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Hyaluronic acid-decorated liposomal nanoparticles for targeted delivery of 5-fluorouracil into HT-29 colorectal cancer cells

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Author Contribution:

B. M. developed the hypotheses for this study. B. B. planned the methodology. B. B. and B. M. organized, supervised and were responsible for the course of the project and the article. B. B. provided personnel, environmental, and financial support, tools, and instruments that were vital for the project. B. M. and A. M. provided biological materials, reagents, and referred patients. B. M., F. A. G, S. A, M. G, R. Y, and S. S were responsible for the execution of the experiments, patient follow up, data management and reporting. B. B, G. D. and M. H were responsible for the interpretation and presentation of the results. B. B. was responsible for overall supervision of this study. B. M. was responsible for the construction of the whole or body of the manuscript. M. H, and B. B reviewed and edited the article before submission.

Data availability statement

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of interest

All the authors declare no conflict of interest and Dr Hamblin is on the following Scientific Advisory Boards:

Transdermal Cap Inc, Cleveland, OH

BeWell Global Inc, Wan Chai, Hong Kong

Hologenix Inc, Santa Monica, CA

LumiThera Inc, Poulsbo, WA

Vielight, Toronto, Canada

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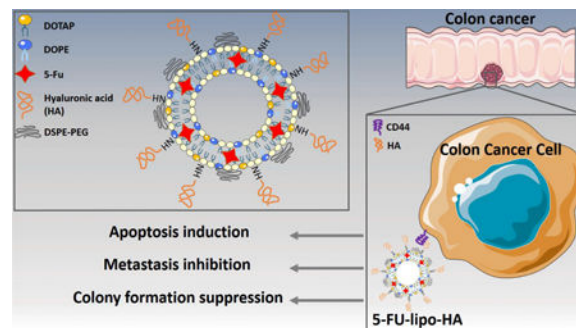
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Abstract

The use of liposomes as drug carriers improves the therapeutic efficacy of anti-cancer drugs, while at the same time reducing side effects. Hyaluronic acid (HA) is recognized by the CD44 receptor, which is over-expressed in many cancer cells. In this study, we developed HA-modified liposomes encapsulating 5-fluorouracil (5-FU) and tested them against a CD44 expressing colorectal cell line (HT29) and a non-CD44 expressing hepatoma cell line (HEPG2). The average size of 5-FU-lipo and 5-FU-lipo-HA nanoparticles were 112 ± 28 nm and 144 ± 77 nm respectively. The MTT assay showed selective cancer cell death depending on CD44 expression in a time-dependent manner. Apoptosis assays and cell cycle analysis indicated that G0/G1 arrest occurred. The colony formation study revealed that cells treated with 5-FU-lipo and 5-FU-lipo-HA had reduced colony formation. QRT-PCR study showed that the oncogenic mRNA and miRNA levels were significantly reduced in the 5-FU-lipo-HA treated group, while tumor suppressors were increased in that group. We suggest that optimal targeted delivery and release of 5-FU into colorectal cancer cells, renders them susceptible to apoptosis, cell cycle arrest and decreased colony formation.

Graphical Abstract



Keywords

Colorectal cancer; 5-Fluorouracil; Hyaluronic acid; CD44; Liposomes; Apoptosis; Clonogenicity; Cell cycle; microRNA

1. Introduction

Colorectal cancer is one of the most common cancers in the world, although its prevalence is higher in developed countries (Janout & Kollárová, 2001; Wilmink, 1997; Winawer et al., 1997). Surgery, Chemotherapy, Radiotherapy are common treatment methods for colorectal

cancer. Although chemotherapy agents are widely used for many types of cancers, their efficacy and selectivity are limited due to low bioavailability, poor solubility, development of resistance, and non-specific bio-distribution between cancer and normal cells/tissues (Abedi-Gaballu et al., 2018; Arnold et al., 2017; Singh, Palombo, & Sinko, 2008; Watanabe et al., 2012). For example, 5-fluorouracil (5-fluoro-2, 4-pyrimidinedione or 5-FU) is one chemotherapeutic agent that was first reported for colorectal cancer in 1960. It is still widely used to manage colorectal cancer. 5-FU is an antimetabolite and an analogue of the Pyrimidine, Uracil that disrupts DNA replication, but is metabolized through identical pathways to Uracil (Arias, 2008). In addition, 5-FU is an effective treatment for advanced colorectal cancer with poor prognosis and improves patient survival and quality of life (Fanciullino et al., 2007; Zhang, Yin, Xu, & Chen, 2008). Furthermore, both oral administration or intravenous injection of 5-FU have problems such as rapid removal from the blood due to a short biological half-life, and rapid catabolism in the liver leading to the need for larger doses that cause side effects, such as diarrhea, mucositis, hand-foot syndrome and leucopenia (Cheng et al., 2012; Othman et al., 2017). To overcome these deficiencies, drug delivery systems have been developed to deliver 5-FU. Their main purposes are: (a) the protection of 5-FU from the degradation within the body before reaching the desired target sites (Abedi-Gaballu et al., 2018; Ghaffari et al., 2018); (b) to preserve the normal cells/tissues from non-specific cytotoxicity; (c) to allow controlled 5-FU released at predictable rates; (d) to carry a suitable amount of 5-FU to overcome poor drug permeation; (e) to deliver 5-FU to the target tumor site by a passive targeting process; (f) to deliver 5-FU by an active targeting process. The passive targeting process is a strategy whereby nanocarriers that are loaded with drugs can accumulate into specific tissues, relying on the specific properties of the delivery system and the particular pathophysiology of the disease to be treated. This process is well known as the “enhanced permeability and retention” (EPR) effect. Active targeting is, however, another type of delivery in which the nanocarrier is covalently attached to specific ligands, such as hyaluronic acid (HA), folic acid (FA), or transferrin (Tf). These molecular ligands can be recognized by cognate receptors, which are overexpressed on the target tissues or cells. Recognition of ligands by their receptors leads to the gradual accumulation of functionalized nanocarriers at the target site, which eventually enhances the internalization of drugs via increasing ligand-receptor mediated cellular uptake (Adams et al., 2001; Kannagi, Izawa, Koike, Miyazaki, & Kimura, 2004). For instance, HA is a naturally occurring polysaccharide that can specifically bind to the CD44 receptor that is overexpressed by several types of cancer cells. HA has been widely employed as an active targeting moiety for various drug delivery systems (Hayward, Wilson, & Kidambi, 2016). Several nanocarriers, such as solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC), polymeric nanoparticles, liposomes, graphene oxide, gold nanoparticles etc, have been developed and used as drug delivery systems. Most of these nanoparticles have been examined for delivery of 5-FU for cancer therapy via passive or active targeting strategies (Amasya, Badilli, Aksu, & Tarimci, 2016; Barzegar Behrooz et al., 2017; Paliwal, Paliwal, Agrawal, & Vyas, 2016; Safwat, Soliman, Sayed, & Attia, 2016; Thomas et al., 2011; Vinothini & Rajan, 2017). Among the mentioned nanoparticles, liposomes have been considered as a potential vehicle for therapeutic agents and imaging agents (Kim, 2016; Malam, Loizidou, & Seifalian, 2009). The present study aimed to prepare liposomes-decorated with HA as nanoparticles for targeted delivery of 5-FU, and to evaluate its

