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Research paper

Folate-tethered emulsion for the target delivery of retinoids to cancer cells

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Abstract

Folic acid, conjugated to poly(ethylene glycol)-distearoylphosphatidylethanolamine (folate-PEG-DSPE), was used to target emulsions of all-*trans* retinoic acid (ATRA) to folate receptor-overexpressing tumor cells. Two kinds of ATRA-incorporated folate-tethered emulsions (ATRA-FTE$_{2000/3400}$) were prepared using soybean oil, egg phosphatidylcholine and folate-PEG-DSPE with different PEG length. As a control, ATRA-incorporated non-tethered emulsion (ATRA-NTE) was prepared by using PEG2000-DSPE without folate instead of using folate-PEG-DSPE. The mean particle diameters of ATRA-FTE$_{2000/3400}$ were about 100–130 nm. The cellular uptake in KB cells of fluorescence-labeled ATRA-FTE$_{3400}$ was determined with HPLC (for ATRA) and confocal microscopy (for lipid emulsion). The growth inhibitory activity of ATRA was evaluated by MTT assay. The folate ligands in emulsion increased the cellular uptake of ATRA about 3-fold and 1.6-fold in ATRA-FTE$_{3400}$ and ATRA-FTE$_{2000}$, respectively. Growth inhibitory activity of ATRA-FTE$_{3400}$ in KB cells was higher than that of ATRA-NTE at the same dose. Whereas the growth inhibitory effect in MCF-7 cells of ATRA was similar between ATRA-NTE and ATRA-FTE$_{3400}$. The addition of free folate significantly reduced the uptake of ATRA regardless of the length of PEG attached to folate. Folate-tethered lipid emulsion showed effective and selective delivery to the folate receptor-abundant carcinomas, suggesting a potential for targeted delivery of anticancer agents.

Keywords: Folic acid; Folate receptor; All-*trans* retinoic acid; Folate-tethered emulsion

1. Introduction

The effective cancer therapy is used to selectively destroy cancer cells while sparing normal tissues [1]. In this regard, a ligand-mediated drug delivery system plays a crucial role in achieving target delivery [2]. Of various ligands, the vitamin folate ligand targets the folate receptor which has been known to be frequently overexpressed in a wide range of tumor types, such as malignant ovarian, endometrial and brain tissue [3,4] and thus, presents an attractive target for tumor-selective drug delivery. The efficient delivery of folate conjugate to tumor cell surface is attributed to smaller size of folate [5], the high affinity of folate for its receptor on cancer cells [6] and stability against lysozyme [7]. Then, liposome targeting the folate receptor is the useful means to alter liposome biodistribution with potential pharmacodynamic implications that may favorably tilt the therapeutic index [8]. Moreover, folate is a natural substance and thus can be repeatedly administered without eliciting immunogenicity which is engendered by various proteins and ligands [9].

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In recent years, it has been demonstrated that receptor-mediated uptake of vitamin folate could also be exploited to facilitate entry of attached molecules such as liposomes, emulsions, and so on [10,11]. It has been reported that the covalent conjugation of folate to liposomes through a PEG spacer permitted avid uptake of liposomes by folate receptor-abundant carcinomas [6]. The binding of these folate-grafted liposomes to carcinomas could be competitively inhibited by free folate or by an anti-serum against the folate receptor, demonstrating that the interaction is mediated by the cell surface folate receptor [12,13]. Moreover, a variety of anticancer drugs and macromolecular therapeutics were efficiently delivered by folate receptor targeting [14–16].

To investigate the folate receptor target delivery of drug, all-*trans* retinoic acid (ATRA) was employed as a model compound. ATRA, an active metabolite of retinol, has high potential as an anticancer drug for acute promyelocytic leukemia and other tumors [17–19]. Our previous reports show that emulsion has been described as carrier of water-insoluble drugs for parenteral delivery and emulsion did not impair the pharmacokinetics [20,21]. As a drug carrier, emulsions prepared with ethyl oleate and Tween 20 could have a limited use because of uncertainty of long-term stability of emulsion and toxicity of Tween 20 [22]. Therefore, there is a need to develop a stable and non-toxic emulsion formulation without using surfactant such as Tween 20.

Here, we prepared the lipid-based emulsion formulations of ATRA by using the folate-conjugated lipid with low toxicity and evaluated the physicochemical properties. This study included the cellular uptake and anticancer activity of ATRA in human cancer cell lines.

2. Materials and methods

2.1. Materials

Folic acid was purchased from Fluka (Switzerland). Poly(ethylene glycol) (PEG) 2000, PEG 3400, all-*trans* retinoic acid (ATRA) and 3-(4,5-dimethylthialzol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Soybean oil was obtained from Green Cross Co. (Yongin, Korea). Distearoylphosphatidylethanolamine (DSPE), PEG2000-DSPE and egg phosphatidylcholine (PC) were purchased from Avanti Polar lipids (Alabaster, AL, USA). RPMI 1640, folate-free RPMI 1640, fetal bovine serum (FBS) and trypsin-EDTA were purchased from Life Technologies (Gaithersburg, MD, USA). All other reagents were used without further purification.

2.2. Synthesis of folate-PEG-DSPE

Two types of conjugates, folate-PEG2000-DSPE and folate-PEG3400-DSPE, were synthesized as described previously [5] with slight modification. To convert one terminal hydroxyl group of PEG (HO–X–OH) 2000 and 3400 Da to amino group, PEGs were tosylated by *p*-tosylchloride and aminated via phthalimide derivatives into α-amino-ω-hydroxy PEG [23].

