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Targeting human clonogenic acute myelogenous leukemia cells via folate conjugated liposomes combined with receptor modulation by *all-trans* retinoic acid

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ABSTRACT

Our previous data demonstrated that folate receptor β (FR-β) targeted liposomal doxorubicin (FT-L-DOX) showed enhanced cytotoxicity relative to non-targeted liposomal doxorubicin (CON-L-DOX), and the effect was enhanced by selective FR-β upregulation by *all-trans* retinoic acid (ATRA) in AML blast cells. In this study, the enhanced cytotoxicity was investigated in the proliferating human AML clonogenic cells by combining FT-L-DOX with ATRA. Also, pharmacokinetic properties by pretreatment of ATRA were evaluated using FR-targeted liposomal calcein (FT-L-Calcein). Pharmacokinetic study showed that the area under the concentration curve (AUC) of FT-L-Calcein was decreased and total clearance was increased by pretreatment with ATRA. Meanwhile, the volume of distribution was significantly increased by pretreatment of ATRA. Moreover, calcein level in the liver, spleen and kidney was increased following intravenous administration of FT-L-Calcein by pretreatment of ATRA. In vitro cytotoxicity of FT-L-DOX was higher than that of CON-L-DOX and was increased by pretreatment with ATRA. Colony formation in AML cells was lower due to treatment with FT-L-DOX compared with CON-L-DOX and colony formation further decreased upon pretreatment with ATRA. Moreover, FT-L-DOX was more toxic to AML clonogenic cells than to AML blast cells. The results demonstrate that the efficiency of FR-mediated targeting of FT-L-DOX was preferentially enhanced by ATRA induced FR-β upregulation in AML clonogenic cells.

1. Introduction

Acute myelogenous leukemia (AML) is a clonal disorder involving a hierarchy of leukemic cells that differ in their phenotypic characteristics and proliferation potential. Similar to normal hematopoietic stem cells, leukemic stem cells supposedly reside within the CD34+/CD38− fraction of the leukemic clone (Bonnet and Dick, 1997; Lapidot et al., 1994). They are characterized by indefinite self-renewal and give rise to a population of extensively proliferating progenitor cells which produce the vast pool of aberrantly differentiated and arrested blasts (Passegué et al., 2003; Jordan and Guzman, 2004). Thus, the efficiency of any molecular therapy will ultimately depend on the treatment's ability to eradicate the leukemic stem and progenitor cell compartment.

Standard cytarabine and anthracycline-based chemotherapy results in approximately 70% complete remission rate and 30–40% long-term survival in AML patients (Bishop, 1999). However, treatment with drugs such as anthracycline, is associated with severe side effects such as myelosuppression and dose-limiting cardiotoxicity and also with a high incidence of relapse (Hortoagyi, 1997). Relapsed disease is frequently refractory to chemotherapy due to multidrug resistance (MDR) (Hortoagyi, 1997; List, 1997). Moreover, it has been reported that relapse of AML is associated with survival of leukemic stem cells and progenitor cells (Sperr et al., 2004). Liposomal delivery of anthracycline drugs has been shown to overcome drug efflux in resistant AML cells (Michieli et al., 1999a,b; Booser and Hortobagyi, 1994). In addition, liposomal drug delivery could selectively target malignant tissues (Pan and Lee, 2004). Therefore, therapeutic strategies for targeting leukemic stem cells or progenitor cells by tissue-targeted liposomal vehicles

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are promising improvements in the treatment of AML (Taussig et al., 2005; Hong et al., 1999).

Human folate receptor (FR) type α and type β are high-affinity folate binding proteins with a glycosyl phosphatidylinositol (GPI) anchor (Kamen and Smith, 2004; Yan and Ratnam, 1995). Because of their selective expression in solid tumors and in leukemia, these receptors have been investigated as cellular markers for targeted drug delivery (Salazar and Ratnam, 2007; Jackman et al., 2004). 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 (Ross et al., 1999; Nakashima-Matsushita et al., 1999). However, FR-β in neutrophils are unable to bind folate due to aberrant post-translational modifications (Nakamura et al., 2002). FR-β is expressed in approximately 70% of the cases of acute AML blast cells and is frequently co-expressed with CD34 (Pan et al., 2002), a common marker used to enriched populations of human hematopoietic stem cells (HSCs) and progenitors. FR-β is also expressed in bone marrow mononuclear cells of human AML engrafted NOD/SCID mice (Blaser et al., 2007), suggesting that FR-β is expressed in the AML progenitor cells and/or the AML stem cells. In addition, the expression of FR-β can be specifically upregulated by *all-trans*-retinoic acid (ATRA) in FR-β (+) KG-1 AML cells, MV4-11 AML cells, and primary AML cells (Pan et al., 2002; Blaser et al., 2007; Wang et al., 2000; Qi and Ratnam, 2006). Moreover, enhanced cytotoxicity of FR-targeted liposomal doxorubicin relative to non-targeted control liposomes was further enhanced by selective FR-β upregulation using ATRA (Pan et al., 2002; Lu et al., 2007).

