2.8 ± 0.43

[a] Cytotoxicity was determined using the MTT assay as described in *Materials and Methods*. IC_{50} is the half-maximal inhibitory concentration. Values are means $\pm$ the standard deviation ($n = 3$). [b] Comparison between 0.5% f-PEG-DSPE and 0.2% f-PEG-DSPE ($p < 0.05$). Comparison between 0.5% f-PEG-DSPE and 1–2% f-PEG-DSPE ($p > 0.05$).

basis of these results, we selected f-L-DOX containing 0.5% f-PEG-DSPE for the subsequent experiments.

3.3. Pharmacokinetic Properties of f-L-DOX Formulations. Plasma clearance kinetics of the various liposomal formulations in mice were compared. The results are listed in Table 3.

All of the liposomal formulations including L-DOX and f-L-DOX with various percentages of f-PEG-DSPE exhibited circulation times much longer than those of free DOX and a biphasic clearance kinetics. In general, f-L-DOX exhibited faster clearance than L-DOX (Table 3). This was consistent with previous reports and probably due to FR-β expression in the phagocytic cells of the reticuloendothelial system (RES).[11] When the f-PEG-DSPE content was increased from 0 to 0.5 mol %, there was a significant increase in plasma clearance (CL) and a reduction in mean residence time (MRT) and area under the curve (AUC). However, a further increase in the level of f-PEG-DSPE from 0.5 to 2 mol % did not produce significant changes in these pharmacokinetic parameters. This suggested that the increase in the level of f-PEG-DSPE beyond 0.5 mol % produced no additional affinity for the FR-β that is expressed by phagocytic cells.

3.4. Enhanced FR Targeting Efficiency of f-L-DOX in MV4-11 Cells following Pretreatment with ATRA. To determine the role of FR upregulation, MV4-11 cells were treated with 1 μM ATRA, previously known to produce the maximum increase in the level of expression of the endogenous FR-β.[20] In the presence of ATRA, the increase in the level of FR-β expression was observed at both the mRNA level and the protein level [measured by real-time RT-PCR and Western blot (Figure 2A)].

Cellular uptake of f-L-DOX (0.5% f-PEG-DSPE) was assessed by flow cytometry in MV4-11 cells that were untreated or pre-exposed to 1 μM ATRA for 5 days (Figure 2B). The results showed that the 5 day pretreatment with ATRA significantly increased the rate of uptake of f-L-DOX by MV4-11 cells ($p < 0.05$). Furthermore, in cytotoxicity studies, f-L-DOX was found to be 4.8 times more cytotoxic than L-DOX to MV4-11 cells without pretreatment with ATRA and 8.6 times more cytotoxic with ATRA pretreatment. However, no effect of ATRA on cytotoxicity was observed for free DOX and L-DOX in MV4-11 cells. This suggested that the observed effect of ATRA on f-L-DOX cytotoxicity was due to FR-β upregulation.

4. Discussion

The strategy of using FR-targeted liposomal DOX combined with the receptor induction using ATRA to treat AML has gained support in a murine ascitic leukemia model using KG-1 AML cells. This study was undertaken to optimize the formulation of f-L-DOX for further in vivo therapeutic studies in a different AML model, i.e., MV4-11 cells that are more suitable for engraftment in mouse bone marrow to better simulate human leukemia. Accordingly, liposomal formulations were characterized for their in vitro efficacy of drug delivery and for their in vivo pharmacokinetics.

Optimal specificity and sensitivity of FR-dependent cellular uptake and cytotoxicity, along with a long systemic circulation time, were achieved with f-L-DOX by optimizing the folate ligand and the PEGylation densities on the surface of the liposomes. Previous studies showed that maximal FR-dependent uptake of the liposomes could be obtained with a density of 0.2–0.5% f-PEG-DSPE.[21–24] In the study presented here, data on cellular uptake and in vitro cytotoxicity indicated that a molar fraction of 0.5% f-PEG-DSPE was optimal for targeting FR-β on MV4-11 cells. If the content of f-PEG-DSPE were more than 0.5 mol %, the uptake efficiency and cytotoxicity exhibited no further increase. The effect of densities of f-PEG-DSPE on the plasma clearance kinetics of f-L-DOX was evaluated in mice. Previous studies indicated that f-L-DOX had faster clearance times than nontargeted L-DOX,[11] possibly due to expression of FR by phagocytic cells. Our data confirmed this observation. The

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Table 3. Pharmacokinetic Parameters of Various f-L-DOX Formulations in Mice after Intravenous Bolus Administrations[a]

drug formulation	C_{max} (μg/mL)	AUC (μg mL^{-1} h)	MRT (h)	$t_{1/2\alpha}$ (h)	$t_{1/2\beta}$ (h)	CL (mL/h)
L-DOX	75.90	1619 ± 63	44 ± 6	0.36 ± 0.06	43 ± 6	0.062 ± 0.00
f-L-DOX (0.2% f-PEG-DSPE)	85.97	1006 ± 56	28 ± 5	0.42 ± 0.10	27 ± 3	0.10 ± 0.04
f-L-DOX (0.5% f-PEG-DSPE)	84.26	671 ± 36[b]	22 ± 2[b]	0.33 ± 0.10	22 ± 3	0.15 ± 0.02[b]
f-L-DOX (1% f-PEG-DSPE)	83.33	588 ± 34	18 ± 3	0.33 ± 0.09	18 ± 2	0.17 ± 0.02
f-L-DOX (1.5% f-PEG-DSPE)	83.77	577 ± 29	20 ± 2	0.37 ± 0.03	20 ± 2	0.17 ± 0.02
f-L-DOX (2% f-PEG-DSPE)	90.20	651 ± 25	19 ± 3	0.27 ± 0.031	20 ± 1	0.15 ± 0.02
free DOX	44.19	111 ± 19	4.5 ± 0.6	0.096 ± 0.01	5.3 ± 0.7	0.90 ± 0.17

[a] The values are means ± the standard deviation ($n = 5$). [b] Comparison between 0.5% f-PEG-DSPE and 0.2% f-PEG-DSPE ($p = 0.001$). Comparison between 0.5% f-PEG-DSPE and 1–2% f-PEG-DSPE ($p > 0.05$).