In the last step, the polymer product was obtained a mixture of polyethylene glycol derivatives containing 0–2 amino groups at the terminal groups. These products were separated by silica gel column chromatography. After loading on the silica gel column, the product was loaded in a very sticky state by using CH_2Cl_2–methanol (8:1) mixture. Then the column was eluted with 1% triethylamine solution of CH_2Cl_2–methanol (8:1). In turn, PEG (HO–X–OH), monoamino-PEG (HO–X–NH_2) can be identified with TLC. To separate the monoamino-PEG product, the mixture was purified in such a manner. At first, it was evaporated and redissolved in CH_2Cl_2 then filtered to remove the remaining silica and very few PEGs with the monoamino-PEGs. Product in CH_2Cl_2 was dried with anhydrous Na_2SO_4 and properly evaporated then poured with ethyl ether. Then the solution was filtered again, and continuously freeze dried.

The amino group of the products was protected by Boc_2O so that DSPE attached to the other hydroxyl group [24]. The final product, folate-PEG-DSPE, was synthesized from the conjugation process of amino-PEG-DSPE and folic acid by dicyclohexyl carbodiimide-mediated coupling reaction and intensive dialyzed purification using cellulose ester membrane followed [5]. The conjugates, folate-PEG2000/3400-DSPE, were characterized by ^{1}H NMR, HPLC and MALDI–TOF.

2.3. Preparation of ATRA-incorporated emulsion bearing folate

Using the synthesized lipids, three types of ATRA-incorporated emulsion systems were prepared such as ATRA-incorporated non-tethered emulsion with PEG2000-DSPE (ATRA-NTE), ATRA-incorporated emulsion with folate-PEG2000-DSPE (ATRA-FTE_{2000}) and ATRA-incorporated emulsion with folate-PEG3400-DSPE (ATRA-FTE_{3400}). Egg PC (48 mg) and ATRA (7 mg) were mixed with PEG2000-DSPE, folate-PEG2000-DSPE or folate-PEG3400-DSPE (12 mg) followed by soybean oil (300 mg). Adding 4 ml of *t*-butylalcohol, the mixture was stirred for a few minutes at 45 °C and the solution was frozen at −20 °C. In 40 min, the solution was placed on liquid nitrogen and dried under reduced pressure for 24 h. Distilled water (1.63 ml) was added and the solutions were sonicated at 50 °C for 3 h using bath type sonicator until crude and milky emulsions were obtained. These crude emulsions were homogenized for 8 cycles at 150 MPa and 50 °C using a high pressure homogenizer (Emulsiflex® EF-B3, Avestin Inc., Canada) wired with heating tape (Thermolyne®) to reduce the droplet size to the submicron range [21]. After homogenization, the precipitated drug was removed from emulsions by filtration through a 0.45 μm membrane filter.

2.4. Measurement of particle diameter and zeta potential

Mean particle diameter and zeta potential of the emulsions were determined using electrophoretic light scattering spectrophotometer (ELS-8000, OTSUKA Electronics Co. Ltd., Japan) at room temperature. Prior to measurements, the emulsions were diluted with filtered distilled water to an intensity of 300 Hz.

2.5. Cell culture

KB (human mouth epithelial carcinoma) and MCF-7 (human breast carcinoma) cells were purchased from Korea Cell Line Bank (Seoul, Korea). The cells were cultured in RPMI 1640 supplemented with 10% heat inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. In cellular uptake study, we used folate-free RPMI 1640 supplemented with 5% heat inactivated FBS to minimize the effect of folic acid in RPMI 1640. All cell lines were maintained at 37 °C in a water-saturated atmosphere containing 5% CO_2.

2.6. Cellular uptake

To evaluate a cellular uptake of ATRA, the cells were seeded at 5×10^5 cells/well and cultured in 6-well plates for 2 days. All the wells were treated with 10 μM of ATRA for each formulation and the cells were harvested at designated time intervals. On other prepared plates, each well was inoculated by ATRA-NTE and ATRA-FTE_{3400} dose-dependently from 1 to 20 μM then harvested. No significant changes in ATRA were found during the washing process. Then, the cell pellets were stored at −20 °C until analysis. After cellular uptake, ATRA in cells was extracted by liquid/liquid extraction method as reported previously [25]. The concentration of ATRA was determined by high performance liquid chromatography (HPLC) method [26].

2.7. Confocal microscopy

In an attempt to confirm the internalizations into cells, confocal microscopy was performed to visually monitor the cell association of fluorescence-labeled emulsions. At first, the fluorescence-labeled emulsions were prepared with same formulations except adding the fluorescence-labeled DOPE. A fluorescence-labeled DOPE was added at the 0.1% ratios of total constituent lipid, and the emulsions were prepared as per a previously described procedure [27]. KB cells were grown on sterilized coverslips for confocal microscopy. After inoculation for 24 h, the cells were washed four times with PBS (pH 7.4). A confocal microscope (Leica TCS NT, Leica Microsystems, Germany) was used to obtain images of these samples [28,29].

2.8. Anticancer activity

The cytotoxicity of ATRA was determined by MTT assay, indicating the viability and proliferation of cells against ATRA-incorporated emulsions. The cells were seeded in 96-well plate at a density of 2×10^3 cells/well and were incubated with different concentrations of each formulation for 80 min to enable all the folate receptors expressed on the cell surface to be saturated. Then, the excess drug-containing medium was removed. After the cells were rinsed and incubated for 3 days at 37 °C with fresh medium without drug, we examined the effect of each formulation on cytotoxicity. Twenty microliters of 0.5% (w/w) MTT in PBS solution was added to each well and then incubated for 3 h to allow producing formazan crystals. The formazan crystals were dissolved by adding 180 μl of dimethylsulfoxide (DMSO) and further by adding 20 μl of PBS. The plates were shaken for 20 min and the quantity of formazan products was measured at 570 nm in a microplate reader (MCC340, Multiskan, Belgium). The 100% value was obtained from the OD value measured in non-treated wells.