anticancer effects on proliferation, apoptosis, cell cycle, and colony formation of CD44 expressing HT-29 colorectal cancer cells.

2. Materials and methods

2.1. Materials

1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-dioleoyl-sn-glycerol-3 phosphoethanolamine (DOPE) and 1-ethyl-3-[3-dimethyl aminopropyl] carbodiimide hydrochloride (EDAC) were purchased from CAIMAN. DSPE-PEG₂₀₀₀ was provided from Lipoid GmbH (Ludwigshafen Germany). 5-Fluorouracil (2, 4-Dihydroxy-5-fluoropyrimidine, PubChem CID: 24278439), 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) and hyaluronic acid (HA) were obtained from Sigma Aldrich (St. Louis, Missouri, United States). Fetal bovine serum (FBS), penicillin-streptomycin (10,000 U/ml), Roswell Park Memorial Institute 1640 (RPMI), 2-(4-amidinophenyl)-6-indolecarbamidine dihydroformaldehyde (DAPI) and trypsin-EDTA (0%25) were provided from Gibco. Dimethyl sulfoxide (DMSO) and phosphate-buffered saline (PBS) was obtained from Merck Company, Germany.

2.2. Methods

2.2.1. Preparation of liposomes and liposome decoration with HA—Liposomal nanoparticles were prepared by incorporating DOPE, DOTAP, and des PEG₂₀₀₀ at a 1:1:1.5 molar ratio using the thin layer film hydration method, which was previously described (Hayward et al., 2016). Briefly, 4.46mg DOPE (600 μ M), 4.19 mg DOTAP (600 μ M) and des PEG₂₀₀₀ (9 μ M) were dissolved in 10 ml of chloroform, then 1mg 5-FU was added to the lipid solution. In the next step, the organic solvent was removed using a rotary evaporator under vacuum at 37 °C. The resulting thin film was hydrated with 1ml HEPES buffer and sonicated in order to achieve size reduction of the liposomes. After preparing the liposomes, the amine groups of the DOPE phospholipids were covalently conjugated to the carboxylic acid groups of EDC-activated HA to form an amide bond according to a previously described method with minor modification (Zarebkohan et al., 2015). To achieve this, 7mg HA was dissolved in 2.5 ml in water and preactivated with 3 mg EDC for 2h at 37 °C, followed by titration by 1 N HCL to the final pH 4. Next, the activated HA was mixed with prepared formulation liposomes (1:1 molar ratio liposomes: HA).

2.2.2. Size, Zeta Potential, and Morphology of 5FU-Loaded HA-liposomes—The particle size of the liposomes was measured by dynamic light scattering technique (DLS) using a particle size analyzer (Malvern Instruments Ltd, UK) at room temperature. All samples were diluted 10 times with deionized water. In addition, the morphology of 5-FU loaded bare liposome (5-FU-lipo) and 5-FU loaded HA decorated liposome (5-FU-lipo-HA) nanoparticles was investigated using a scanning electron microscope (SEM) (MV2300, Vega Tescan, Czech Republic).

2.2.3. Encapsulation efficiency and 5-FU loading measurement—Encapsulation efficiency (EE) of 5-FU in liposomes and liposomes-HA was measured using the following equation $EE = W_o/W_i \times 100\%$, where W_o and W_i are the weight of the drug before and after

separation respectively. To evaluate the weight of non-entrapped 5-FU, 1 ml of the formulation was filtered via Amicon® Ultra-Centrifugal filters (MWCO 30 KD, Millipore, USA) for 15 min at 3000rpm. The concentration of unloaded 5-FU was measured via ultraviolet-visible spectroscopy (UV-vis) method. The drug loading (DL) ratio was calculated as the weight of encapsulated 5-FU divided by the weight of lipids.

2.2.4. *In vitro* 5-FU release assay: The dialysis bag diffusion technique was employed to evaluate the rate of 5-FU release from 5-FU-lipo and 5-FU-lipo-HA nanoparticles. Briefly, 2ml of each 5-FU loaded liposomes and liposome-HA nanoparticles were separately diluted with 2 ml PBS and then placed into a dialysis bag (MWCO 20 kDa) and immersed into 25 ml of PBS (10 mM, pH7.4 and 5.5), containing 1% (v/v) Tween 80 as a surfactant. The release system was kept at 37°C and 300 rpm. At a predetermined time interval, 1 ml of the samples were withdrawn and replaced with fresh buffer solution. The amount of released 5-FU was measured using UV-Vis spectrophotometry at 266nm.

2.2.5. Cell culture—Human colorectal cancer cells (HT-29) (CD44^{high}) and HEPG2 (CD44^{low}) hepatoma cells were purchased from the Pasteur Institute (Tehran, Iran). The cells were maintained in RPMI 1640 with 10% FBS (GIBCO, Carlsbad, CA, USA) and cultured at 37C with 5% CO₂. The cells were sub-cultured 24–48 h later with an initial concentration of 4×10⁴ cells/ml and used in the logarithmic phase in all experiments.