In the present study, we aimed to investigate the targeting of FT-L-DOX on proliferating AML clonogenic cells after pretreatment with ATRA. The targeting efficiency was evaluated by MTT and colony forming unit (CFU) assay. The CFU assay was used to identify a subpopulation of AML cells that comprise a proliferating pool of leukemic cells that presumably include broadly defined progenitors of leukemic blasts. In addition, it was suggested that prolonged blood circulation time of FT-L-DOX may be important to leukemia cell targeting in vivo (van Etten et al., 1998; Duncan, 2006). Therefore, the effect of ATRA pretreatment on the pharmacokinetics and tissue distribution was also investigated.

2. Materials and methods

2.1. Reagents

ATRA, folic acid, cholesterol, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), calcein and doxorubicin hydrochloride (DOX) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Iscove's modified Dulbecco's medium (IMDM) and MethoCult™ GF H4434 were obtained from StemCell Technology Inc. (Vancouver, Canada). Interleukin 3 (IL-3), human stem cell factor, and granulocyte-macrophage colony-stimulating factor (GM-CSF) were purchased from StemCell Technologies Inc. Hydrogenated phosphatidylcholine from soybean (HSPC) and methoxy-polyethylene glycol (MW = 2000) distearoyl phosphatidylethanolamine (mPEG-DSPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Folate-polyethylene glycol (MW = 3350)-cholesterol hemisuccinate (F-PEG-CHEMS) was synthesized as described previously (Xiang et al., 2008). All other chemicals used were of analytical grade.

2.2. Cell culture and treatment with ATRA

MV4-11 (human acute myelocytic leukemia cell line), K562 (human erythromyeloblastoid leukemia cell line) and KB (human epidermoid carcinoma cell line derived from HeLa) cells were purchased from the American Type Culture Collection (Rockville, MD, USA). K562 and KB cells were grown in folate-free RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS, Gibco-BRL, Grand Island, NY, USA), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C in a 5% CO_2/95% air humidified atmosphere. MV4-11 cells were grown in folate-free medium containing 20% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. To determine ATRA effect on the upregulation of FR-β expression, cells were treated with 1 μmol/L ATRA for 5 days.

2.3. Treatment of AML patient cells with ATRA

AML patient cells were obtained from OSU Leukemia Tissue Bank. Each of the patients signed an informed consent to storing and using his/her leukemia tissue for discovery studies according to institutional guidelines from OSU. Fresh AML samples were fractionated by Ficoll-Hypaque (Nygaard, Oslo, Norway) gradient centrifugation and grown in folate-free RPMI 1640 medium containing with 20% of human serum with the inclusion of 20 ng/ml of interleukin 3 (IL-3), 20 ng/ml of human stem cell factor, and 10 ng/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF) at 37 °C. For the FR-β upregulation, the cells were treated with ATRA for 24 h (Qi and Ratnam, 2006).

2.4. Reverse transcription polymerase chain reaction (RT-PCR) analysis for FR-β mRNA

Total RNA from K562, MV4-11 and AML patient cells were treated with vehicle or ATRA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) reagent. Real-time RT-PCR was used to measure endogenous mRNAs for FR-β as well as 36B4 for control in the same sample. The reverse transcription step was carried out using the SuperScript™ III First-Strand Synthesis System from Invitrogen (Carlsbad, CA, USA) following the manufacture's protocol. One microliter of the resulting cDNA was added to 20 μl PCR containing 10 μl of SYBR green PCR Mastermix (Applied Biosystems, USA) and FR-β or 36B4 (acidic ribosomal phosphoprotein) primer. For FR-β, forward primer (GACTGAACTCAGCCAAGGAGCCAGAGTT) and reverse primer (AGAAAGACATGGTCTGGAAATGGATG) were used. PCR amplification as follow: 10 min at 95 °C, followed by 30 cycles at 95 °C for 15 s, at 60 °C for 1 min and 72 °C for 90 s. Fluorescence data generated were monitored and recorded by the Gene Amp 5700 sequence detection system (Applied Biosystems, USA). All data were set up in triplicate and normalized to 36B4 housekeeping gene.

2.5. Liposome preparation

Liposomes containing DOX were prepared by polycarbonate membrane extrusion methods following by pH-gradient remote loading, as described previously (Mayer et al., 1990; Pan et al., 2003). The lipid composition of CON-L-DOX was HSPC/cholesterol/mPEG-DSPE at molar ratios of 55:40:5. The composition of FT-L-DOX was HSPC/cholesterol/mPEG-DSPE/F-PEG-CHEMS at molar ratios of 55:40:4.5:0.5. For the liposome preparation, lipids were dissolved in chloroform and dried under vacuum. The lipid film was then hydrated with 0.25 M ammonium sulfate $(NH4)_2SO_4$ at 65 °C and 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., Vancouver, B.C., Canada). The $(NH_4)_2SO_4$ outside of the liposomes was removed by tangential flow diafiltration against phosphate-buffered saline (PBS, pH7.4) using a Millipore Pellicon XL cartridge with a MWCO of 30 kDa. Then DOX solution was added to the

empty liposomes and incubated at 65 °C for 30 min to enable pH gradient-driven remote loading. Free DOX was removed by size exclusion chromatography on a Sepharose CL-4B column. For calcein encapsulated liposomes, same lipid composition of DOX was used. Calcein containing liposomes were prepared by polycarbonate membrane extrusion method, as described previously by Lee and Low (1995). Size distribution of liposomes was determined by dynamic light scattering on a NICOMP 370 Submicron Particle Sizer (NICOMP, Santa Barbara, CA, USA). To analyze DOX and calcein concentration, liposomes were lysed with ethanol and the concentration in the lysate was determined by measuring absorption at 480 and 495 nm, respectively, on a Shimadzu UV-Vis spectrophotometer.