3. Results and discussion

ATRA is a promising anticancer agent, which has been investigated in the chemoprevention and treatment of cancer [30]. Cancer targeting is usually achieved by adding a ligand moiety to the delivery system, specifically directed to certain types of binding sites on cancer cells [31]. Among the several ligands, folate would be a preferable one for targeting carcinomas with high densities of folate receptor [6,7,13,29,32]. Moreover, many groups also reported both uptake enhancement and in vivo cytotoxicity enhancement in folate-linked emulsions. Stevens and Lee reported that the folate-linked emulsion loading paclitaxel showed a significant difference in the IC_{50} values between the targeted emulsions and those that were non-targeted [16]. Shiokawa et al. also reported that the folate-linked microemulsion loading aclacinomycin A showed the higher accumulation in solid tumor and greater tumor growth inhibition [33]. In this study, to give an ability of targeting carcinomas with ATRA formulation, folate-lipid emulsions were prepared using the synthesized folate-PEG-DSPE conjugates.

3.1. Characterization of folate-conjugated lipids

From an HPLC analysis, both folic acid and folate-PEG-DSPE were eluted as a single peak at retention time 2.1 min. ^1H NMR analysis was performed at 400 MHz, scanned from 0 to 10 ppm. The analysis conditions under a linear mode and data for 2-ns pulses of the 337 nm nitrogen laser were averaged for each spectrum. Their purities were also confirmed with the MALDI–TOFMS data. The data exhibited a bell-shaped distribution of MALDI–TOF spectra as that centered at 3280 Da (theoretical mass of 3200 Da) for folate-PEG2000-DSPE and 4480 Da (theoretical mass of 4540 Da) for folate-PEG3400-DSPE, respectively (data not shown).

Table 1
Zeta potential and loading capacity of emulsions ($n = 3$)

Formulations	Particle diameter (nm)		Zeta potential (mV)	Loading capacity (mg/ml)
	At preparation	After 4 weeks		
ATRA-NTE	117.2 ± 5.2	84.5 ± 8.6	−26.0 ± 2.2	0.924
ATRA-FTE$_{2000}$	129.3 ± 8.6	109.3 ± 8.6	−22.8 ± 1.9	0.988
ATRA-FTE$_{3400}$	105.2 ± 5.2	167.2 ± 9.7	−20.7 ± 1.7	0.931

3.2. Particle diameter and zeta potential

The particle diameter of emulsion was 100–130 nm immediately after preparation (Table 1) and slightly changed in the range of 80–160 nm for 4 weeks. Therefore, the compositions of these emulsions such as soybean oil, egg PC, folate-PEG-DSPE and ATRA are favorable for constituting very stable systems. The change in particle diameter is one of the important parameters by varying surfactant components to get a stable emulsion formulation applicable for parenteral administration [21]. Particle diameters of emulsions were within the value range reported for parenteral emulsion (under 200–400 nm of mean particle diameter) [34] and emulsions were also stabilized by the presence of PEG-DSPE [22,35].

The zeta potential and loading capacity of the formulations are presented in Table 1. The zeta potential is an electrostatic value measured by surface electrostatic double layer and is used to predict storage stability of colloidal particles. Our emulsion formulations have similar zeta potential values and, a slight increase was observed in particle diameter of ATRA-FTE$_{3400}$ during the storage for 4 weeks compared to both ATRA-NTE and ATRA-FTE$_{3400}$.

The concentrations of ATRA in the emulsion formulations were 0.924, 0.988 and 0.931 mg/ml, respectively. The loading capacity of these emulsions was lower than that of folate-free PEG-DSPE emulsion [21].

3.3. Cellular uptake of ATRA-FTE

KB cells bearing high densities of folate receptor were incubated with 10 μM ATRA in the three emulsion formulations. Folate ligands on emulsion increased the cellular uptake of ATRA (Fig. 1). Intracellular concentration of ATRA was increased with time-dependence (Fig. 1a). When the cells were treated with ATRA-FTE$_{3400}$, the cellular uptake of ATRA was more rapid and higher than any other formulations. In the time-dependent cellular uptake study, the concentration of ATRA in the cells was linearly increased up to about 40 min and gradually declined to the almost extinction of ATRA in cells. To compare the dose-dependent cellular uptake of NTE and FTE formulations, ATRA-NTE and ATRA-FTE$_{3400}$ were selected among the formulations. ATRA-FTE$_{3400}$ shows a more highly increased uptake as varying concentrations of ATRA (Fig. 1b). The data showed more than 1.7-fold higher intracellular concentration of ATRA in the ATRA-FTE$_{3400}$ at each concentration. These emulsions were found to rapidly

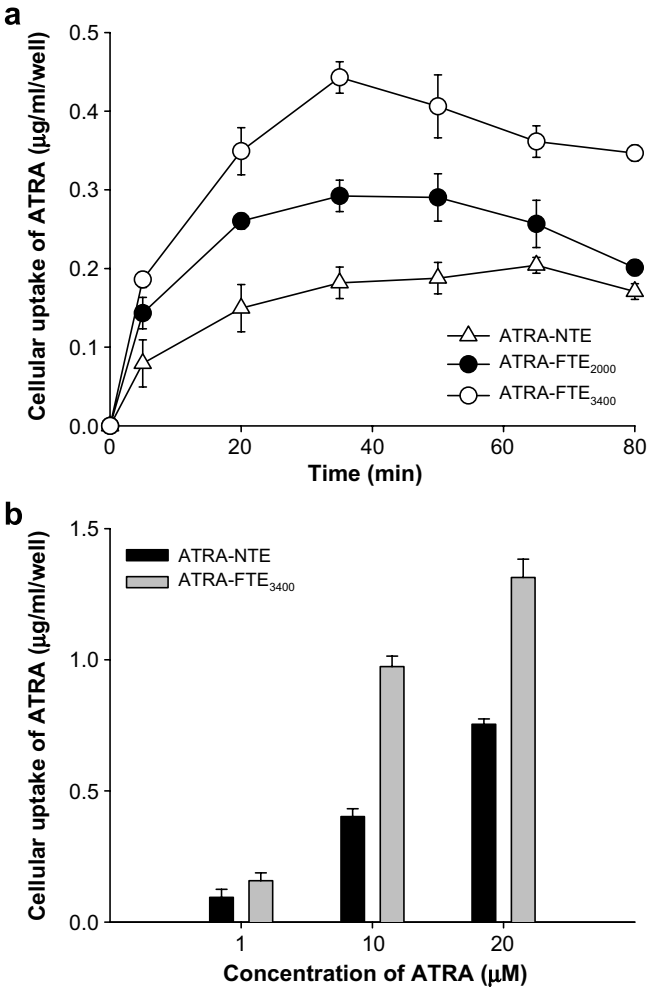

Fig. 1. Time-dependence (a) and dose-dependence (b) of ATRA cell uptake.

associate with folate receptor-positive KB cells, and saturation was typically reached within 1 h incubation [36]. The maximum uptake by folate binding was achieved at 30 min and from that moment the uptake was decreased [37]. In addition, Matarese and Lodish reported that the peak cellular uptake by retinol-binding protein was obtained at 2 h, thus the incubation for 2 h was followed by each experiment [38]. Of course, it is possible that folate receptor would be saturated by the folate-lipid emulsions and half-life of ATRA within the cell would be controlled by cell environments [37].

Furthermore, the PEG length affected the cellular uptake of ATRA. It is thought that longer spacer length

can induce the sterical hindrances made by clusters of folate receptors on carcinoma surface. Our results disagree with a previous report that PEG length over 1000 in molecular weight showed no significant difference. However, it was also reported that folate-conjugated lipid formulations have a higher tendency to target carcinoma compared to non-targeted formulations [36]. Moreover, folate ligands of the folate-tethered emulsions can interact with the folate receptors at the same time in the possible docking areas; therefore PEG spacer length would be important. Actually, the circulation of the conjugates was prolonged with increased molecular weight of the polymer used, since polymer inhibited serum protein binding and substantially increased aqueous solubility [39]. As a result, intravenous administration to mice of polymer-coated complexes extended systemic circulation [39]. The biodistribution study of poly(ethylene glycol) derivatives for preparation of ribosome-inactivating protein conjugates also showed lower organ uptake after PEGylation, in particular by the liver and spleen [40].

3.4. Internalization of fluorescence-labeled ATRA-FTE in KB cells

When ATRA was incorporated with folate-tethered emulsions, significant amount of ATRA-FTE_{3400} was associated with the KB cells (Fig. 3). Large and numerous green fluorescent spots were found in all observed cells (Fig. 2d

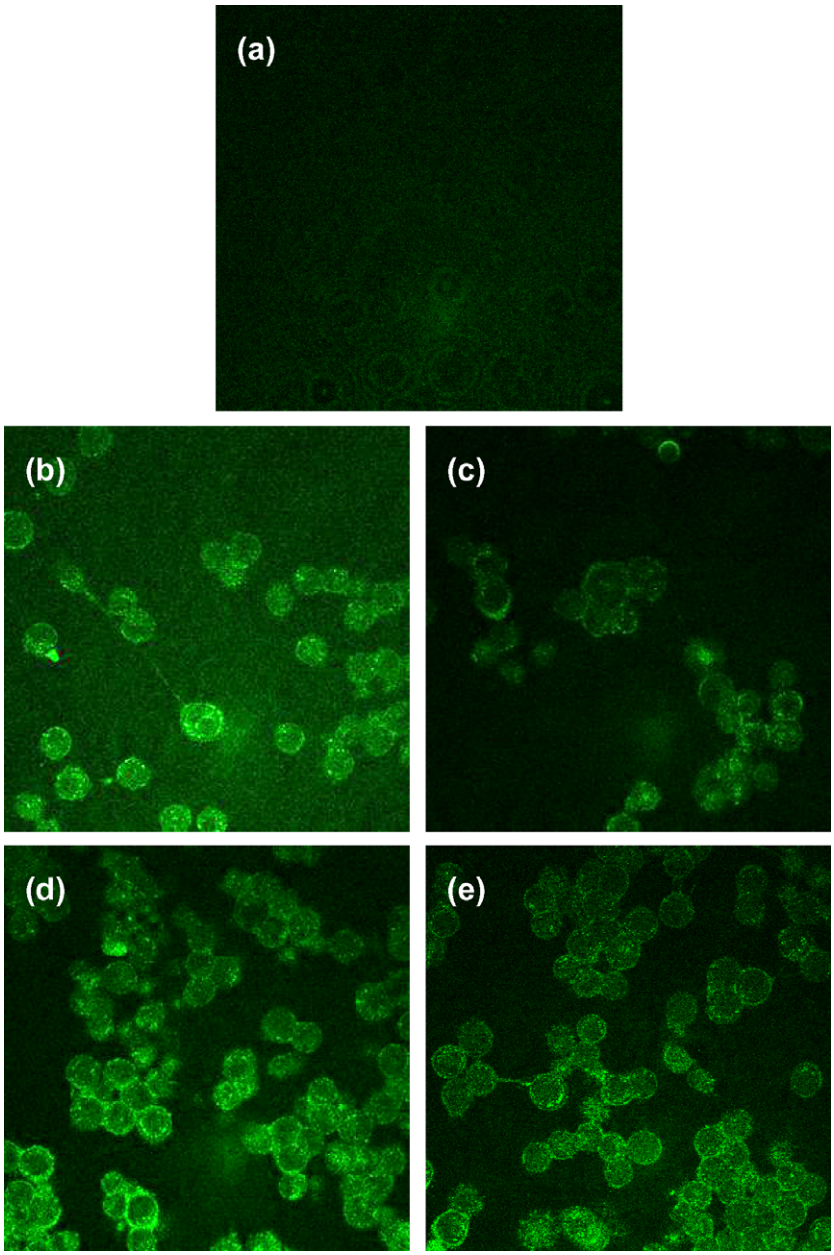

Fig. 2. Confocal microscopy. KB cells were preincubated for 24 h to encourage strong coverslip adherence. Ten micromolars of fluorescence-containing ATRA-NTE and ATRA-FTE_{3400} were incubated with the cells for 30 min and 1 h at 37 °C. Following extensive rinsing, cell-associated fluorescence was imaged by confocal laser scanning microscope. (a) Control (b) 30 min ATRA-NTE (c) 1 h ATRA-NTE (d) 30 min ATRA-FTE_{3400} (e) 1 h ATRA-FTE_{3400}.

and e). Moreover, a reduction in cell association was observed for the ATRA-NTE that lacked the folate ligand (Fig. 2b and c). These data support our conclusion that folate emulsions can efficiently target the incorporated retinoids to folate receptor-positive cells.

In the process of endocytosis, ATRA-FTE was immediately accumulated on the surface of the cancer cells as shown in Fig. 2. We observed the appearance of the time-dependent cellular uptake from 35 to 80 min. However, the difference of fluorescent spots at between the cell surface and intracellular region was not found over the time interval, suggesting that the folate receptors on the cell surface were very rapidly saturated by folate ligand on the ATRA-FTE and they maintained similar patterns of endocytic process for a few hours. In this respect, our results show that short time is necessary for saturation by folate-tethered emulsion below about 1 h. Moreover, these results mean that ATRA-FTE_{3400} is an effective formulation for cellular uptake than any other formulations and this study undoubtedly demonstrates folate receptor-mediated intracellular distribution of folate-tethered emulsion [28,29].

3.5. Competitive binding of ATRA-FTE and free folate

The binding competition of ATRA-FTE_{2000} and ATRA-FTE_{3400} was studied to evaluate the folate ligand affinity for the folate receptor in KB cells. The ATRA-FTE binding competition profiles show that the formulation competes with free folate for the cell receptor (Fig. 3). As a result, the cellular uptakes of ATRA were reduced by increasing the concentration of folate as a competitor. As concerning about in vivo mechanisms of competition with free endogenous folate, folate-tethered emulsions may be superior due to their higher affinities for the target cell resulting from multivalent bindings to targeted carcinomas relatively. Furthermore, if the formulations have proper size for attaching to the cell surface, the in vivo efficiency will be increased to the maximum. Also the longer spacer length of PEGs probably enables a better interaction with the folate receptor in order to overcome the sterical hindrance which prevents interactions [41].

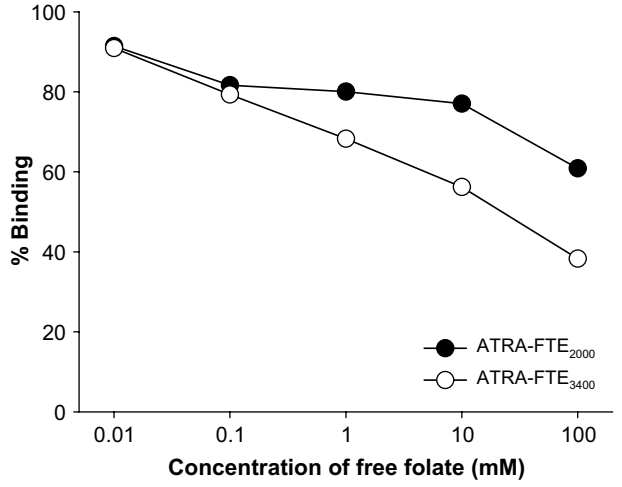

Fig. 3. Inhibition of ATRA-FTEs uptake by free folate. The cellular uptake was compared between folate-added cells and folate-free cells treated with same formulation. KB cells was incubated for 1 h with 1 μM of ARA-FTE_{2000} and ATRA-FTE_{3400}, varying concentrations of free folic acid from 0.01 to 100 mM [6]. After thorough washing by PBS, the cells were harvested using trypsin (1×) and then the cellular ATRA concentration was determined with HPLC.

3.6. Growth inhibitory effect of FTE-ATRA

ATRA has anticancer activity in KB cells and MCF-7 cells depending on dose (Fig. 4). On the KB cells with high densities of folate receptors, ATRA-FTE_{3400} showed the lowest cell viability which means the maximum growth inhibition effect compared to the other formulations (growth inhibitory activity on KB cells: ATRA-FTE_{3400} > ATRA-FTE_{2000} > ATRA-NTE) (Fig. 4a). On the contrary, on the MCF-7 cells with low level of folate receptors, all formulations showed similar cell growth inhibition which was dose-dependent (Fig. 4b). KB cells are known to

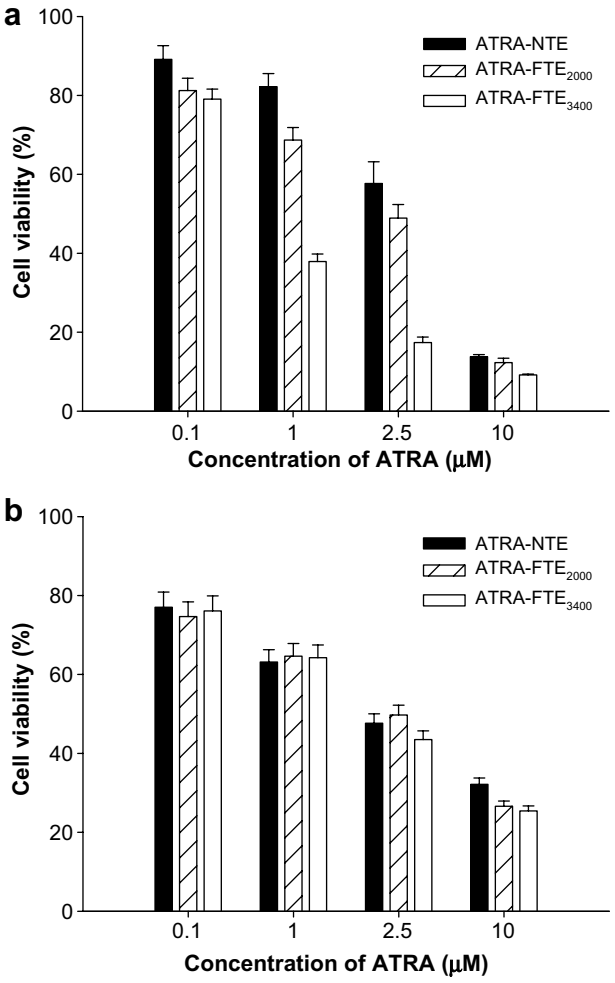

Fig. 4. Growth inhibitory effect on KB cells (a) with high densities of folate receptor and on MCF-7 cells (b) with low level of folate receptor (no additional free folate).

express 4 million folate receptors/cell in normal condition and express 90,000 folate receptors/cell when grown under low conditions of folate in the medium [6,36,41]. Meanwhile, MCF-7, human breast carcinoma cells, express undetectable levels of folate receptor [43,44]. Therefore, the cell viability of KB cells was ~2 times lower than MCF-7 cells. Although treated with different emulsion formulations such as FTE and NTE, MCF-7 cells did not show the significant changes in cytotoxicity at the same dose of ATRA because of low expression level of folate receptors in the cell surfaces. This is clear evidence that these folate-tethered emulsions have a potential for folate receptor-mediated uptake. However, our results showed only moderate increase in cytotoxicity in KB cells because ATRA could be transported to the cell via non-specific mechanisms under these incubation conditions. There are several reports which show that folate-targeted drugs in liposomes exhibit cytotoxicity which is 50–100 times (about 2-logs) higher than non-folate-targeted drug administration [6,45]. In each instance, the cells were conditioned for several weeks in low folate medium (folate-free medium containing serum which provides nM concentrations of folate to the cells) to allow for maximal expression of folate receptors on the cell surface.

Moreover, since most tissue culture media contain micromolar concentrations of folate, it is essential to use medium with no folate but contain 10% heat-inactivated bovine serum, so that the folate concentration is about 9 nM in the culture medium which is close to the circulating concentration of folate in human serum. This would also mean that cytotoxicity experiments should be conducted with cells adapted to grow in low folate medium before incubation with folate-tethered liposomes. Perhaps by doing this way the difference in magnitude of cytotoxicity between folate-receptor expressing cells and non-expressing cells would be very high.

3.7. Inhibition of anticancer activity by free folate

Continuously, the addition of 1 mM free folate into the ATRA-FTE$_{3400}$ reduced the anticancer activity of ATRA to the level of ATRA-NTE (Fig. 5). Folate-tethered emulsions had little anticancer effect on the growth inhibition under conditions by competition with free folate. It means that free folate influences both the cellular uptake and growth inhibition of ATRA by preventing folate-tethered emulsion from binding the carcinomas bearing folate receptors. Figs. 1b and 5 show a good correlation between cellular uptake and cytotoxicity of ATRA-FTE with dose-dependence. Compared with the numerical differences with IC_{50} and cellular uptake efficiency, almost similar patterns showed with ATRA-NTE and ATRA-FTE$_{3400}$. It was a different results with the folate-liposomal formulations using doxorubicin in vitro test already published report [42], which the 86-fold increase in cytotoxicity due to folate-targeting seems to exceed the 45-fold increase in cellular uptake.

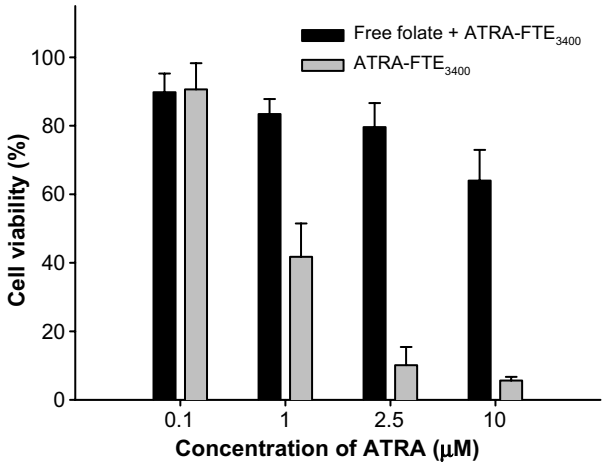

Fig. 5. Inhibition of ATRA activity in ATRA-FTE$_{3400}$ by adding folate in KB cell cultures.

4. Conclusions

In this study, we developed folate-tethered emulsions of ATRA for targeting folate receptor-abundant cancer cells and improved the cellular uptake and growth inhibition by altering the polymer length. Therefore, folate-tethered emulsion has a potential for target delivery of poorly water-soluble retinoids to cancer cells. However, to confirm this suggestion, the further study needs to be carried out in maintained cells in folate-free medium or low folate medium to overexpress folate receptors.

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References

[1] J.S. Chawla, M.M. Amiji, Biodegradable poly (ε-caprilactone) nanoparticles for tumor-targeted delivery of tamoxifen, Int. J. Pharm. 249 (2002) 127–138.
[2] P. Sapra, T.M. Allen, Ligand-targeted liposomal anticancer drugs, Prog. Lipid Res. 42 (2003) 439–462.
[3] A. Gabizon, H. Shmeeda, A.T. Horowitz, S. Zalipsky, Tumor cell targeting of liposome-entrapped drugs with phospholipid-anchored folic acid-PEG conjugates, Adv. Drug Deliv. Rev. 56 (2004) 1177–1192.
[4] T.L. Andresen, S.S. Jensen, K. Jorgensen, Advanced strategies in liposomal cancer therapy: problems and prospects of active and tumor specific drug release, Prog. Lipid Res. 44 (2005) 68–97.
[5] A. Gabizon, A.T. Horowitz, D. Goren, D. Tzemach, F. Mandelbaum-Shavit, M.M. Qazen, S. Zalipsky, Targeting folate receptor with folate linked to extremities of poly (ethylene glycol)-grafted liposomes: in vitro studies, Bioconjug. Chem. 10 (1999) 289–298.
[6] R.J. Lee, P.S. Low, Delivery of liposomes into cultured KB cells via folate receptor-mediated endocytosis, J. Biol. Chem. 269 (1994) 3198–3204.

[7] S. Wang, P.S. Low, Folate-mediated targeting of antineoplastic drugs, imaging agents, and nucleic acids to cancer cells, J. Control. Release 53 (1998) 39–48.
[8] A. Gabizon, A.T. Horowitz, D. Goren, D. Tzemach, H. Shmeeda, S. Zalipsky, In vivo fate of folate-targeted polyethylene-glycol liposomes in tumor-bearing mice, Clin. Cancer Res. 9 (2003) 6551–6559.
[9] R.J. Lee, L. Huang, Folate-targeted, anionic liposome-entrapped polylysine-condensed DNA for tumor cell-specific gene transfer, J. Biol. Chem. 271 (1996) 8481–8487.
[10] X. Pan, R.J. Lee, Tumour-selective drug delivery via folate receptor-targeted liposomes, Expert Opin. Drug Deliv. 1 (2004) 7–17.
[11] P.J. Stevens, M. Sekido, R.J. Lee, A folate receptor-targeted lipid nanoparticle formulation for a lipophilic paclitaxel prodrug, Pharm. Res. 21 (2004) 2153–2157.
[12] C.T. Okamoto, Endocytosis and transcytosis, Adv. Drug Deliv. Rev. 29 (1998) 215–228.
[13] J. Sudimack, R.J. Lee, Targeted drug delivery via the folate receptor, Adv. Drug Deliv. Rev. 41 (2000) 147–162.
[14] Y. Lu, P.S. Low, Folate-mediated delivery of macromolecular anticancer therapeutic agents, Adv. Drug Deliv. Rev. 54 (2002) 675–693.
[15] X.Q. Pan, H. Wang, R.J. Lee, Antitumor activity of folate receptor-targeted liposomal doxorubicin in a KB oral carcinoma murine xenograft model, Pharm. Res. 20 (2003) 417–422.
[16] P.J. Stevens, R.J. Lee, A folate receptor-targeted emulsion formulation for paclitaxel, Anticancer Res. 23 (2003) 4927–4931.
[17] P.G. Montaldo, G. Pagnan, F. Pastorino, V. Chiesa, L. Raffaghello, M. Kirchmeier, T.M. Allen, M. Ponzoni, *N*-(4-hydroxyphenyl) retinamide is cytotoxic to melanoma cells in vitro through induction of programmed cell death, Int. J. Cancer 81 (1999) 262–267.
[18] E.H. Estey, F.J. Giles, H. Kantarjian, S. O'Brien, J. Cortes, E.J. Freireich, G. Lopez-Berestein, M. Keating, Molecular remissions induced by liposomal-encapsulated all-trans retinoic acid in newly diagnosed acute promyelocytic leukemia, Blood 94 (1999) 2230–2235.
[19] S.L. Hsu, W.S. Wu, Y.S. Tyan, C.K. Chou, Retinoic acid-induced apoptosis is prevented by serum albumin and enhanced by Lipiodol in human hepatoma Hep3B cells, Cancer Lett. 129 (1998) 205–214.
[20] K.M. Park, C.K. Kim, Preparation and evaluation of flurbiprofen-loaded microemulsion for parenteral delivery, Int. J. Pharm. 181 (1999) 173–179.
[21] S.R. Hwang, S.J. Lim, J.S. Park, C.K. Kim, Phospholipid-based microemulsion formulation of all-trans-retinoic acid for parenteral administration, Int. J. Pharm. 276 (2004) 175–183.
[22] K.M. Park, M.K. Lee, K.J. Hwang, C.K. Kim, Phospholipid-based microemulsions of flurbiprofen by the spontaneous emulsification process, Int. J. Pharm. 183 (1999) 145–154.
[23] S. Furukawa, N. Katayama, T. Iizuka, I. Urabe, H. Okada, Preparation of polyethylene glycol-bound NAD and its application in a model enzyme reactor, FEBS Lett. 121 (1980) 239–242.
[24] S. Zalipsky, E. Brandeis, M.S. Newman, M.C. Woodle, Long circulating, cation liposomes containing amino-PEG-phosphatidylethanolamine, FEBS Lett. 353 (1994) 71–74.
[25] A. Agadir, M. Cornic, P. Lefebvre, B. Gourmel, M. Jerome, L. Degos, P. Fenaux, C. Chomienne, All-trans retinoic acid pharmacokinetics and bioavailability in acute promyelocytic leukemia: intracellular concentrations and biologic response relationship, J. Clin. Oncol. 13 (1995) 2517–2523.
[26] S.J. Lim, C.K. Kim, Formulation parameters determining the physicochemical characteristics of solid lipid nanoparticles loaded with all-trans retinoic acid, Int. J. Pharm. 243 (2002) 135–146.
[27] R. Parthasarathy, P.G. Sacks, D. Harris, H. Brock, K. Mehta, Interaction of liposome-associated all-trans-retinoic acid with squamous carcinoma cells, Cancer Chemother. Pharmacol. 34 (1994) 527–534.
[28] K. Na, K.H. Park, S.W. Kim, Y.H. Bae, Self-assembled hydrogel nanoparticles from curdlan derivatives: characterization, anti-cancer drug release and interaction with a hepatoma cell line (HepG2), J. Control. Release 69 (2000) 225–236.
[29] R. Tachibana, H. Harashima, M. Shono, M. Azumano, M. Niwa, S. Futaki, H. Kiwada, Intracellular regulation of macromolecules using pH-sensitive liposomes and nuclear localization signal: qualitative and quantitative evaluation of intracellular trafficking, Biochem. Biophys. Res. Commun. 251 (1998) 538–544.
[30] M. Orlandi, B. Mantovani, K. Ammar, E. Avitabile, P. Dal Monte, G. Bartolini, Retinoids and cancer: antitumoral effects of ATRA, 9-cis RA and the new retinoid IIF on the HL-60 leukemic cell line, Med. Princ. Pract. 12 (2003) 164–169.
[31] M. Ratnam, H. Hao, X. Zheng, H. Wang, H. Qi, R. Lee, X. Pan, Receptor induction and targeted drug delivery: a new antileukaemia strategy, Expert Opin. Biol. Ther. 3 (2003) 563–574.
[32] C.P. Leamon, R.B. DePrince, R.W. Hendren, Folate-mediated drug delivery: effect of alternative conjugation chemistry, J. Drug Target. 7 (1999) 157–169.
[33] T. Shiokawa, Y. Hattori, K. Kawano, Y. Ohguchi, H. Kawakami, K. Toma, Y. Maitani, Effect of polyethylene glycol linker chain length of folate-linked microemulsions loading acalacinomycin A on targeting ability and antitumor effect in vitro and in vivo, Clin. Cancer Res. 11 (2005) 2018–2025.
[34] R.H. Muller, S. Heinemann, Fat emulsions for parenteral nutrition I: evaluation of microscopic and laser light scattering methods for the determination of the physical stability, Clin. Nutrit. 11 (1992) 223–272.
[35] M.S. Hong, S.J. Lim, M.K. Lee, Y.B. Kim, C.K. Kim, Prolonged blood circulation of methotrexate by modulation of liposomal composition, Drug Deliv. 8 (2001) 231–237.
[36] C.P. Leamon, S.R. Cooper, G.E. Hardee, Folate-liposome-mediated antisense oligodeoxynucleotide targeting cancer cells: evaluation in vitro and in vivo, Bioconjug. Chem. 14 (2003) 738–747.
[37] S.B. Kurlandsky, M.V. Gamble, R. Ramakrishnan, W.S. Blaner, Plasma delivery of retinoic acid to tissues in the rat, J. Biol. Chem. 270 (1995) 17850–17857.
[38] V. Matarese, H.F. Lodish, Specific uptake of retinol-binding protein by variant F9 cell lines, J. Biol. Chem. 268 (1993) 18859–18865.
[39] C.M. Ward, M. Pechar, D. Oupicky, K. Ulbrich, L.W. Seymour, Modification of pLL/DNA complexes with a multivalent hydrophilic polymer permits folate-mediated targeting in vitro and prolonged plasma circulation in vivo, J. Gene Med. 4 (2002) 536–547.
[40] S. Arpicco, F. Dosio, A. Bolognesi, C. Lubelli, P. Brusa, B. Stella, M. Ceruti, L. Cattel, Novel poly(ethylene glycol) derivatives for preparation of ribosome-inactivating protein conjugates, Bioconjug. Chem. 13 (2002) 757–765.
[41] A.L. Klibanov, K. Maruyama, A.M. Beckerleg, V.P. Torchilin, L. Huang, Activity of amphipathic poly(ethylene glycol) 5000 to prolong the circulation time of liposomes depends on the liposome size and is unfavorable for immunoliposome binding to target, Biochim. Biophys. Acta 1062 (1991) 142–148.
[42] R.J. Lee, P.S. Low, Folate-mediated tumor cell targeting of liposome-entrapped doxorubicin in vitro, Biochim. Biophys. Acta 1233 (1995) 134–144.
[43] P.C. Elwood, Molecular cloning and characterization of the human folate-binding protein cDNA from placenta and malignant tissue culture (KB) cells, J. Biol. Chem. 264 (1989) 14893–14901.
[44] K.N. Chung, Y. Saikawa, T.H. Paik, K.H. Dixon, T. Mulligan, K.H. Cowan, P.C. Elwood, Stable transfectants of human MCF-7 breast cancer cells with increased levels of the human folate receptor exhibit an increased sensitivity to antifolates, J. Clin. Invest. 91 (1993) 1289–1294.
[45] J.M. Saul, A. Annapragada, J.V. Natarajan, R.V. Bellamkonda, Controlled targeting of liposomal doxorubicin via the folate receptor in vitro, J. Control. Release 92 (2003) 49–67.