2.2.6. *In Vitro* Cellular Uptake—To confirm and compare the internalization of bare liposomes and liposome-HA nanoparticles into the HT-29 and HEPG2 cells, rhodamine B (RhoB) was used as a fluorescent marker according to a previous report (Sabzichi et al., 2017). *In vitro* cellular uptake was performed via encapsulation of RhoB (as a model drug) into the liposomes. To separate, the free RhoB from entrapped RhoB, a purification process was applied using an Amicon® tube (Ultra-30kDa molecular weight cut-off membrane, Millipore, Germany). The fluorescent liposomes were placed into the upper chamber of Amicon® Falcon and centrifuged at 5000 rpm for 15 min. Unloaded RhoB molecules were passed through the filter and gathered in the lower chamber of the Amicon tube. The upper chamber that comprised pure fluorescent liposomes was diluted with PBS and then centrifuged again five times to ensure the total removal of non-entrapped RhoB from the nanoparticles. The cell lines were seeded in six-well plates with 4×10⁵ cells per well for 24 h. After that, the cells were treated with fluorescent dye-labelled liposomes for 1, 3, 6, 12 and 24 hours; they were washed three times with sterile PBS and collected by centrifugation at 1000 rpm for 10min. Finally, quantitative cellular uptake of the formulations was measured using flow cytometry. Moreover, nuclear staining using DAPI was done to image qualitative cell uptake using fluorescence microscopy at 6 and 12 h.

2.2.7. Cell viability assay—The MTT (3-[4, 5-dimethyl-2-thiazolyl]- 2, 5 diphenyltetrazolium bromide) assay was used to measure cell viability after incubation with free 5-FU, 5-FU-lipo and 5-FU-lipo-HA [23]. Briefly, 15×10³ HT-29 and HEPG2 cells at passage 3 were seeded into each well of a 96-well plate. Then, the cells were treated with different concentrations (10–70 µg/ml) of free 5-FU and its nanoformulations. After 24, 48 and 72 h, MTT solution (2mg/ml PBS) was added to each well. The incubated medium was

replaced with 200 μ l of DMSO after 4 h. Then, the absorption of the plates was read at a wavelength of 570 nm using TCAN Elisa Reader.

2.2.8. DAPI staining—DAPI staining is a method that reveals chromatin fragmentation. HT-29 cells were seeded into six-well plates, including 12mm cover-slips at 5×10^5 cells per well. The cultivated cells were treated with free 5-FU, blank liposomes, 5-FU-lipo and 5-FU-lipo-HA over 48 h. Then, the cells were fixed with 5% formaldehyde for 3 h and permeabilized with 0.1% Triton X-100 in PBS for 5 min. Following that, they were stained with DAPI 0.1% for 10 min and finally monitored by a fluorescence imaging system (Cytation 5, Biotek, USA).

2.2.9. Determination of Apoptotic Cells by Flow Cytometry—HT-29 cells at a density of 4×10^5 cells per well were seeded and incubated overnight in medium containing the IC₅₀ concentrations of free 5-FU, 5-FU-lipo and 5-FU-lipo-HA at 37°C for 48 h. Then, they were detached by diluted trypsin three times, washed twice in PBS, and re-suspended in binding buffer at ice-cold temperature. Finally, the cells were detached and stained using an Annexin V/PI apoptosis detection kit (Exbio, Czech Republic) according to the manufacturer's protocol. Then, the rate of the apoptosis was analyzed by flow cytometry (MACS Quant 10, Miltenyi Biotech GmbH). The achieved data were analyzed by using FlowJo software package (Treestar, Inc., San Carlos, CA).

2.2.10. Cell cycle assay—HT-29 cells were cultivated at a density of 2×10^6 cells/well in 6-well plates in 4 groups: blank liposome, free 5-FU, 5-FU-lipo and 5-FU-lipo-HA. The cells were harvested 48 h after the incubation, fixed with 50% ethanol, treated with 5 mg/ml RNase A (Bioneer, Daedeok-gu, Daejeon, Korea), stained with 50 mg/ml propidium iodide, and subsequently analyzed by flow cytometry for DNA synthesis and cell cycle status (MACS Quant 10, Miltenyi Biotech GmbH).

2.2.10. Clonogenicity Assays—The colony formation assay was performed to investigate the effect of the blank liposome, free 5-FU, 5-FU-lipo and 5-FU-lipo-HA on the clonogenicity of HT29 cells. 5×10^3 HT29 cells were seeded in 6-well plate after incubation and then followed up for two weeks. The colonies were fixed, stained with crystal violet, and then photographed. They were counted manually and plotted as the number of colonies.

2.2.11. qRT-PCR—Total cellular RNA were isolated by the RiboEX reagent after 48 h treatment from 1×10^6 HT-29 cells (GeneAll, GeneAll biotechnology, Seoul, Korea). Complementary DNA (cDNA) was synthesized from 1 μ g of total RNA via thermal cyclor system (Bio-Rad, Hercules, CA). qRT-PCR was performed with the Light Cycler 96 instrument (Roche Diagnostics, Mannheim, Germany) using a 2X SYBR green master mix.

The primer sequences were as follows in Table 1. The evaluation of ALDH, KLF4, HMGA2, C-Myc, Nanog, CD44, Kras, miR-181a, miR181b, Let-7, miR-21, miR-155, miR-200c and miR-34c were carried out using an initial denaturation step at 94 °C for 10 min, followed by 45 cycles at 94 °C for 10 sec, and then 60 °C. Beta-actin and U6 were used as reference gene for mRNA and miRNA. The relative expression of miRNA and mRNA measured by $2^{-\text{ct}}$.

2.2.12. Statistical analyses—All of the obtained parameters were expressed as the mean \pm standard deviation (SD). All the experiments were repeated three times independently. Statistical analyses were carried out using a one-way and two-way analysis of variance (one-way and two-way ANOVA) with Dunnett's multiple comparisons test (Prism, version 6.0, GraphPad software, INC). A p-value less than 0.05 was considered significant.

Results and discussion

3.1. Preparation and characterization of nanoparticles

To investigate the possibility of using HA as a targeting ligand for colorectal cancer, liposomal nanoparticles were firstly prepared using the thin layer film hydration method. Empty liposomes and HA-decorated liposomal nanoparticles were prepared by incorporating DOTAP: DOPE: des PEG at a 1:1:1 molar ratio and DOTAP: DOPE: des PEG: HA with 1:1:1:0.5 molar ratio, respectively. The optimized ratios of phospholipids of the nanoparticles were obtained according to pilot study results (data not shown). The conjugation of HA to DOPE in the liposomal nanoparticles was done using EDC to facilitate amide linkage formation between the amine group of DOPE and the carboxyl group of the HA and this was confirmed by FTIR spectroscopy. The conjugation of HA with DOPE was confirmed by IR bands at 729–500 cm^{-1} out of plane N-H wagging, 1644 cm^{-1} aromatic C=C stretch of HA, and 3454 cm^{-1} N-H stretch of amide. Therefore, the presence of these bands in HA and DOPE IR spectrum (Figure 1 a and b) indicated that DOPE-HA conjugation had been formed. This result is in accordance with several previous reports showing conjugation of HA to DOPE through covalent amide bond formation (Hayward et al., 2016; Taetz et al., 2009).

The nanoparticles were prepared and then characterized by measuring their size, zeta potential and PDI. It was reported that the *in vivo* fate of nanoparticles can be influenced by several parameters such as their size and surface charge. The particle size of bare liposomes and HA-decorated liposomes were 112 \pm 28 and 144 \pm 77nm with acceptable PDI values of 0.15 and 0.2, respectively (Figure 2 a, b c, d). Therefore, surface functionalization of liposomal nanoparticles with HA increased the particle size in agreement with a report by Ravar et al in 2016 (Ravar et al., 2016). They showed that HA, as a hydrophilic molecule, is able to uptake water and swell, increasing the size several folds higher than the original size in aqueous media (Ravar et al., 2016). They concluded that the increased particle size of HA-modified liposomes is related to the presence of HA. On the other hand, the measured size for the nanoparticles should be suitable for 5-FU delivery due to their appropriate size. Previously, it was reported that nanoparticles with an average size of 70 to 200 nm have longer blood circulation time than free drugs, because they are able to effectively escape clearance by the reticuloendothelial system (RES), whereas if the particle size was larger than 200nm, they can be eliminated by the RES (Ossipov, 2010; T. Yang et al., 2007). The zeta potential was found to be +4mV and -19mV for bare liposomes and HA-decorated liposomes, respectively. These results indicated that the positive charge of bare liposomes had been converted to a negative charge in HA-decorated liposomes due to the presence of excess carboxylic acid of HA on the surface of the liposomes. Overall, these results

confirmed the surface functionalization of liposomal nanoparticles with HA (Hayward et al., 2016).

The % value for EE and DL were found to be $92\pm 4.54\%$ and $4.6\pm 0.22\%$ for bare liposomal nanoparticles, respectively, while these values were calculated to be 91% and 4.21% for HA-liposomes. These results showed that there was no significant difference between EE and DL % for both bare and HA-modified liposomes consistent with several previous studies (Assanhou et al., 2015; Han et al., 2016; Paliwal et al., 2016). SEM was employed to monitor the morphology of nanoparticles. The result showed that both bare and HA-decorated liposomes had a spherical shape (Figure 3 a and b).

The amount of 5-FU released from liposomal nanoparticles (bare liposomes and HA-liposomes) was measured after complete separation of non-entrapped 5-FU from the liposomes. Figure 4 shows that the release of 5-FU from HA-liposomes was about 40% after 10 h in an acidic environment (pH 5.5 similar to the endosomal pH). Furthermore, at physiological (pH 7.4, similar to blood pH), 5-FU release was about 19% over 10 h. Moreover, in the case of bare liposomes after 2h, 5-FU release at pH 5.5 and pH 7.4 was about 40% and 20%, respectively. According to these results, 5-FU release from bare liposome more rapid than that from HA-decorated liposomes. Therefore, surface modification of liposomes with HA decreased 5-FU release when compared with bare liposomes. This can be explained by the surface covering of the liposomes with HA, which reduces the bilayer fluidity or membrane permeability. Our release profile results are consistent with recent reports. In one study, Tian et al 2018 studied the DOX release profile from bare liposomes and HA-modified liposomes. Their results showed that DOX release from bare and HA-modified liposomes was prolonged and that DOX release from bare liposomes was about two-fold higher in comparison to HA-modified liposomes (Tian, Liu, Li, Garamus, & Zou, 2018). Elsewhere, Qu et al 2015 could successfully modify the surface of nanostructured lipid carrier (NLC) nanoparticles with HA to allow combination dual delivery of 5-FU and cisplatin. Their results showed that NLC modified with HA had significantly lower 5-FU and cisplatin release than the plain NLC nanoparticles (Qu et al., 2015).

3.2. Cellular uptake of liposomal nanoparticles

The quantitative cellular uptake of bare liposomes and HA-liposomes was measured by flow cytometry on HT-29 cells, which overexpress CD44, and HEPG2 cells, which have low expression of CD44. The fluorescent intensity of RhoB was proportional to the amount of the liposomes that were internalized. HT-29 and HEPG2 cells were incubated at 1, 3, 6, 12 and 24 h with RhoB loaded bare liposomes and HA-liposomes. The results showed that both nanoparticles were successfully internalized into HT-29 cells in a time-dependent manner, but RhoB/HA-liposomes had a significantly higher uptake ($P < 0.001$) than RhoB/ bare liposomes. However, the HEPG2 cells incubated with lipo/RhoB and lipo-HA/RhoB also showed cell uptake. However, there was no significant ($P > 0.005$) difference in HEPG2 cell uptake between targeted (lipo-HA/RhoB) and non-targeted (lipo/RhoB) nanoparticles. The results showed that liposomes decorated with HA had cellular uptake related to CD44 receptors that mediated the endocytosis process (active targeting). It should be mentioned

that CD44 receptors are overexpressed on some target cells; therefore, conjugation of HA as their cognate ligand on the surface of liposomes not only increases the specific binding of HA-liposomes to CD44 receptors but also can increase cellular uptake (Paliwal et al., 2016). In a similar study, Hayward et.al compared DOX loaded plain liposomes and HA surface-modified liposomes internalization into glioblastoma multiform cells by flow cytometry. The result of this study showed that the cell uptake of DOX/HA-liposomes was significantly higher than DOX/bare liposomes related to CD44 receptor-mediated endocytosis (Hayward et al., 2016). To further verify cellular uptake of the formulations, the qualitative uptake of RhB loaded bare liposomes and HA-liposomes were monitored by fluorescence microscopy. The studied cells were treated with RhoB loaded liposomes and HA-liposomes for 6 and 12 h. Figure 5 shows that the red colour (RhoB) intensity was increased from 6 to 12 h, demonstrating the time-dependent accumulation of the nanoparticles into the cells. The observed red colour in the cytoplasmic area of the cells revealed that the nanoparticles could successfully enter the cells, while the difference between targeted and non-targeted nanoparticles was shown by the intensity of the red colour. Lipo-HA/RhoB nanoparticles had a stronger fluorescent intensity when compared with RhB/liposomes in HT-29 cells, which may be related to CD44 receptor-mediated internalization. However, this was not observed for HEPG2 cells due to the lower expression of CD44 receptors on the surface of HEPG2 hepatoma cells. It seems that an active targeting process did not play an essential role in HEPG2 uptake of lipo-HA/RhoB nanoparticles (Figure 6). RhoB/HA-liposomes cellular uptake by HT-29 cancer cells occurred based on CD44 receptor-mediated endocytosis (active targeting); however, non-specific internalization could be the main mechanism of cellular uptake for lipo/RhoB and, lipo-HA/RhoB by HEPG2 cancer cells. These results are an agreement with several recent studies (Hayward et al., 2016; Paliwal et al., 2016; Sabzichi et al., 2017).

3.3. Liposomal formulation effects on the cell cytotoxicity

The effects of free 5-FU, 5-FU-lipo and 5-FU-lipo-HA nanoparticles on the HT-29 and HepG2 cell viability were measured using an MTT assay. After 24, 48 and 72 h of treatment, a significant reduction in the cell viability was observed. It was calculated that the IC_{50} of free 5-FU, was 93, 63, and 35 $\mu\text{g/ml}$ and the IC_{50} of 5-FU-lipo was 59, 48, and 8 $\mu\text{g/ml}$ and 5-FU-lipo-HA was 44, 25, 4 $\mu\text{g/ml}$ during 24, 48 and 72 h of incubation, respectively (Figure 7A, B, and C) for HT-29 cancer cells. Notably, 5-FU-loaded HA-decorated liposomes showed a higher reduction of cell viability in a time-dependent manner over 24, 48 and 72 h; however, blank liposomes did not show any cell death during 24, 48 and 72 h (Figures 7A, B, and C). On the other hand, the cell cytotoxicity effects of the targeted system (5-FU-lipo-HA) and non-targeted (5-FU-lipo) nanoparticles were tested on the HEPG2 cancer cells, with low expression of CD44 (HA receptor). The result of the MTT assay indicated that both nanoparticles were significantly able to reduce the cell viability in comparison with the control cells. However, there was no significant difference in cytotoxicity between 5-FU-lipo and 5-FU-lipo-HA nanoparticles. The IC_{50} values of free 5-FU were found to be 100, 70, 40 $\mu\text{g/ml}$ over 24, 48 and 72 h, respectively, while the IC_{50} values of the non-targeted and targeted liposomes were calculated to be 78, 65, 28 and 65, 55, 22 $\mu\text{g/ml}$ over 24, 48 and 72 h, respectively. Overall, according to the obtained data the 5-FU-lipo-HA nanoparticles had superior cytotoxicity against the HT-29 cells than 5-FU-

lipo and free 5-FU. By contrast, there was no significant difference between the effects of 5-FU-lipo and 5-FU-lipo-HA on the cell viability of HEPG2 cells. These results are consistent with the cellular uptake findings, which showed higher cellular uptake of HA-decorated liposome compared to bare liposomes on HT-29 cancer cells. Moreover, it was reported that liposome nanoparticles decorated with HA had higher binding affinity to CD44 receptors; therefore, HA-decorated liposomes are able to recognize CD44 receptors and internalize into the cells by CD44 receptor-mediated endocytosis (Paliwal et al., 2016). On the contrary, bare liposomes also could enter into the cells through non-specific endocytosis. It can be concluded that the active targeting of 5-FU liposomes through HA binding to CD44 receptors resulted in higher cytotoxicity and greater selectivity against HT-29 cancer cells. These results are consistent with a recent study by Paliwal et al. They modified pH-sensitive liposomal nanoparticles with HA to specifically deliver DOX into MCF-7 breast cancer cells (Paliwal et al., 2016). The results of this study showed that HA-modified liposomes loaded with DOX showed higher cytotoxicity than bare liposomes/DOX. It was suggested that this observation was associated with the presence of HA molecules on the surface of the nanoparticles, which generally increase the cellular uptake of DOX (Paliwal et al., 2016). Moreover, the prepared nanoparticles had time-dependent cell cytotoxicity on both cell lines that may be related to the release profile of 5-FU from the nanoparticles (Qu et al., 2015).

3.4. 5-FU-loaded liposome nanoparticles increase apoptosis in colorectal cells

To investigate quantitative apoptosis, HT-29 cells were stained with annexin V/PI after 48 h of incubation with free 5-FU, 5-FU-lipo and 5-FU-lipo-HA. In this study, the cells treated with blank liposomes were considered as a negative control group. As shown in Figure 8, the blank liposome HT-29 cells were negative in this assay. According to the obtained results, free 5-FU showed $7.55 \pm 1.09\%$ apoptosis (P value < 0.01), while 5-FU-lipo and 5-FU-lipo-HA showed $9.25 \pm 0.53\%$ (P value < 0.001) and $14.83 \pm 0.3\%$ (P value < 0.0001) apoptosis compare to blank liposome group, respectively (Figure 8A, B). Therefore, 5-FU-lipo-HA nanoparticles are able to induce more apoptosis than 5-FU (P value < 0.05) and 5-FU-lipo (P value < 0.0001). In addition, the rate of apoptosis also was qualitatively considered. DAPI staining assay was used for recognition of nuclear fragmentation in treated HT-29 cells with free 5-FU, 5-FU-lipo and 5-FU-lipo-HA (Figure 8C). From the morphological assessment of DAPI stained cells, free 5-FU resulted in less deformation and nuclear fragmentation, while nuclear fragmentation was increased when the cells were treated with 5-FU-lipo and 5-FU-lipo-HA. This result is in accordance with the Tian et al. study, which reported that surface modification of liposomal nanoparticles with HA could increase the rate of apoptosis relative to DOX/liposome nanoparticles (Tian et al., 2018). Another active targeting drug delivery strategy was developed to deliver 5-FU into HT-29 cancer cells through conjugation of transferrin (Tf) as a ligand on the surface of liposomal nanoparticles. The results of this study showed that 5-FU/Tf-liposomal nanoparticles were able to induce apoptosis more effectively than bare 5-FU/liposomes, which supports our results (Moghimpour et al., 2018).

3.5. 5-FU-lipo and 5-FU-lipo-HA arrest the cell cycle in the G0/G1 phase

After the synchronization of the cells in different phases, the effects of free 5-FU, 5-FU-lipo and 5-FU-lipo-HA nanoparticles were studied at different phases of the cell cycle via the

DAPI staining method. The results showed a significant cell cycle arrest in the G0/G1 phase in 5-FU-lipo (P value<0.01) and 5-FU-lipo-HA (P value<0.001) groups after 48h (Figure 9 A and B). Encapsulation of 5-FU by the liposomes led to cell cycle arrest at the G0/G1 phase of the cell cycle, significantly more compared to when 5-FU was used alone (Figure 9 B). In addition, flow cytometry analysis confirmed the inhibitory effects of 5-FU-lipo-HA on the proliferation of HT29 cells via stimulation of cell cycle arrest at the G0/G1 phase along with apoptosis. A study in 2012 showed that the cell cycle was arrested at G1-phase when human colorectal cancer cells were treated with PEG-liposomal oxaliplatin (C. Yang, Liu, & Fu, 2012). In another study in 2005, it was revealed that, compared with free 5-FU, liposomal 5-FU demonstrated inhibitory effects on the cell cycle of hepatic cancer cells (Jin et al., 2005). On the other hand, Venkatesan et al, at 2011 showed encapsulation of celecoxib by liposomes showed no difference in cell cycle arrest, and cell cycle analysis showed that apoptosis induced by liposomal celecoxib was somewhat lower than treatment with free celecoxib (Perumal, Banerjee, Das, Sen, & Mandal, 2011).

3.6. 5-FU-lipo and 5-FU-lipo-HA reduce colony formation by HT-29 cells

In order to understand if the free 5-FU, 5-FU-lipo and 5-FU-lipo-HA nanoparticles could decrease the colony-forming ability, a clonogenic assay was performed on HT-29 colorectal cancer cells. The colony number significantly decreased (more than 70 fold) in 5-FU-lipo and 5-FU-lipo-HA treated cells compared with the control, blank liposomes, and free 5-FU groups (Figure 10A and B). In a study, Han and et al. successfully modified the surface of liposomal nanoparticles with HA encapsulating gemcitabine to target CD44 receptors that are overexpressed on the surface of the MCF-7 cell line. The results of this study were in agreement with our results and showed that gemcitabine/HA-liposomal nanoparticles showed colony formation reduction compared with bare gemcitabine/liposome and free gemcitabine (Han et al., 2016). Additionally, we observed that the encapsulation of 5-FU by liposomes led to a much greater reduction of colony formation compared to free 5-FU. In another study in 2005, the inhibition of tumor colony formation with liposomal 5-FU was significantly more than with free 5-FU. This suggests that liposomal compounds may be a valuable agent against tumors (Jin et al., 2005). The results of a study by Baumgartner et al suggested that the anti-cancer effects of 5-FU were mediated via colony formation inhibition (Udofot, Affram, & Bridg'ette Israel, 2015).

3.7. 5-FU-lipo-HA inhibited the genes and miRNAs related to oncogenesis and increased the expression of tumor suppressors in HT-29 cells

To confirm the effect of delivery of 5-FU via the HA-liposomes on the gene expression and miRNA expression related to oncogenesis, a qRT-PCR assay was performed to measure ALDH, KLF4, HMGA2, cMyc, Nanog, CD44, Kras mRNA, miR-181a, miR-181b, Let-7, miR-21, miR-155, mir-200c and miR-34c. The results showed that the expression of ALDH, KLF4, HMGA2, cMyc, Nanog, CD44 and Kras mRNA were significantly decreased compared with the blank liposome (Figure 11A). Further, miR-181a, miR-181b, Let-7 and miR-200c expression was significantly increased compared with the blank liposome group (Figure 11B). In contrast, a decrease in miR-21 in the 5-FU-lipo-HA group was observed compared to the blank liposome (Figure 11B). Our results did not show any significant changes in miR-155 and miR-34c expression between groups (Figure 11B).

There are some stem cell markers for colon cancers, such as CD44 and ALDH that have a critical role in the formation and development of tumors (Kozovska, Gabrisova, & Kucerova, 2014). Also, Nanog is a transcription factor that is involved in tumorigenesis and is related to the poor outcome of tumors (IV Santaliz-Ruiz, Xie, Old, Teknos, & Pan, 2014). HMGA2 is a member of the high-mobility group A family, that is associated with tumorigenicity of different malignancies. In addition, it seems that HMGA2 causes the resistance to 5-FU to develop in colon cancer cells (D'Angelo, Mussnich, Arra, Battista, & Fusco, 2017). The relative expression level of these genes in tumor cells could provide useful insights into liposomal 5-FU therapy. Our gene expression results suggested that liposomal 5-FU might provide better treatment of colorectal cancer. In other words, cells which were treated with 5-FU-lipo-HA had lower expression levels of CD44, ALDH, HMGA2 and Nanog compared with the cells treated with free 5-FU. Additionally, our results showed that treatment with 5-FU-lipo-HA nanoparticles could better inhibit the expression levels of oncogenes, such as c-Myc and Kras. We also investigated expression of a tumor suppressor gene, KLF4. It was observed that the expression level of KLF4 mRNA was reduced in HT29 cells after treatment with 5-FU-lipo-HA nanoparticles. Kruppel-like factor 4 (KLF4 or GKLf) is an inhibitor of cell cycle colony formation and migration in colorectal cancer (Zhao et al., 2004). KLF4 can act as inhibitor or inducer of proliferation depending on the type of cell, but in a study on colorectal cancer cells, KLF4 inhibited colony formation, migration and invasion (Dang et al., 2003). In a similar study, Mohan et al investigated apoptosis gene expression cascade in head and neck cancer cell lines, and their results showed that the encapsulation of 5-FU by liposomes had better anti-proliferative effects than free 5-FU on the gene expression (Mohan, Narayanan, Sethuraman, & Krishnan, 2014).

MicroRNAs (miRNA) are a group of small, noncoding RNA molecules that contribute to the developmental control of gene expression. Therefore, any change in miRNA expression can lead to physiological changes, such as proliferation, migration, and apoptosis that are important in malignant cells (Shah, Pan, Fix, Farwell, & Zhang, 2011). In the current study, we investigated the expression level of some miRNAs that are involved in colorectal cancer, after treatment with 5-FU-lipo-HA. Our data showed that miR-181a, miR-181b, Let-7 and miR-200c were overexpressed, while miR-21 was down-regulated after 5-FU treatment. Also, we observed no significant difference in the expression levels of miR-15 and miR-34c. MiR-21 is a miRNA that has been shown to be overexpressed in colorectal cancer in different studies, and a higher miR-21b expression level has been associated with 5-FU resistance (Schetter, Okayama, & Harris, 2012). Therefore, it seems that the down-regulation of miR-21 in the present study could show that the efficacy of 5-FU could be markedly enhanced by encapsulation in liposomes. By contrast, the results of a study in 2006 revealed that miR-181b, Let-7g and miR-200c were overexpressed in colorectal cancer and were correlated with a good response to an oral 5-FU prodrug called S-1. miR-181b was down regulated especially in the patients who responded to S-1 treatment (Nakajima et al., 2006). Rossi et al investigated the effect of 5-FU treatments on some miRNA expressions. Their results showed that 5-FU up-regulated miRNA genes that were previously over-expressed in malignant tissues (Rossi, Bonmassar, & Faraoni, 2007).

Conclusion

In the present study, HA surface-modified liposomes encapsulating 5-FU were prepared for targeting the CD44 receptor in colorectal cancer. The prepared nanoparticles had optimal physicochemical characteristics, such as particle size, zeta potential, and PDI that enables them to be effectively internalized compared to non-targeted nanoparticles. Moreover, the HA-decorated drug delivery systems showed slightly higher (but not markedly) EE and DL % values with sustained 5-FU release compared to bare liposomal nanoparticles, with good cytotoxicity against colorectal cancer cells. Our results also suggested that the targeted delivery system of 5-FU rendered colorectal cancer cells susceptible to apoptosis and arrested the cell cycle in G0/G1 phases. The clonogenicity study revealed that the effective delivery of 5-FU could decrease colony formation in colorectal cancer cells. The molecular experiments also suggested that 5-FU could have a significant effect on the down-regulation of oncogenes and up-regulation of tumor suppressors.

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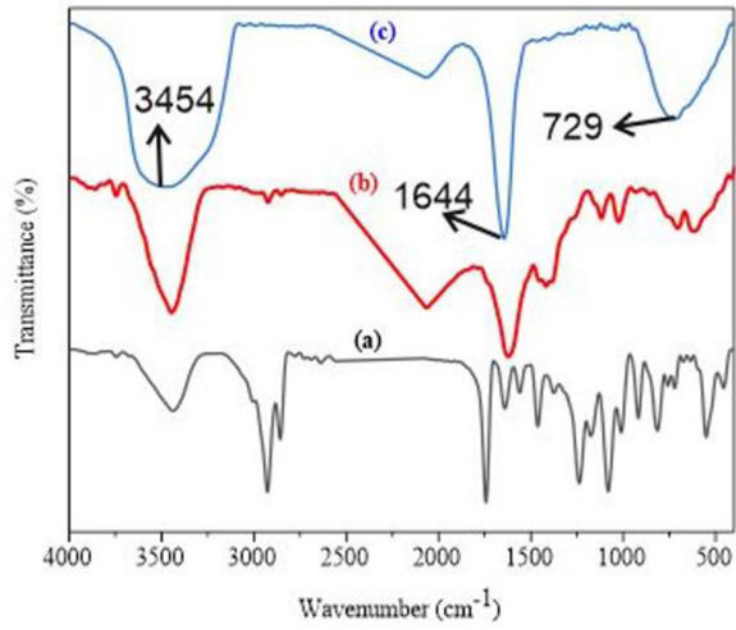


Figure 1. Shows the FTIR spectrum for DOPE (a), HA (b) and DOPE-HA (c).

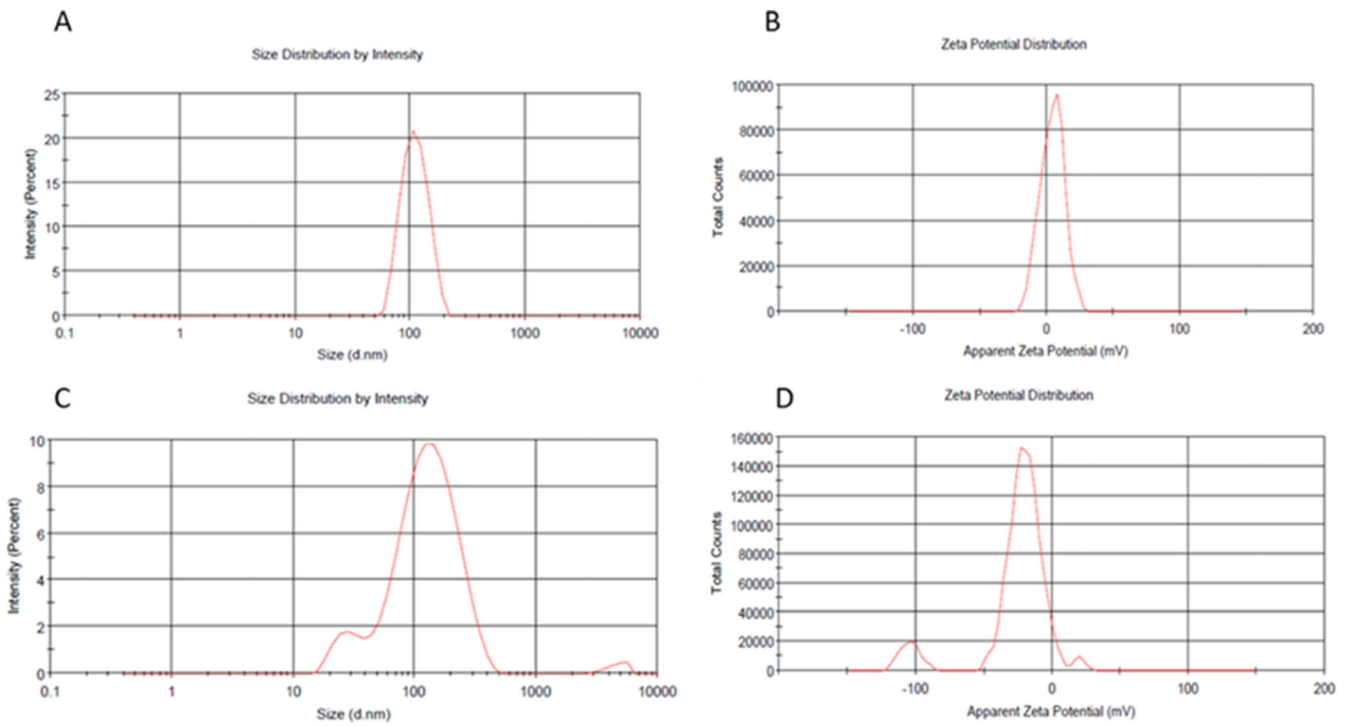


Figure 2. DLS obtained particle size for 5-FU-lipo (A), zeta potential of 5-FU-lipo (B), particle size of 5-FU-lipo-HA (C), and zeta potential of 5-FU-lipo-HA (D).

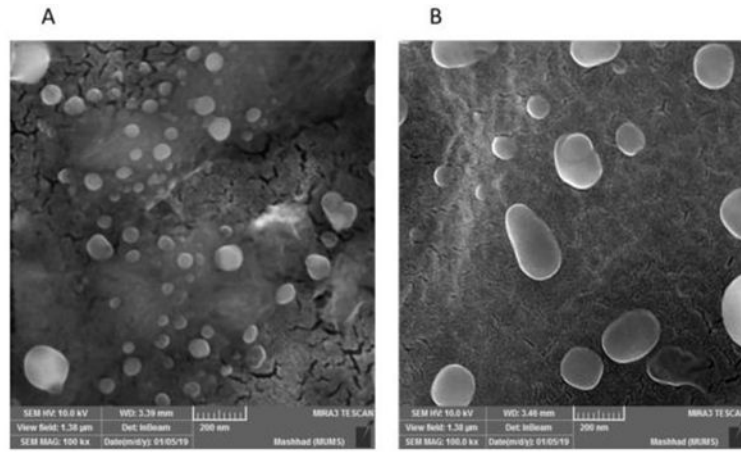


Figure 3. SEM visualized the morphology of (A) 5-FU-lipo and (B) 5-FU-lipo-HA nanoparticles

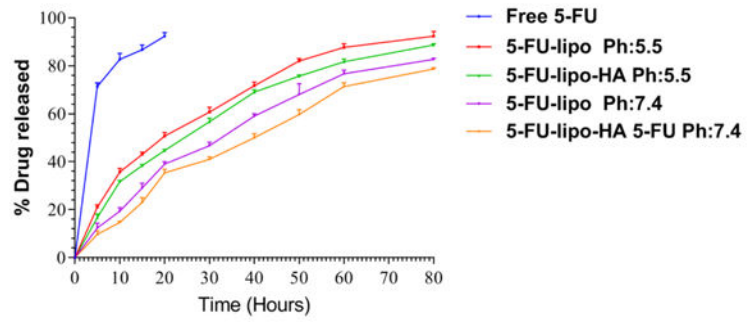


Figure4.
The in vitro release of 5-FU from 5-FU-lipo and 5-FU-lipo-HA nanoparticles at pH = 7.4 and pH = 5.5.

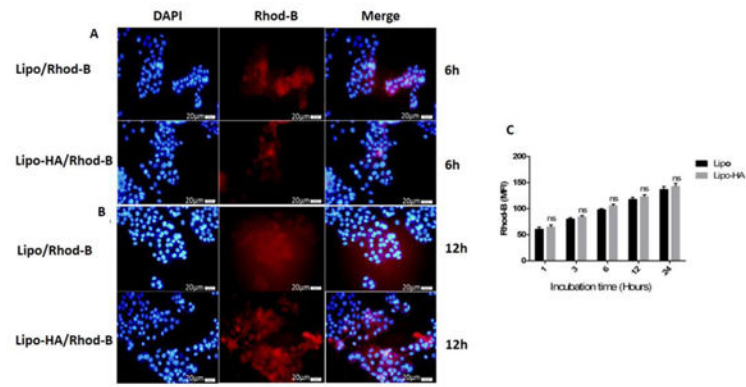


Figure 5. Fluorescence microscopy images of HT29 (CD44^{high} expressed) cancer cells treated with lipo/RhoB and lipo-HA/RhoB nanoparticles (with 1 μ M concentration of RhoB in each nanoparticle) showing the cellular uptake at 6 and 12 h incubation times (A-B). Uptake of lipo/RhoB and lipo-HA/RhoB nanoparticles measured by flow cytometry (C). Blue and red fluorescence are referred to the DAPI and RhoB respectively (magnification 40 \times). * p <0.001 versus lipo/RhodB. Data represent mean \pm SD (n=3). MFI: Mean fluorescence intensity.

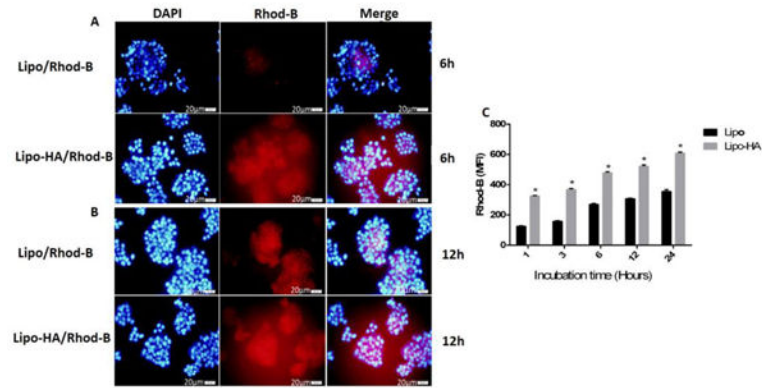


Figure 6.

Fluorescence microscopy images of HEPG2 ($CD44^{low}$ expressed) cancer cells treated with lipo/RhoB and lipo-HA/RhoB nanoparticles (with $1\mu\text{M}$ concentration of RhoB in each preparation) showing the cellular uptake at 6 and 12 h incubation times (A-B). Uptake of lipo/RhoB and lipo-HA/RhoB nanoparticles measured by flow cytometry (C). