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Original article

Synthesis of octadecylamine-retinoic acid conjugate for enhanced cytotoxic effects of 5-FU using LDL targeted nanostructured lipid carriers

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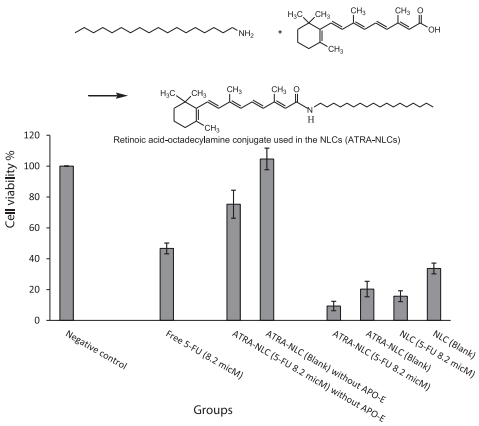
HIGHLIGHTS

- Retinoic acid was conjugated to octadecylamine.
- 5-FU and retinoic acid conjugate were loaded in cholesterol NLCs.
- NLCs targeted LDL receptors of HT29 cells.
- IC_{50} of NLCs of retinoic acid conjugate and 5-FU was more than 5 fold of free 5-FU.
- NLCs of 5-FU and retinoic acid conjugate seems promising in colorectal cancer.

GRAPHICAL ABSTRACT



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ABSTRACT

The aim of the present study was to reduce 5-FU side effects by targeted nanostructured lipid carriers (NLCs) to LDL receptors that are over expressed in colorectal carcinoma and also use of a new synthesized conjugate of retinoic acid as a cytotoxic agent. Fatty acyl amide derivative of retinoic acid was synthesized by its conjugation to octadecylamine with the expectation to improve its loading capacity in NLCs of 5-FU. The NLCs were prepared by an emulsification-solvent evaporation method using cholesterol and cholesteryl stearate. Physical properties and drug release were studied in NLCs. The cytotoxicity of NLCs loaded with 5-FU and retinoic acid conjugate was studied on colon cancer cells (HT29) using MTT assay. To confirm that drug targeting has been done through LDL receptors, APO-E was omitted from the cell culture and the MTT assay was repeated. FTIR and ^{1}H NMR spectra confirmed successful production of the conjugate. Results showed the IC_{50} of free 5-FU was about 7.6 μM while in comparable concentration, the cytotoxicity of 5-FU loaded in NLCs containing the retinoic acid conjugate was nearly 2 fold of NLCs just loaded with 5-FU and more than 5 fold of free 5-FU. The retinoic acid conjugate loaded NLCs prepared by cholesterol can target LDL receptors of HT29 cells and seems promising in reducing 5-FU dose in colorectal cancer.

1. Introduction

Cancer is the second cause of death after cardiac disease in America [1]. Colorectal cancer is the third most common form of

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cancer and cause of death due to cancer in the United States. Colorectal cancer by incidence of 1 million new cases in each year affects men and women in world wide. Adenomatus polyps and malignant cells that are formed in colon replicate so fast and become tumors that can spread to other sites [2] indicating an urgent need for more effective colon cancer chemotherapy. Targeted therapy for colorectal cancer allows for the local high concentration of chemotherapeutic drugs and reduces their side effects [2].

Nanostructured lipid carriers (NLCs) are a new generation of colloidal drug carriers. These nanostructures contain liquid lipid that improves drug loading and stability of nanoemulsion [3,4] due to an unjustified, imperfect lipid matrix having the space for the molecules of drug.

5-FU is a pyrimidine analog that is used in different solid tumors of colon, liver, neck, head and breast cancers. 5-FU is that inhibits the activity of thymidylate synthetase. Unfortunately, 5-FU shows many disadvantages such as: inactivation by dihydropyrimidine dehydrogenase that results in inadequate absorption by gastrointestinal tract, very short half life and the toxic effects on bone marrow and un-selectivity on normal cells. For these reasons scientists try to enhance the efficacy of this drug by increasing the time of circulation and reducing side effects by localizing the drug to the infected cells by target therapy [1,2].

Retinoids are a family of chemicals containing vitamin A and its natural and synthetic derivatives that are some important chemotherapeutic and chemo preventive agents for a lot of cancers like leukemia and some forms of skin cancer [5,6]. Between retinoic acid receptors that are types of α, β and γ, the type β is necessary for sensitivity to retinoic acid in cell lines and mediates retinoid activity [7]. It is shown that the expression rate of RAR-β in tumor tissues is significantly lower than those of both normal and adjacent tissues. The expression rate of RAR-β decreases in human colorectal tumor tissues, suggesting RAR-β may be involved in the formation of human colorectal cancer. RAR-β may become a new prognostic indicator of human colorectal cancer [8].

Unfortunately, chemotherapy with retinoic acid results in undesirable side effects and retinoic acid-resistant tumors are very common. Retinol (vitamin A) is thought to exert its effects through the actions of its metabolite, *all-trans*-retinoic acid (ATRA). The ability of retinol to inhibit the growth of ATRA-sensitive and ATRA-resistant human colon cancer cell lines was examined by Park et al. [9]. Retinol inhibited cell growth in a dose-responsive manner.

Conjugation of some drugs to fatty acids has been reported to enhance their stability, solubility, short half life, and their cellular uptake. For example the conjugate of paclitaxel to a natural fatty acid has enhanced its tumor accumulation [10] or fatty acyl amide derivative of doxorubicin i.e., dodecanoyl-doxorubicin is consistently the most effective derivative which inhibited the proliferation of colon and ovarian cancer cells [11]. Also fatty acyl derivatives of cytarabine improved cellular uptake with a longer duration of action [12].

Expression of LDL receptor is higher in some cancer cells like leukemia, breast cancer and human lung cancer tissues [13]. Also in human colon carcinoma LDL receptor mRNA expression is significantly increased so using the cholesterol as a drug carrier for treatment of colorectal carcinomas seems logic [14]. Apolipoprotein E (Apo-E) is a class of apolipoprotein found in the chylomicron, that binds to LDL receptors on liver cells and peripheral cells. It is essential for the normal catabolism of triglyceride-rich lipoprotein constituents [15]. For these reasons in the present study the LDL receptors of colorectal cancer cells were targeted by cholesterol. RAR-independent inhibitory effect of retinoic acid on cell growth on colon cancer was also used to enhance the cytotoxic effect of 5-FU. To do so retinoic acid was conjugated to octadecylamine first to enhance its solubility in the mixture of solvents used for the production of NLCs, so higher entrapment of retinoic acid caused better uptake of retinoic acid by the cells.

2. Materials and methods

2.1. Materials

5-FU was provided from Sigma (USA), Retinoic acid was purchased from Solmag Chemical Company (Italy), cholesterol, oleic acid, octanol, Tween 20, Tween 80, chloroform and silica gel F_{254} 60 were from Merck Chemical Company (Germany). Soy lecithin S100 was from Lipoid (Germany), Dicyclohexyl carbodiimide (DCC) from Fluka (USA), cholesteryl stearate (CS) from Aldrich (US), N-hydroxysuccinimide (NHS), dimethyl sulfoxide (DMSO), methanol, thriethyl amine, thiazolyl blue tetrazolium bromide (MTT) and octadecylamine were from Sigma (USA). HT_{29} cell line from Pasture institute (Iran), Tripsin/EDTA, and RPMI 1640 from PAA Company (Austria). Steptomycin/Pencilline from Gibko (US), 96 well plate from Nunck Company (Denmark).

2.2. Conjugation of retinoic acid and octadecylamine

Fig. 1 shows the chemical structure of retinoic acid and octadecylamine. For preparation of this conjugate 500 mg of retinoic acid, 330 mg of DCC and 400 mg of NHS were added to 15 mL of DMSO. This solution was shaken at room temperature for 72 h until retinoic acid was activated. After 72 h the precipitate of dicyclohexyl urea was separated by filtration. Then this solution was dripped into the chloroform solution of octadecylamine (425 mg/17 mL). After 24 h shaking, the solvents were evaporated under vacuum distillation.

2.3. Characterization of synthesized derivative of retinoic acid

For the purification and identification of the product a preparative TLC (silica gel f_{254} 60) was used. Fifty mg of the solid mixture collected from the previous stage was dissolved in the minimum amount of ether and was implanted on the preparative TLC with the mobile phase of chloroform. The middle spot with the $R_f = 0.5$ was carved and the silica gel was removed.

FTIR and ^{1}H NMR spectra were used for analysis of synthesized retinoic acid-octadecylamine. The samples were measured at 298°k with about 5 wt% $CDCl_3$ or $(CD_3)_2SO$ solution using NMR Spectrometer (AC-80, Bruker Biospin, Germany).

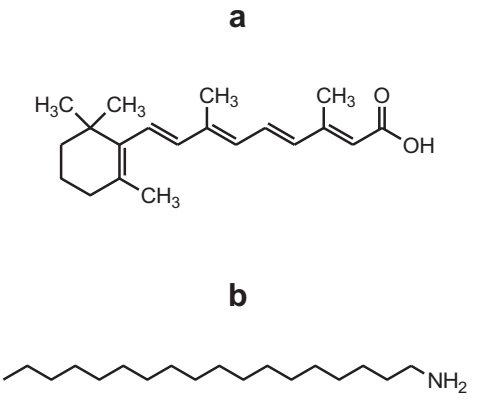


Fig. 1. Chemical structure of a) retinoic acid and b) octadecylamine.

Table 1
Different formulations of prepared NLCs (containing 20 mg 5-FU in 25 mL of NLCs dispersion).

Formulation code	Octanol (OC) %	Oleic acid (OA) %	Cholesterol (C) %	Cholesteryl stearate (CS) %
$CS_{64.5}OA_{15}$	–	15	–	64.5
$CS_{59.5}OA_{20}$	–	20	–	59.5
$CS_{54.5}OA_{25}$	–	25	–	54.5
$C_{64.5}OA_{15}$	–	15	64.5	–
$C_{59.5}OA_{20}$	–	20	59.5	–
$C_{54.5}OA_{25}$	–	25	54.5	–
$CS_{64.5}OC_{15}$	15	–	–	64.5
$CS_{59.5}OC_{20}$	20	–	–	59.5
$CS_{54.5}OC_{25}$	25	–	–	54.5
$C_{64.5}OC_{15}$	15	–	64.5	–
$C_{59.5}OC_{20}$	20	–	59.5	–
$C_{54.5}OC_{25}$	25	–	54.5	–

2.4. Preparation of NLCs

Among all liquid lipids selected for solubility screening, 5-FU had the highest solubility in octanol and oleic acid. Therefore, these two liquid lipids were selected for preparation of NLCs. In a preliminary test seven factors each in 2 levels including: lipid type and content, oil type and content, lecithin and PEG 40 stearate contents and the ratio of acetone/ethanol were first screened by the Taguchi design using an L_8 orthogonal array to find the most effective factors on 3 responses of particle size, drug loading and release efficiency. This screening test showed the most important effective factors were the lipid and oil type and their content (data are not shown here). Therefore, they were selected for further optimization studies. The content of 5-FU was fixed at 20 mg as higher amount was not soluble in the organic phase. Retinoic acid-octadecylamine conjugate concentration was also fixed at 10 weight percent of the NLCs as this study was a preliminary study to see whether it can increase cytotoxicity of 5-FU or not and of course further studies are needed to optimize its cytotoxic concentrations which has synergistic effect with 5-FU. According to the formulation type (Table 1) 59.5% ± 5% of lipid (cholesteryl stearate or cholesterol), 0.5% of lecithin, 10% of PEG 40 stearate (as the pegylated lipid to help escaping NLCs from reticuloendothelial system), 10% of the synthesized derivative i.e., retinoic acid-octadecylamine, 20% ± 5% of oil (oleic acid or octanol) and 20 mg of 5-FU were dissolved in 5 mL of the mixture of acetone/ethanol by the ratio of 3:1. Then this organic phase was slowly added to 25 mL of distilled water containing 0.5% of Tween 80 while stirred on a magnetic stirrer during 15 min. The organic solvent was allowed to evaporate for 1 h using a magnetic stirrer with a minimum speed. The effects of oil type, lipid type and their contents were investigated and nanoparticles with the greatest drug loading efficiency, the least particle size, the greatest absolute value of zeta potential and the greatest drug release were considered as the optimum formulation.

2.5. Particle size and zeta potential measurements

The mean particle size and zeta potential of NLCs were measured by photon correlation spectroscopy (PCS) at a fixed angle of 90° (Zetasizer 3000HS, Malvern Instrument, UK). Nanodispersion was suitably diluted to measure mean particle size and polydispersity index.

For this purpose the samples were diluted 10 fold with de-ionized water before measurements of particle size and zeta potential.

2.6. Entrapment efficiency and drug loading

For the quantitative determination of 5-FU a spectrophotometric method was used. The amount of the loaded 5-FU into the NLCs was determined indirectly as follows: 600 μL of sample emulsion and 600 μL of blank emulsion were centrifuged (Microcentrifuge Sigma 30k, UK) at 10000 rpm for 5 min to separate nanoparticles. The supernatant containing the free drug was diluted 40 times and analyzed using a spectrophotometer (RF-5301 PC, Shimadzu, Kyoto, Japan) at $\lambda_{max} = 267$ nm. The difference between the total and free drug shows the amount of the encapsulated drug. The encapsulation efficiency (EE) of 5-FU in NLCs was determined as the ratio between the actual and theoretical loading by using the following equation:

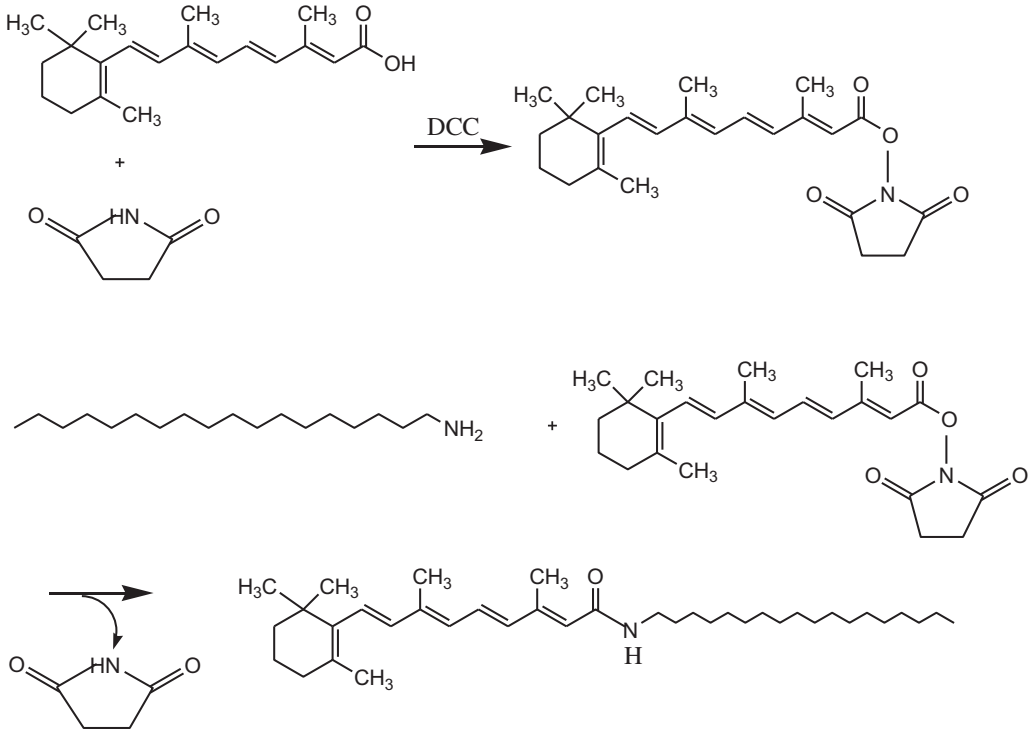


Fig. 2. Schematic representation of chemical reaction for preparation of retinoic acid-octadecylamine conjugate.

$$EE\left(\%\right) = \frac{\text{Entrapped drug in NLCs}}{\text{Total amount of drug added}} \times 100 \quad (1)$$

Drug loading capacity (DL) was calculated according to the Eq. (2). The total amount of the drug, lipid and oil excipients added during preparation were considered as the total weight of NLCs:

$$DL\left(\%\right) = \frac{\text{Entrapped drug in NLCs}}{\text{Weight of NLCs}\,(\text{amount of drug} + \text{amount of lipid})} \times 100 \quad (2)$$

The validity of this method was checked by directly measuring the entrapped drug in the separated NLCs. So that they were dissolved in a mixture of acetone/ethanol and the content of loaded 5-FU in NLCs was measured. It was in accordance to the indirect method. For this reason the indirect method was used throughout the study due to its easiness.

2.7. Drug release studies

To study the drug release from nanoparticles, 1 mL of dispersion was transferred to a dialysis tube (molecular weight cutoff 12000, Membra-Cel®, Viskase, USA), then the sealed tube was put into a beaker of 70 mL of phosphate buffer solution (pH 7.4) containing 2% Tween 20. Samples were shaken horizontally in a shaker bath (Lab tech, Korea) at 37 ± 1 °C with 40 strokes per minute. 600 μL of the medium was taken at predetermined time intervals and the

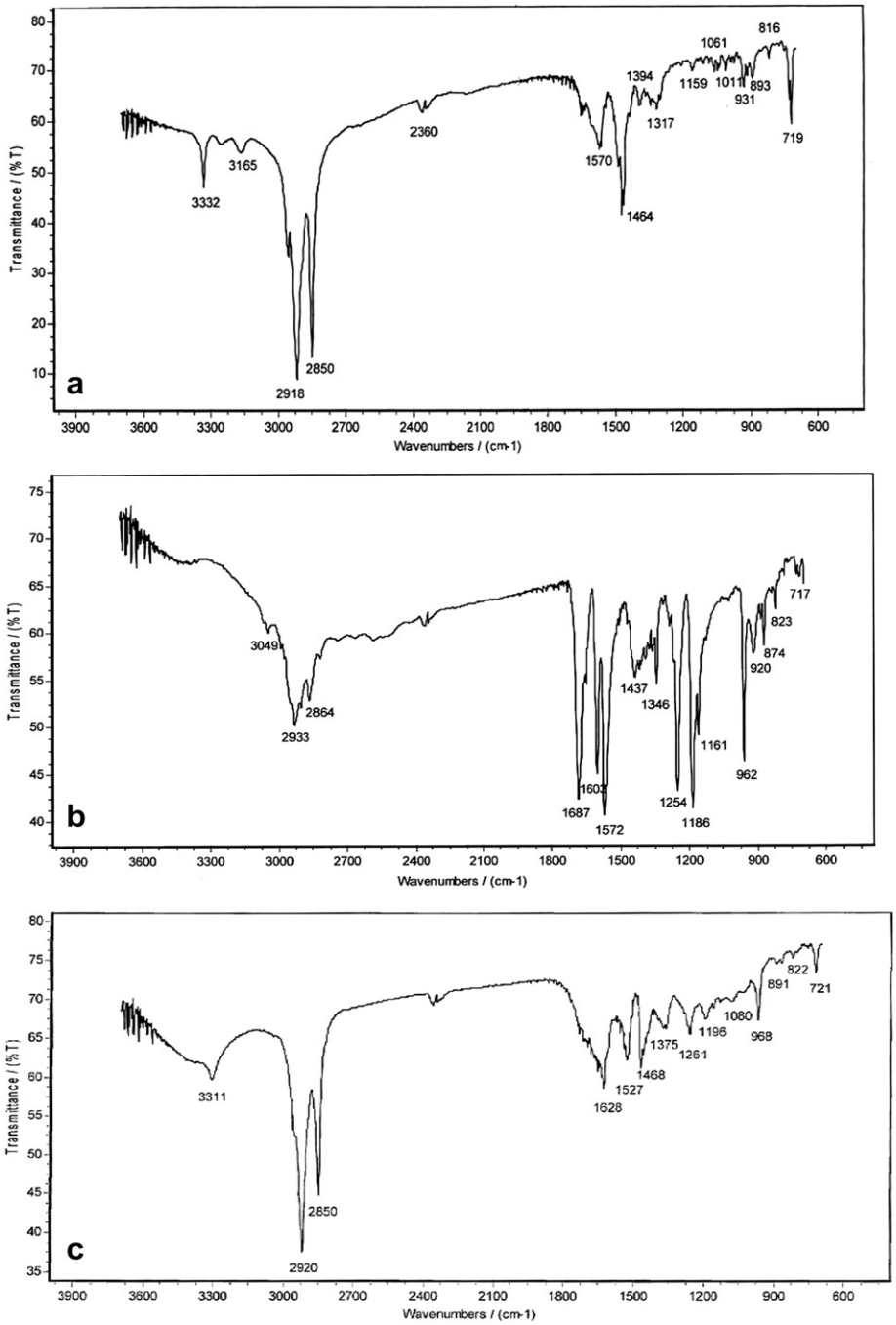


Fig. 3. FTIR spectra of a) octadecylamine, b) retinoic acid and c) octadecylamine-retinoic acid conjugate.

absorbance of free 5-FU was measured at λ_{max} = 268 nm. The samples were returned to the test medium again. The parameter of release efficiency within 20 h (RE_{20}%) was used to compare the release profiles [16]:

$$RE\% = \frac{\int_0^t ydt}{y_{100}t} \times 100 \quad (3)$$

2.8. Cell culture

Human colon cancer cell line HT29 was used in this study. The cells were cultured on RPMI 1640 containing 10% FBS (Fetal Bovine Serum) and 1% antibiotics mixture of penicillin (10000 U/mL) and streptomycine (10000 μg/mL) at 37 °C and in 5% CO_2. HT29 cells are from colon epithelial like cells in culture medium. First 180 μL of the suspension of cells at a density of 5×10^4 cells/ml were seeded into each well of a 96-well culture plate (SPL Lifescience, Korea) and incubated for 24 h at 37 °C in 5% CO_2 and 100% humidity before cell viability test.

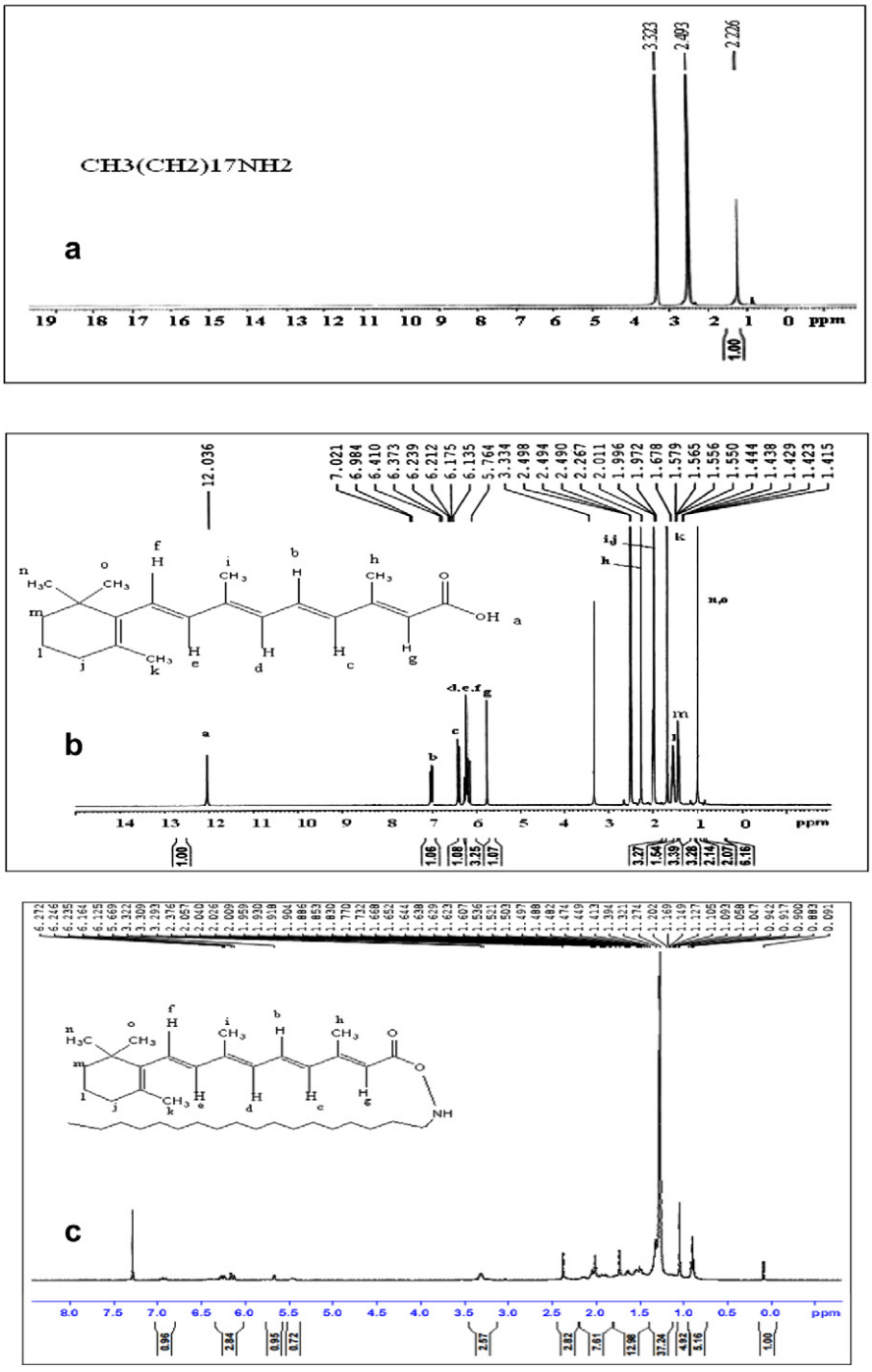


Fig. 4. ^{1}H NMR spectra of a) octadecylamine, b) retinoic acid and c) octadecylamine-retinoic acid conjugate.

Table 2
Physical properties of different NLCs.

Formulation code	Intensity Z-average particle size (nm)	PdI	Zeta potential (mv)	Drug loading efficiency %	RE_{20} %
$CS_{64.5}OA_{15}$	226.5 ± 2.1	0.28	−14.2 ± 2.3	48 ± 2.3	51.53 ± 3.20
$CS_{59.5}OA_{20}$	130.9 ± 2.4	0.42	−26.0 ± 1.9	30 ± 1.7	48.37 ± 1.03
$CS_{54.5}OA_{25}$	135.3 ± 3.9	0.30	−9.1 ± 0.6	34 ± 1.0	48.35 ± 0.78
$C_{64.5}OA_{15}$	119.0 ± 0.9	0.12	−20.8 ± 1.1	30 ± 1.3	35.33 ± 0.89
$C_{59.5}OA_{20}$	102.0 ± 1.3	0.18	−29.0 ± 3.0	30 ± 1.7	32.69 ± 2.00
$C_{54.5}OA_{25}$	105.8 ± 1.9	0.23	−25.1 ± 0.7	38 ± 3.1	37.98 ± 1.98
$CS_{64.5}OC_{15}$	257.4 ± 27.9	0.39	−19.3 ± 1.4	38 ± 1.3	40.59 ± 1.77
$CS_{59.5}OC_{20}$	150.6 ± 8.8	0.44	−18.3 ± 1.4	51 ± 2.9	44.46 ± 2.11
$CS_{54.5}OC_{25}$	284.1 ± 16.8	0.48	−7.7 ± 1.6	29 ± 1.9	51.53 ± 2.82
$C_{64.5}OC_{15}$	105.5 ± 1.0	0.24	−20.1 ± 0.7	38 ± 2.3	32.94 ± 2.26
$C_{59.5}OC_{20}$	80.6 ± 0.5	0.21	−8.6 ± 1.0	22 ± 1.8	10.21 ± 1.31
$C_{54.5}OC_{25}$	110.1 ± 1.5	0.40	−14.5 ± 1.0	36 ± 2.6	48.37 ± 0.87

Considering that cholesterol is fitted to the LDL receptors via Apo-E protein, the cellular viability test was carried out when Apo-E was removed from the cell culture medium to study if the targeting of nanoparticles is through the LDL receptors or just passive penetration of NLCs causes the cellular uptake of drug. Actually we expected that if Apo-E peptide is omitted from the culture medium the intermediate ligand for linking cholesterol NLCs is absent and consequently they are not taken up by the LDL receptors. To show this the cells were treated by the RPMI medium that was without serum, for 48 h.

2.9. Cytotoxicity assessment-MTT assay

After cells were seeded on 96 well plate each raw was treated with: 20 μL of three concentrations (1/2, 1/4, 1/8) of blank NLCs with or without retinoic acid-derivative, 20 μL of three concentrations (1/2, 1/4, 1/8) of dialyzed NLCs loaded with 5-FU with or without retinoic acid-derivative, 20 μL of 8.2 μM solution of free 5-FU (as positive control) to reach the same concentration of 5-FU loaded in NLCs, and 20 μL of culture medium (as negative control). Then the plate was incubated for 72 h and after that 20 μL of MTT was added. After 3 h incubation the cell medium was removed cautiously while not allowing the produced Formazan crystals to pour. Then 180 μL of DMSO was added to the crystals until they were dissolved. Immediately after pippeting each raw was separately analyzed by ELISA method. Cell viability for each sample was calculated using Eq. (4):

$$\text{Cell survival} = \frac{\text{Mean of each group} - \text{mean of blank}}{\text{mean of negative control} - \text{mean of blank}} \quad (4)$$

2.10. Atomic force microscopy (AFM)

Observation of the morphology and particle size of NLCs was performed by an Atomic Force Microscope (Bruker, Nanos 1.1, Germany).

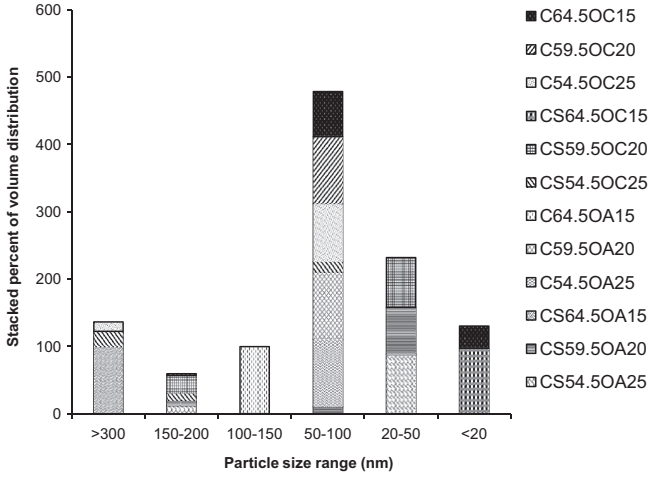


Fig. 5. Stacked percent of volume distribution of different particle size ranges of NLC formulations.

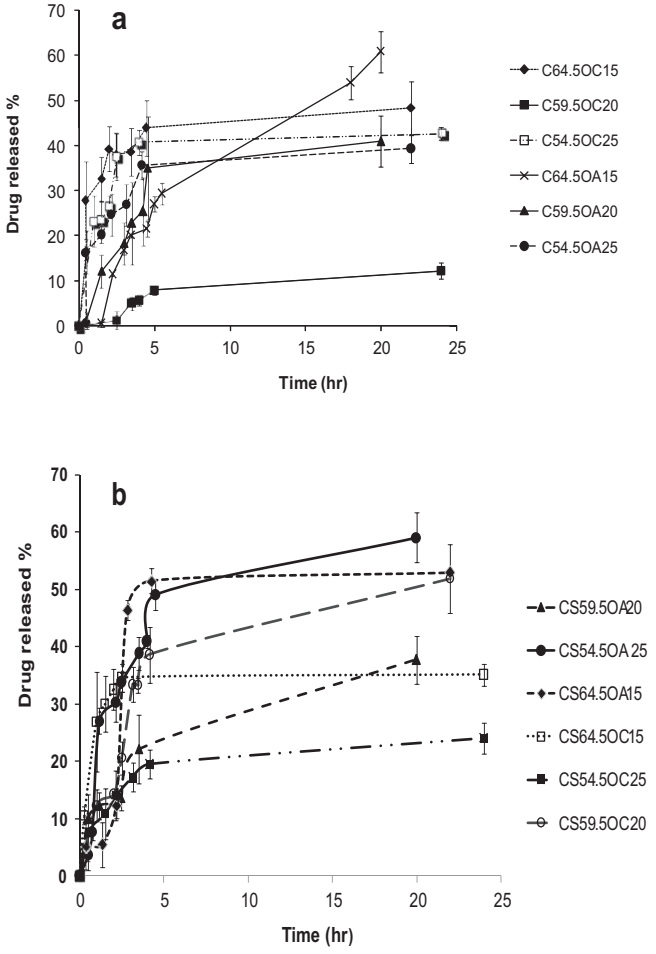


Fig. 6. Release profiles of 5-FU from NLC formulations containing a) cholesterol, b) cholesteryl stearate and different amounts of oils ($n = 3$).

AFM images were obtained by measurement of the interaction forces between the tip and the sample surface. The experiments were done in air at room temperature (25 °C) operating in contact mode. Droplets of the final suspension (20 μL) were deposited onto a small mica disk. After the drop was dried, the contact mode was used at room temperature. The measurements were performed in different sample locations. The mean size of NLCs was obtained by processing the topographical AFM images with the AFM Nanos 1.1 software.

2.11. Statistical analysis

SPSS software version 11.5 was used for all statistical analysis. Univariate analysis of data by a full factorial design was used for comparison between particle size, zeta potential, loading and release efficiency percent of 5-FU in different NLC formulations. The cell culture data were expressed as mean ± SEM and were compared by analysis of variance (ANOVA) test followed by the post hoc test of LSD. A significant level of $p < 0.05$ was considered in all cases.

3. Results and discussion

Considering the cytotoxic effects reported for 5-FU especially on the colorectal carcinoma and other types of malignancies [17,18] by directly promoting apoptosis, we tried to see if it is possible to increase cytotoxic effect of this drug by using retinoic acid containing NLCs targeted to LDL receptors of HT29 cell line.

3.1. Conjugation of retinoic acid and octadecylamine

Chemical reaction of retinoic acid and DCC, NHS and octadecylamine are shown in Fig. 2.

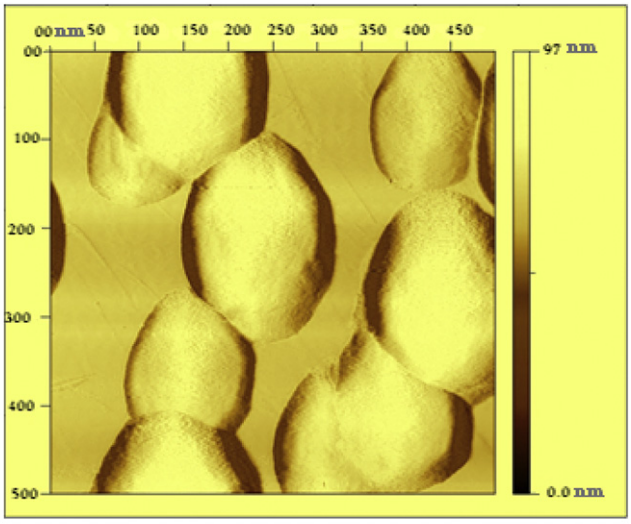
Conjugation of retinoic acid and octadecylamine was confirmed by FTIR spectra as seen in Fig. 3. In this figure the peak of amine group of NH_2 in octadecylamine is seen in 3332 cm^{-1} which is shifted to 3311 cm^{-1} in the product and this means the free NH_2 group is not present in the product. The peak of C=O in retinoic acid that was seen in 1687 cm^{-1} is not present in the product but is seen in 1628 cm^{-1}. This shift in the peak location means the production of an amide bond.

Fig. 4 shows the ^1H NMR spectra of octadecylamine, retinoic acid and their conjugate. ^1H NMR ($CDCl_3$) results were as follows: δ (ppm): 6.92 (1H, dd, Hb) 6.25 (2H, m, Hc, Hd), 6.12 (2H, m, Hc, Hf), 5.66 (1H, s, Hg), 5.45 (1H, br5, −NH−), 2.4 (3H, s, Hh), 2.05 (5H, m, Hi, Hj), 1.72 (3H, s, Hk), 1.65 (2H, m, HL), 1.55 (2H, CH_2−CH_2−NH−C=O), 1.45 (2H, m, Hm), 1.32 (2H, m, CH_2−CH_2−NH−C=O), 1.25–1.31 (30H, m, CH_2 Stearyl amine), 2.04 (2H, t, $J = 6$, Hj), 2 (3H, s, Hi), 1.05 (6H, s, Hn, Ho), 0.9 (3H, m, CH_2−CH_3).

3.2. Physical properties of NLCs

After the preliminary study which showed the most effective variables on the physicochemical properties of NLCs, different formulations were prepared from 5-FU by two types of lipids and two types of oils each in three different percentages by emulsification-solvent evaporation method containing retinoic acid-octadecylamine conjugate. Table 2 shows the physical properties of NLCs.

Different particle sizing techniques report primary results based on number, volume, weight, surface area, or intensity. As a general rule specifications should be based in the format of the primary result for a given technique. Laser diffraction generates results based on volume distributions and any specification should be volume based. Likewise, an intensity basis should be used for DLS specifications, volume for acoustic spectroscopy, and number for image analysis. Table 2 shows the intensity Z-average values of particles size of different NLCs while the results of stacked percent of volume distribution in each particle size range for different NLCs are shown in Fig. 5. These data are according to volume and not intensity measurements. As this figure shows the most popular volume distribution for all formulations is seen between 50 and 100 nm. $CS_{64.5}OA_{15}$ NLCs have the highest volume distribution at size range of more than 300 nm. Considering this figure it may be concluded that the higher range of volume particle size distribution in NLCs prepared by combination of CS and OA is higher than those prepared by cholesterol probably due to the higher molecular weight of cholestryl stearate compared to cholesterol (653.12 g/mol versus 386.7 g/mol for cholesterol) [19]. The other reason is that cholestryl stearate has no surfactant characteristic compared to cholesterol [20] which reduces the surface tension and consequently decreases the particle size distribution [21]. As Table 2 indicates almost all NLCs have polydispersity index lower than 0.3 which is quite acceptable and just NLCs of $CS_{64.5}OC_{15}$, $CS_{59.5}OC_{20}$, $CS_{54.5}OC_{25}$, $CS_{59.5}OA_{20}$ and $C_{54.5}OC_{25}$ have PdI>0.3. It seems that the combination of octanol and cholesteryl stearate causes the higher PdI than the mixture of oleic acid and cholesterol. This may be due to higher viscosity of the



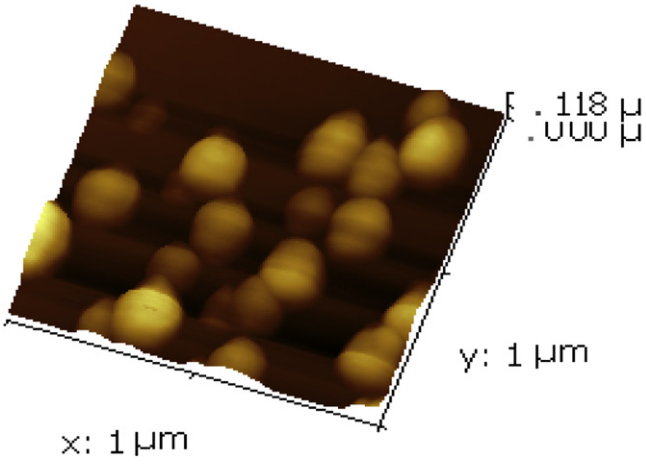


Fig. 7. Atomic force microscopy of NLCs of 5-FU.

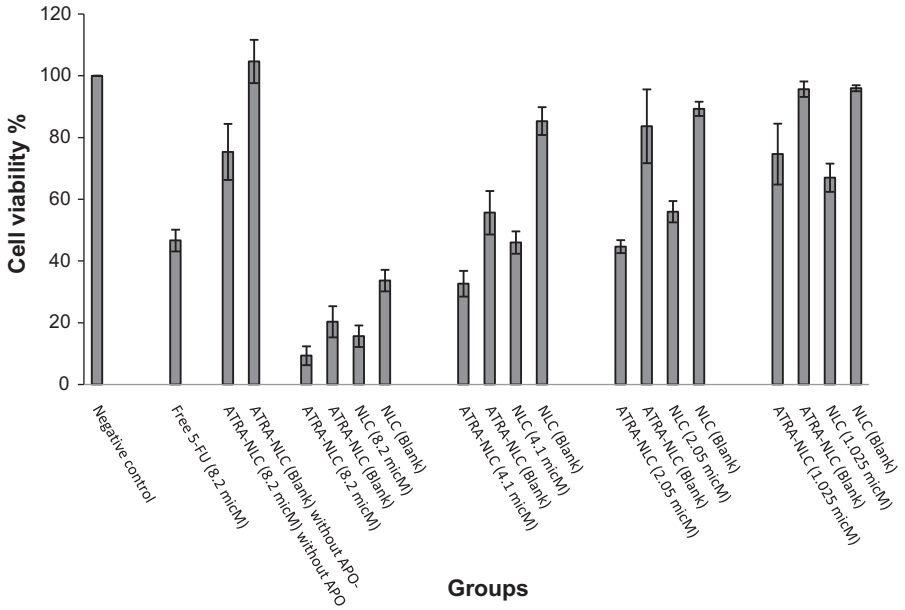


Fig. 8. Percent of viable cells of HT29 cells after treatment with different concentrations of 5-FU loaded NLCs with or without retinoic acid conjugate in comparison with free 5-FU and also in presence or absence of Apo-E by MTT assay ($n = 3$).

combination of CS and OC than the mixture of C and OA which causes continuous increase of the surface tension and production of more particles with different sizes, their aggregation, size growth and making heterogeneous systems [22,23].

Zeta potential is a key factor in stability of nanoparticles. Zeta potential of NLCs changed between -29.0 ± 3.0 mV ($CS_{59.5}OA_{20}$) and -7.7 ± 1.6 mV ($CS_{54.5}OC_{25}$) (Table 2). All NLCs had negative charge. Concerning the effect of lipid type on zeta potential, it was noticed that the absolute value of zeta potential was increased when the lipid type changed from cholestryl stearate to cholesterol possibly due to reduction in particle size which increases the negative charge density of charges on NLCs [24]. However, the differences were not significant between $CS_{59.5}OA_{20}$, $C_{59.5}OA_{20}$ and $C_{54.5}OA_{25}$ ($P > 0.05$).

Changing the oil type from oleic acid to octanol had a reverse effect on zeta potential may be because of negative zeta potential of oleic acid which has a carboxylic acid group compared to octanol [25]. However, the differences between $CS_{64.5}OC_{15}$, $CS_{59.5}OC_{20}$ and $C_{64.5}OC_{15}$ were not significant ($P > 0.05$).

The greatest loading efficiency of 5-FU was observed in $CS_{59.5}Oc_{20}$ (Table 2) which may be due to higher partitioning of drug to this lipid/oil mixture. It was noticed that by changing cholestryl stearate to cholesterol and octanol to oleic acid the loading efficiency percent reduces due to the reduction of the particle size of NLCs. Increasing the oil content of NLCs from 20 to 25% caused many imperfections offering space to accommodate the drug (Table 2).

3.3. In vitro drug release

5-FU release profiles from different NLCs are seen in Fig. 6. As this figure shows after 20 h of release test about 35–45% of the drug is released by a sustained behavior.

As it can be seen from 5-FU release profile from NLCs, there is an initial burst diffusion of drug which is attributed to the drug molecules adsorbed onto the surface of NLCs which can be released instantaneously by contacting to the release medium. This follows by a sustained release profile which is attributed to the release of drug from the core of NLCs. The diffusion of drug out of the core of NLCs is affected by the amount of the drug loading capacity. Table 2 shows that changing the lipid type from cholestryl stearate to cholesterol decreases the RE_{20} which may be because of the hydrogen bond between the fluorine atom of 5-FU and OH of the cholesterol. This bond is more probable than hydrogen bond between the fluorine of 5-FU and O atom of ketone group of cholestryl stearate. Octanol reduces the RE_{20} comparing to oleic acid probably because of the enlargement of particle size of NLCs and consequently reduction of their surface area. Increasing the oil % from 20 to 25% increased the RE_{20} due to reduction of the viscosity of the NLCs and so making them leaky.

The optimum formulation chosen for further studies was NLCs of $C_{54.5}OA_{25}$ due to its logical particle size (105.8 nm), good stability due to a relative high zeta potential (−25 mV), an acceptable drug loading efficiency and release efficiency of 38%.

3.4. AFM

Fig. 7 shows the AFM contact mode atomic force microscopy carried out on optimized NLCs. Imaging shows that the NLCs are spherical and round shaped and are dispersed within the dispersion as discrete particles of about 100 nm in diameter and show little or no aggregation. Photon correlation spectroscopy similarly demonstrates the particle size of NLCs.

3.5. Cytotoxicity assessment-MTT assay

The anti-proliferative activity of cholesterol NLCs containing 5-FU and retinoic acid conjugate (IC50 = 4.5 μM) was smaller than free 5-FU in comparable concentration of 8.2 μM (IC50 = 7.6 μM). Cholesterol NLCs containing 5-FU and the conjugate of retinoic acid showed cell survival of about one fifth of free 5-FU and the NLCs without retinoic acid conjugate showed about half cytotoxicity in respect to free 5-FU that showed 46% cell viability.

Fig. 8 shows the results of cell viability by MTT assay. As this figure shows free 5-FU in concentration of 8.2 μM has caused just about 64% cytotoxicity compared to the same concentration of 5-FU

loaded in NLCs containing the conjugate of retinoic acid-octadecylamine that showed about 91% cytotoxicity and NLCs without the conjugate but the same concentration of free drug that showed about 84.5% cytotoxicity. The results show that the blank NLCs containing retinoic acid conjugate but without drug has shown greater cytotoxicity even compared to free 5-FU ($p < 0.05$). This may indicate the cytotoxicity of retinoic acid conjugate on HT29 cell line in cholesterol containing NLCs which is well targeted to the LDL receptors of these cells. Lower concentrations of 5-FU in test groups showed less cytotoxicity than the concentration of 8.2 μM ($p < 0.05$) but the same trend as the groups treated with 8.2 μM is seen in 4.1 and 1.025 μM concentration.

Apolipoprotein E (Apo-E) is one of protein constituents of plasma lipoproteins that serve various functions, including regulation of the metabolism of several different lipoproteins [15,26]. It is a constituent of liver-synthesized very low density lipoproteins (VLDL), which function primarily in the transport of triglycerides from the liver to peripheral tissues, and of a subclass of high density lipoproteins (HDL), which participate in cholesterol redistribution among cells. In addition, Apo-E becomes a major protein constituent of intestinally synthesized chylomicrons, which transports dietary triglyceride and cholesterol. A major of physiological role for Apo-E in lipoprotein metabolism is its ability to mediate high-affinity binding of Apo-E-containing lipoproteins to the low density lipoprotein (LDL) receptor [26–28]. Lipoprotein binding to the receptors initiates the cellular uptake and degradation of the lipoproteins, which leads to the use of the lipoprotein cholesterol in the regulation of intracellular cholesterol metabolism. The normal plasma concentration of this protein is 5 mg/dL. Omitting the serum from the cell culture of HT29 cells caused cell viability to increase from 9.3% to 75.3% in NLCs containing 8.2 μM 5-FU and the conjugate of retinoic acid-octadecylamine. Therefore, when APO-E that is necessary for transferring cholesterol to LDL receptors is absent the cell viability increases near 8 fold that means targeting of cholesterol NLCs to colon cancer cells has been done by LDL receptors and when APO-E is removed from the serum in the medium of cell culture the targeting to LDL receptors is blocked and cell viability is increased.

Hegg et al. [29] also used a cholesterol-rich microemulsion that binds to LDL receptors and is taken up by malignant cells over-expressing these receptors. This system was further used in Hodgkin's and non-Hodgkin's lymphoma by Pinheiro et al. [30] who resulted that the cholesterol-rich microemulsion loaded with etoposide is suitable for patient use.

4. Conclusions

DCC and NHS can mediate ATRA conjugation to octadecylamine by making an amide linkage. NLC formulations of 5-FU and the new conjugate were prepared in a cholesterol base to fit with LDL receptors of HT29 cells of colon carcinoma. The formulation of $C_{54.5}OA_{25}$ consisting of 54.5% of cholesterol and 25% of oleic acid, 20 mg 5-FU, 0.5% of lecithin, 10% of PEG 40 stearate was selected as the optimum NLC formulation due to its logical particle size (105.8 nm), low polydispersity index (PdI = 0.23), good stability due to relatively high zeta potential (−25 mV), an acceptable drug loading efficiency and release efficiency of 38%. The presence of ATRA conjugate along with 5-FU loaded in cholesterol NLCs targeted to LDL receptors can enhance cellular cytotoxicity of 5-FU. So that when 5-FU was loaded in the NLCs containing retinoic acid conjugate, the cytotoxicity was nearly 2 fold of NLCs just loaded with 5-FU and more than 5 fold of free 5-FU in human colon cancer cell line of HT29 significantly. We postulate that 5-FU and ATRA conjugated octadecylamine loaded in cholesterol NLCs that are targeted to LDL receptors may serve as a potential strategy to improve the treatment outcome of human colorectal carcinoma. Further studies are needed to optimize the ATRA conjugate concentration in presence of 5-FU for obtaining maximum synergistic effect in reducing needed dose of 5-FU. The results should be checked *in vivo* to confirm the promising results on the cell culture.

Declaration of interest

The authors report no conflicts of interest.

Acknowledgments

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