Liposomes containing ATRA were prepared by polycarbonate membrane extrusion methods. The lipid composition of ATRA liposome was EPC/DMPG at molar ratios of 70:30. ATRA encapsulation efficiency was determined by measuring absorption at 395 nm on a Shimadzu UV-Vis spectrophotometer.

2.6. Cellular uptake of liposomal calcein

Cellular uptake of liposomal formulations was assessed using MV4-11 AML cells and KB carcinoma cells. Approximately 10^6 cells were incubated with FT-L-Calcein or CON-L-Calcein in folate-free RPMI 1640 media for 2 h at 37 °C. For receptor blocking studies, 1 mM of free folate was added to media with FT-L-Calcein. Cells were than washed 3 times with cold PBS and analyzed by flow cytometry or fluorescence microscope.

2.7. Pharmacokinetic studies of FT-L-Calcein

The effect of ATRA pretreatment on the pharmacokinetics of FT-L-Calcein was evaluated in female imprinting control region (ICR) mice (18–22 g, purchased from Harlan, USA). Before treatment with liposomal ATRA, mice were placed on a folate-deficient diet (AIN-90G, Dyets, Bethlehem, PA, USA) for at least 1 week. Mice were randomized in 2 experimental groups and treated 5 consecutive days with vehicle (control) or ATRA liposomes at the dose of 10 mg/kg. 3 days after ATRA treatment, mice were intravenously injected with FT-L-Calcein at dose of 1 mg/kg via tail vein. Blood samples were collected in heparin-containing tubes at various time points (5, 60, 240, 360, 480, 960, 1440 min). Plasma was isolated by centrifugation (10 min at $1500 \times g$) and stored at −20 °C. Calcein was extracted with 10% SDS and quantified by fluorescence spectrometry. The excitation and emission wavelength was 490 and 515 nm, respectively, as described previously. In all analysis, a calibration curve relating fluorescence intensity to the plasma calcein concentration was established. Using WinNonlin software, pharmacokinetic parameters were determined, including area under the curve (AUC), mean residence time (MRT), total body clearance (CL), volume in steady state (Vss) and plasma half-life for the distribution and elimination phases.

2.8. Biodistribution of FT-L-Calcein

Female ICR mice were randomized in 2 experimental groups and treated 5 consecutive days with vehicle (control) or ATRA liposomes at the dose of 10 mg/kg. Before treatment, mice were placed on a folate-deficient diet for at least 1 week. 3 days after last ATRA treatment, mice were intravenously injected with FT-L-Calcein at dose of 1 mg/kg via tail vein. After 24 h treatment with FT-L-Calcein, the mice were humanely killed and blood was obtained by cardiac puncture. Subsequently, tissues (lung, liver, spleen and kidney) were dissected and added to the tissue digestion buffer. Tissue digestion buffer consisted of 100 mM Tris/HCl (pH 8.5), 1 mM EDTA, 0.5% sodium dodecyl sulfate, and 200 mM sodium chloride. Prior to tissue digestion, 0.1 U/mg of proteinase inhibitor was added. The tissues were homogenized with tissue homogenizer and the concentration of calcein was determined by fluorescence spectrometry.

2.9. In vitro cytotoxicity by MTT assay

In vitro cytotoxicity of liposomal DOX was determined by seeding 5×10^4 MV4-11, and 1×10^4 K562 in 96 well plates. Cells were incubated with 1:4 serial diluted free DOX, CON-L-DOX and FT-L-DOX for 2 h at 37 °C. After incubation, cells were washed 2 times with cold PBS and cultured for an additional 72 h in fresh medium. For cell viability determination, 20 μl of 5 mg/ml MTT was added to each well, and incubated for 4 h at of 37 °C. Cells were then sedimented by centrifugation at $1000 \times g$ for 8 min, and the formazan crystals were dissolved in 200 μl of DMSO. The absorbance was determined at 570 nm on a Dynatech MR-600 microplates reader. For receptor blocking studies, folic acid (1 mM) was added to media during drug exposure.

2.10. Targeting AML clonogenic cells by CFU assay

The medium for MV4-11 cells and AML patient cells were prepared by StemCell Technologies (Vancouver, BC, Canada) according to manufacture's protocol. Cells were incubated with 50 μM of CON-L-DOX and FT-L-DOX for 2 h at 37 °C. The effect of different concentrations on the colony forming was also investigated in MV4-11 cells. After treatment, cells were washed 3 times with serum-free RPMI medium and harvested by centrifugation and resuspended in 2% IMDM medium. 5000 cells were plated in MethoCult™ GF H4434 medium in 35 mm Petri dishes (Falcon, Cockeysville, MD, USA) in duplicate. For the AML patient cells, 2.5×10^6 cells were plated in MethoCult™ GF H4434 medium in 35 mm Petri dishes. Dishes were incubated at 37 °C with 5% CO_2 and 95% humidity for 10–14 days. Colonies were scored using an inverted microscope.

2.11. Statistical analysis

Data were represented as mean ± standard deviations and analyzed by 2-tailed Student's *t*-test using MiniTAB Program (Minitab Inc., State College, PA). $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of ATRA on the expression of FR-β in MV4-11 and K562 cells

To determine the effect of ATRA on the upregulation of FR-β, FR-β positive AML cells (MV4-11) and FR-β negative AML cells (K562) were used. The FR-β mRNA expression of MV4-11 and K562 cells was determined by RT-PCR after treatment with 1 μM ATRA for 5 days, previously known to produce the maximum increase in the level of expression of the endogenous FR-β. MV4-11 cells express FR-β and the expression was elevated up to 4.8-fold by treatment of ATRA. These results are consistent with previously reported FR-β upregulation by treatment of ATRA in MV4-11 cells (Lu et al., 2007). However, the FR-β expression was not detected in K562 cells, even treated by ATRA for 5 days (Fig. 1A).

3.2. Liposome characterization

The particle size of liposomes was determined by NICOMP 370 Submicron Particle Sizer. The mean particle size used in this study

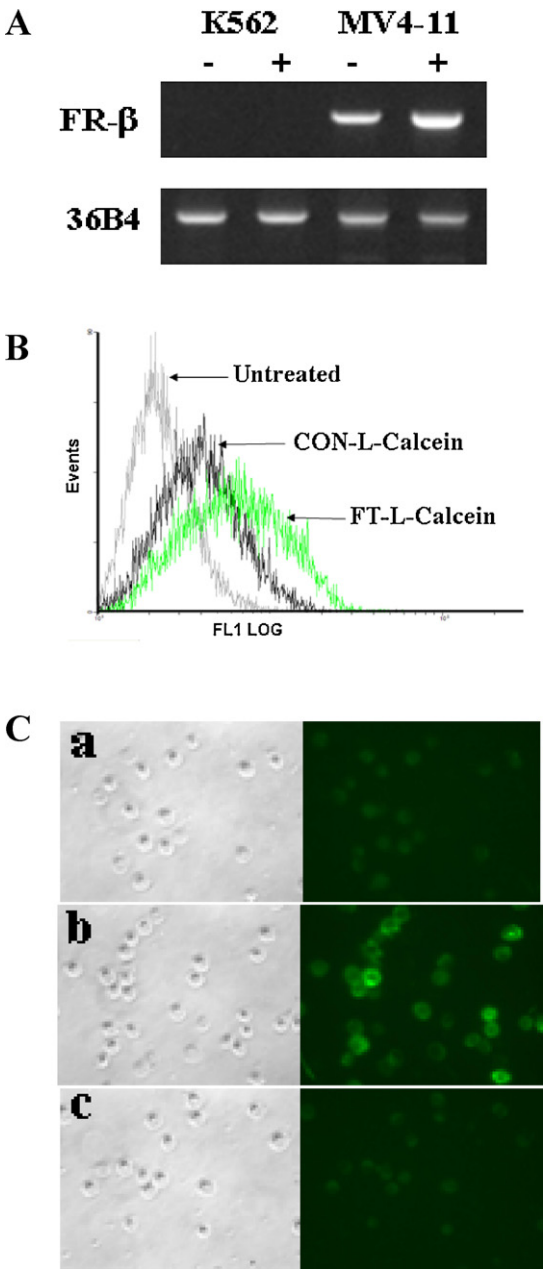

Fig. 1. Effect of ATRA on the FR-β expression (A) and cellular uptake of FT-L-Calcein in MV4-11 (B) and KB (C) cells. (a) CON-L-Calcein, (b) FT-L-Calcein, and (c) FT-L-Calcein plus 1 mM free folate.

was about 120 nm for all liposomes. DOX and calcein loading efficiency were determined by UV absorption at 480 and 495 nm, respectively. The results showed that DOX loading efficiency was greater than 95% and calcein loading efficiency was between 7% and 10%. ATRA encapsulation efficiency was also greater than 95%.

3.3. Effect of ATRA on the cellular uptake

The cellular uptake of FT-L-Calcein was determined in FR-β (+) MV4-11 AML cells and KB carcinoma cells. CON-L-Calcein was used as a non-targeted control. As shown in Fig. 1B, cellular uptake of FT-L-Calcein was significantly higher than that of CON-L-Calcein in MV4-11 cells, and the uptake of FT-L-Calcein was further increased by treatment of 1 μM ATRA. These results indicate that upregulation of FR-β could further increase the cellular uptake of FT-L-Calcein. However, cellular uptake of FT-L-Calcein and CON-L-Calcein did not change upon treatment with 1 μM ATRA in FR-β (−) K562 cells (data not shown). In addition, significantly increased cellular uptake of FT-L-Calcein was observed in KB cells by fluorescence microscopy. The cellular uptake of FT-L-Calcein was blocked by 1 mM free folate (Fig. 1C). These data suggest that the novel ligand, F-PEG-CHEMS could be used for targeting FR-β (+) AML cells.

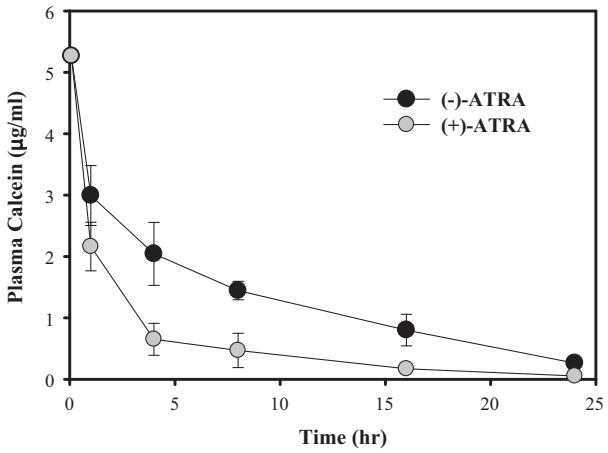

Fig. 2. Temporal plasma profiles of calcein following intravenous administration of FT-L-Calcein at dose of 1 mg/kg by pretreatment of ATRA. Each data represents the mean ± SD ($n = 3$).

3.4. Pharmacokinetic profile of FT-L-Calcein following treatment with ATRA

To assess pharmacokinetic properties of FT-L-Calcein after pretreatment with ATRA, FT-L-Calcein was intravenously administrated in ICR mice. The plasma concentration profiles of calcein after intravenous injection of FT-L-Calcein are shown in Fig. 2 and pharmacokinetic parameters summarized in Table 1. Calcein in folate targeted liposomes showed a biphasic pattern with a rapid distribution phase ($t_{1/2\alpha} = 0.46$ h) and a slow terminal elimination phase ($t_{1/2\beta} = 7.4$ h). The area under the concentration–time curve (AUC) of calcein in FT-L-Calcein was significantly decreased by pretreatment with ATRA ($p < 0.05$). The mean residence time (MRT) and $t_{1/2\beta}$ of calcein was also decreased by pretreatment of ATRA (1.6- and 1.3-fold decrease, respectively). In contrast, the steady-state volume of distribution (Vss) was significantly increased by the treatment of ATRA ($p < 0.05$). In general, folate targeted liposomes exhibited faster clearance compared to non-targeted liposomes, due to FR-β expression in the phagocytic cells of the reticuloendothelial system (RES) (Gabizon et al., 2003, 2004). Therefore, decreased AUC following treatment with ATRA might be due to increased tissue distribution via FR upregulation.

Table 1
Pharmacokinetic parameters of calcein by pretreatment of ATRA following intravenous (i.v.) administration of FT-L-Calcein to ICR mice at a dose of 1 mg/kg.

Group	Control	ATRA
AUC (ng h/ml)	33.4 ± 4.5	12.6 ± 2.9*
$t_{1/2}$ (h)	7.4 ± 2.8	5.9 ± 1.9
CL (ml/h/kg)	29.9 ± 12.4	79.4 ± 9.1
MRT (0–24 h)	10.3 ± 3.5	6.4 ± 1.0
Vss (ml/kg)	307.1 ± 94.0	506.5 ± 101.3*

Data represent the mean ± SD ($n = 3$).
* $p < 0.01$ compared with control.

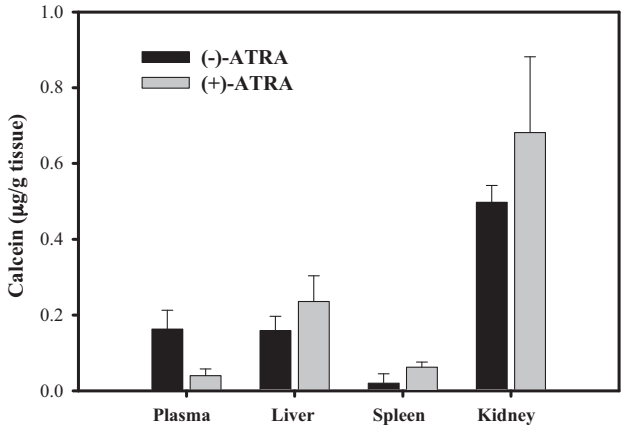

Fig. 3. Tissue distribution of calcein following intravenous administration of FT-L-Calcein at dose of 1 mg/kg by pretreatment of ATRA. Each data represents the mean ± SD ($n = 3$).

3.5. Biodistribution of FT-L-Calcein following treatment with ATRA

In order to investigate the effect of ATRA on the biodistribution of FT-L-Calcein, the tissue concentration of calcein was determined after 24 h intravenous administration of FT-L-Calcein in mice. As shown in Fig. 3, calcein level in the liver and spleen was increased by pretreatment of ATRA. However, no calcein was detected in the lung. In addition, increased calcein level was observed in the kidney by pretreatment of ATRA. Therefore, decreased plasma level of calcein by treatment of ATRA might be due to the increased calcein uptake in the liver, spleen and kidney.

3.6. In vitro cytotoxicity by MTT assay

The cytotoxicity of FT-L-DOX to AML cells and the effect of ATRA induced FR-β upregulation on the cytotoxicity were determined by MTT assay. The half maximal inhibitory concentration (IC_{50}) values of free DOX, CON-L-DOX, FT-L-DOX in MV4-11 and K562 cells are summarized in Table 2. In MV4-11 and K562 cells, free DOX showed the highest toxicity. In contrast, liposomal DOX showed reduced cytotoxicity in both MV4-11 and K562 cells. However, cytotoxicity of FT-L-DOX was significantly increased (3.4-fold) compared with CON-L-DOX and ATRA pretreatment further increased (6.8-fold) cytotoxicity of FT-L-DOX in MV4-11 cells. In addition, the cytotoxicity of FT-L-DOX was significantly reduced by the addition of 1 mM free folate in MV4-11 cells. In FR-β (−) K562 cells, similar cytotoxicity was observed all liposome-treated groups (Table 1). These results demonstrate that increased FR-β expression will increase the cytotoxicity of FT-L-DOX.

Table 2
Cytotoxicity of various liposomal DOX formulations to K562 and MV4-11 cells by treatment of ATRA.

IC_{50} (μM)	K562		MV4-11	
	(−)ATRA	(+)ATRA	(−)ATRA	(+)ATRA
Free DOX	11.4 ± 3.4	11.9 ± 2.9	5.3 ± 1.5	8.0 ± 2.0
CON-L-DOX	203 ± 18	206 ± 12	251 ± 15	242 ± 48
FT-L-DOX	189 ± 17	159 ± 36	74 ± 8[a]	36 ± 5[a,b]
FT-L-DOX + 1 mM folate	196 ± 20	200 ± 12	223 ± 23	216 ± 49

Cytotoxicity was determined using MTT assay as described in Section 2. IC_{50} is the half maximal inhibitory concentration. Data represent the mean ± SD ($n = 4$).
[a] $p < 0.01$ compared with CON-L-DOX treatment.
[b] $p < 0.01$ compared with no ATRA treatment.

3.7. Targeting AML clonogenic cells in MV4-11 cells

To evaluate cytotoxic effect of FT-L-DOX on the leukemic clonogenic cells, colony growth was determined by CFU assay following pretreatment with ATRA in K562 and MV4-11 cells. Both of K562 and MV4-11 cells formed CFU in methylcellulose culture and the number of CFU did not significantly change upon pretreatment with ATRA (Fig. 4). In FR-β (−) K562 cells, liposomal DOX had no effect colony formation even after pretreatment with ATRA (Fig. 4A). In FR-β (+) MV4-11 cells FT-L-DOX significantly decreased the CFU number compared with CON-L-DOX in a manner that was blocked by the addition of 1 mM free folate (Fig. 4B). A similar pattern was observed in ATRA-treated in MV4-11 cells where the effects of the liposomal treatments were more pronounced. The results suggest that FT-L-DOX targets the AML clonogenic cells through to the FR-β. However, ATRA pretreatment significantly decreased the CFU number in all the treatment groups including CON-L-DOX, FT-L-DOX and FT-L-DOX plus free folate, suggesting that ATRA may also enhance the cytotoxicity of DOX on the clonogenic cells. Nevertheless, the CFU number was more effectively decreased in FT-L-DOX group (5.4-fold decrease) than in the CON-L-DOX group (3.8-fold decrease) due to pretreatment with ATRA, presumably reflecting the effect of FR-β upregulation.

Dose–response studies in MV4-11 cells showed that the IC_{50} value of FT-L-DOX to clonogenic cells was 23.2 ± 5.4 and 40.5 ± 3.4 μM with or without pretreatment with ATRA, respectively. The IC_{50} of FT-L-DOX from the CFU assay was 1.6-fold lower than that from the MTT assay by pretreatment with ATRA. In other

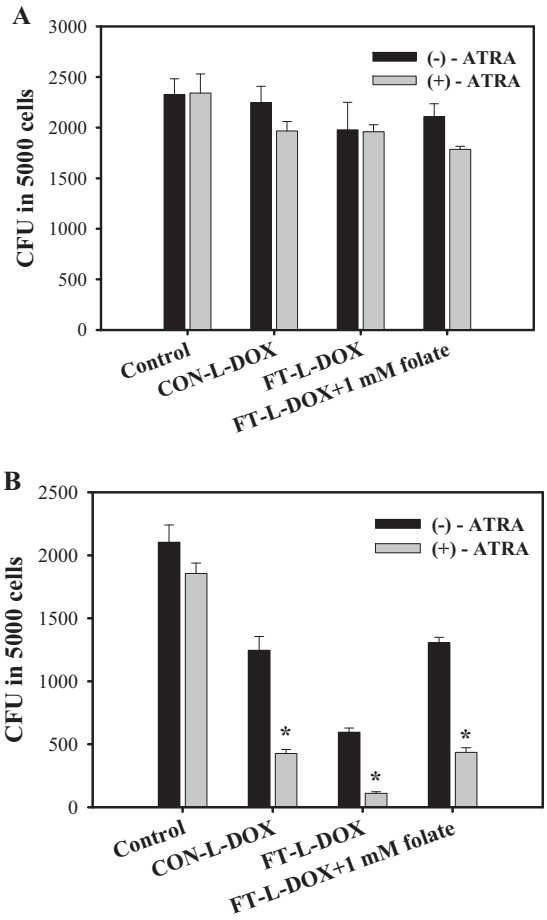

Fig. 4. Effect of ATRA on colony growth in K562 (A) and MV4-11 (B) cells. Each data represents the mean ± SD ($n = 3$). *Significant difference between with or without treatment of ATRA ($p < 0.01$).

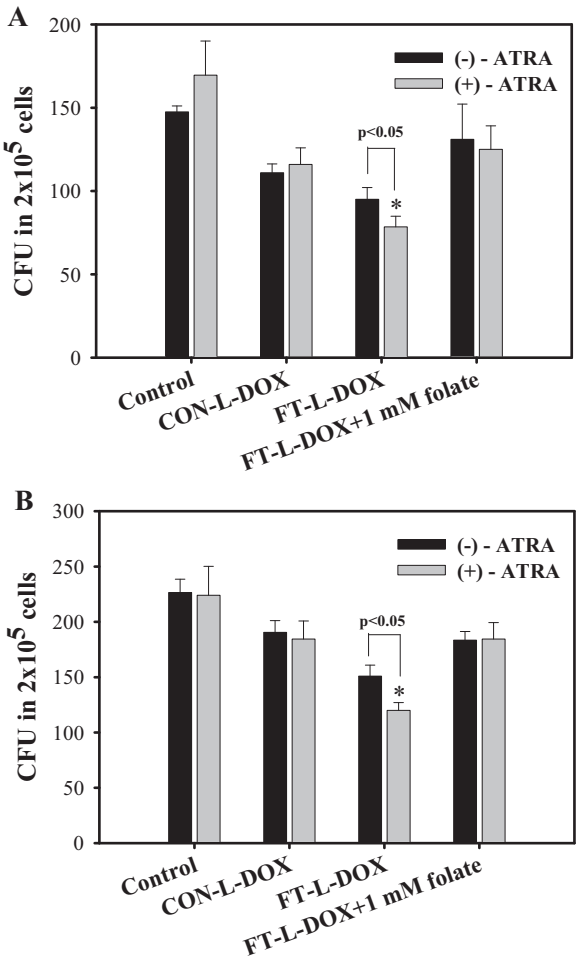

Fig. 5. Effects of ATRA on colony growth in AML patient cells. (A) Patient 1 and (B) Patient 2. Each data represents the mean ± SD ($n = 3$). *Significant difference between with or without treatment of ATRA ($p < 0.05$).

words, targeting efficiency of FT-L-DOX to AML clonogenic cells was more potent than AML blast. The results demonstrate that ATRA pretreatment is more effective in the clonogenic cells than in blast cells.

3.8. Targeting AML clonogenic cells in AML patient cells

FR-β expression in AML patient cells was determined by real-time RT-PCR. Two AML patient samples, in which the leukemic cells expressed FR-β, were selected to evaluate targeting efficiency of FT-L-DOX to the AML clonogenic cells. FR-β expression in two AML patient cells was elevated 1.7- and 2.5-fold by a brief (24 h) pretreatment with ATRA. In the AML patient samples, only 0.075–0.094% of AML cells were able to form CFU in the Methocult medium (Fig. 5). In Patient 1 AML cells, FT-L-DOX significantly decreased the number of CFU compared with CON-L-DOX and the addition of 1 mM free folate blocked this decrease, indicating FT-L-DOX could be use to target AML clonogenic cells in AML patient cells. Moreover, the number of CFU in the FT-L-DOX treatment group was further decreased by pretreatment with ATRA in both the AML patient samples (Fig. 5A). These results are presumably due to ATRA induced FR-β upregulation. Similar results were observed in Patient 2 AML cells (Fig. 5B). However, in contrast to the MV4-11 cells, no additional ATRA effect was evident from the cytotoxicity of CON-L-DOX, possibly because of the relatively brief period (24 h) of the ATRA pretreatment. It may also be noted that the duration of ATRA treatment was sub-optimal for FR-β upregulation but the results provide proof-of-principle for the utility of ATRA treatment in enhancing FT-L-DOX cytotoxicity in primary AML clonogenic cells.

4. Discussion

We have previously reported that FR-β targeted liposomal doxorubicin showed enhanced cytotoxicity relative to non-targeted liposomes, and the effect was enhanced by selective FR-β upregulation by ATRA both in vitro and in vivo (Pan et al., 2002; Lu et al., 2007). In the present study, FT-L-Calcein and FT-L-DOX were prepared by using folate-polyethylene glycol (MW = 3350)-cholesterol hemisuccinate (F-PEG-CHEMS), which have better FR-targeting activity during prolonged storage compared with folate-PEG-cholesterol and folate-PEG-DSPE (Xiang et al., 2008; Zhao et al., 2007). Pharmacokinetic profile and tissue distribution of FT-L-Calcein were investigated in combination with ATRA. Calcein was chosen for the pharmacokinetic and tissue distribution studies because it is membrane impermeable and, therefore, less prone to redistribution in vivo. Furthermore, the effect of ATRA on the targeting of FT-L-DOX on AML clonogenic cells was investigated by using the colony forming unit assay.

Selective folate receptor targeting was observed in FR-positive MV4-11 and KB cells (Fig. 1B and C). The pharmacokinetic study has shown that the AUC was significantly decreased and total clearance was significantly increased by pretreatment of ATRA ($p < 0.05$). Meanwhile, the volume of distribution was significantly increased by pretreatment of ATRA (Table 1). These results indicated that decreased AUC following treatment with ATRA might be due to increased tissue distribution via FR upregulation. Therefore, tissue concentration was determined after intravenously administration of FT-L-Calcein. Calcein level in the liver and spleen was increased by pretreatment of ATRA (Fig. 3). Previous studies demonstrated that folate targeted liposomes had faster clearance compared to non-targeted liposomes possibly due to direct liposome uptake via the liver FR (Gabizon et al., 2003, 2004). Therefore, the increased calcein uptake in the liver by pretreatment of ATRA, might be due to the ATRA induced FR-β upregulation. Although FR-β expression in the spleen is low, ATRA induced FR-β upregulation might be contributed to the increased calcein uptake in the spleen. On the other hand, calcein level in the kidney was also increased by pretreatment of ATRA. Previous studies demonstrated that FR in the kidney should not have contributed to the accelerated clearance of FT-L-Calcein, because FT-L-Calcein does not have access to the luminal side of kidney tubular cells where FR is expressed (Gabizon et al., 2003, 2004). Moreover, FR-α upregulated by ATRA or not is unclear. Recently, Yarali et al. (2008) reported acute renal failure case during ATRA therapy. Taken together, increased calcein uptake in the kidney by pretreatment of ATRA might be explained by impaired kidney function. Thus, patients should be carefully monitored for kidney function before treatment with folate targeted liposomes because of the potential risk of side effect. Although the detailed mechanism needs further investigation, the increased calcein uptake in the liver and spleen might be contributed to the decreased plasma concentration of calcein by pretreatment of ATRA.

Since maintenance of AML is dependent on a smaller population of leukemic stem cells and progenitor cells that have the ability to form colonies, it is important to test whether FT-L-DOX is able to targeting the pool of colony forming cells. Therefore, FR-mediated targeting efficiency of FT-L-DOX combined with ATRA in AML blast and clonogenic cells was determined by MTT and CFU assay. The results showed that FT-L-DOX significantly decreased the number of CFUs compared with CON-L-DOX and the CFU number was further decreased by pretreatment with ATRA in MV4-11

cells and AML patient cells (Figs. 3 and 4). These results suggested that FT-L-DOX could be used to target AML clonogenic cells and that ATRA pretreatment increased the FR-targeting efficiency via selective FR-β upregulation. Interestingly, the IC_{50} from the CFU assay was 2-fold lower than that from MTT assay in MV4-11 cells, indicating FT-L-DOX is more toxic to AML clonogenic cells than AML blast cells.

Prolonged blood circulation and FR-β expression is an important factor for therapeutic efficacy of FT-L-DOX in AML patient. Prolonged blood circulation relative to the DOX free drug was indeed confirmed by pharmacokinetic studies. Although folate receptor targeted liposomes were distributed to normal tissues such as liver and spleen, this was mostly due to the natural RES clearance pathway for liposomes rather than FR-β expression. In contrast, 70% of AML cells expressed FR-β and the expression was upregulated by pretreatment of ATRA. Moreover, ATRA enhanced cytotoxicity of FT-L-DOX was reported in AML KG-1 and MV4-11 cells, suggesting that folate targeted liposomes might be predominantly accumulated FR-β overexpressed leukemia cells. Currently, the most effective treatment of acute promyelocytic leukemia is retinoid differentiation therapy. However, some AML cells are refractory to ATRA-differentiation. Therefore, ATRA induction of FR-β should be particularly beneficial in the therapeutic targeting of FR-β in these AML patients.

In conclusion, the pharmacokinetic profile and tissue distribution was changed by pretreatment of ATRA. Decreased plasma concentration of calcein by treatment ATRA might be due to the increase calcein uptake in the liver and spleen. Moreover, FR-mediated targeting efficiency of FT-L-DOX was enhanced by ATRA induced FR-β upregulation in AML clonogenic cells. Therefore, FR-mediated targeting is a promising strategy for treating human AML in combination with receptor induction using ATRA.

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