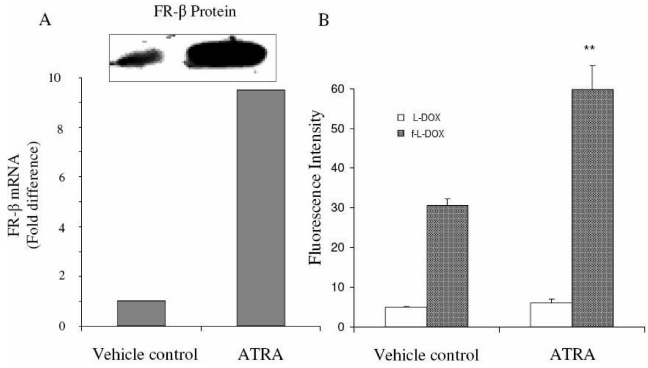

Figure 2. Enhanced FR-β expression and binding of f-L-DOX (0.5% f-PEG-DSPE) due to ATRA treatment. MV4-11 cells were pretreated with or without 1 μM ATRA for 5 days. (A) The amount of FR-β mRNA was measured by real-time RT-PCR analysis as described in Materials and Methods. In the inset, FR-β protein was detected by Western blot analysis of the cell membrane preparations as described in Materials and Methods. Data are representative of three separate experiments. (B) Cells were incubated with 50 μg/ml L-DOX or f-L-DOX for 30 min, and the fluorescence intensity of the cells was detected by flow cytometry. The values are means ± the standard deviation ($n = 3$). Two asterisks indicate the comparison between pretreatment with and without 1 μM ATRA for 5 days ($p < 0.05$).

Table 4. Cytotoxicity of Various DOX Formulations to MV4-11 Cells Pretreated with or without ATRA[a]

	IC_{50} (μM)	
	not pretreated with ATRA	pretreated with ATRA
free DOX	2.8 ± 0.4	2.5 ± 0.4[b]
f-L-DOX (0.5% f-PEG-DSPE)	70 ± 5	36 ± 4[c]
L-DOX	337 ± 29	314 ± 25[b]

[a] Cytotoxicity was determined using MTT assays as described in *Materials and Methods*. MV4-11 cells were pretreated with 1 μM ATRA for 5 days. IC_{50} is the half-maximal inhibitory concentration. Values are means ± the standard deviation ($n = 3$). [b] Comparison between cells treated with ATRA and those with no ATRA treatment ($p > 0.05$). [c] Comparison between cells treated with ATRA and those with no ATRA treatment ($p = 0.001$).

affinity of f-L-DOX for the phagocytic cells showed a pattern of f-PEG-DSPE density dependence similar to that of the MV4-11 cells, i.e., a maximum clearance at 0.5 mol % targeting ligand. Viewed together, the plasma clearance data and the results from the in vitro cellular uptake and cytotoxicity studies indicate that 0.5% f-PEG-DSPE represents an optimal formulation for preparation of f-L-DOX for therapeutics studies in the appropriate in vivo AML models.

To extend the previously observed beneficial effect of FR-β upregulation to f-L-DOX containing 0.5% f-PEG-DSPE, this liposomal formulation was evaluated in MV4-11 cells with or without ATRA pretreatment. The data showed that a 5 day pretreatment with ATRA could further increase the rate of uptake of f-L-DOX in MV4-11 cells. In contrast, the uptake of L-DOX was not altered with ATRA pretreatment. Furthermore, the cytotoxicity of f-L-DOX but not that of free DOX or L-DOX was increased in MV4-11 cells with ATRA-treatment, indicating a strong dependence of f-L-DOX cytotoxicity on FR-β expression.

Similar to other GPI-anchored proteins, FR molecules occur as clusters in specialized microdomains in the plasma membrane. Therefore, an increase in the density of FR molecules by ATRA induction cannot be expected to translate proportionately to an increase in the level of binding of multivalent particulate folate conjugates such as f-L-DOX, in contrast to monovalent folate analogues and folate conjugates. Nevertheless, this study demonstrates a substantial benefit of inducing FR expression is creating more efficient drug delivery in AML cells through an optimal FR-targeted liposomal formulation.

5. Conclusion

By examining various folate-targeted liposomal DOX formulations, we determined that a formulation containing 0.5 mol % f-PEG-DSPE has optimal drug loading properties, efficiency of uptake, and FR-dependent cytotoxicity in FR-β (+) MV4-11 AML cells and also an optimal circulation time relative to the free drug. Drug delivery through the optimal liposomal formulation was enhanced by upregulating FR-β by treating the AML cells with ATRA. Further studies are warranted to investigate the potential therapeutic advantage of this FR-targeted liposomal formulation using various in vivo models of AML.

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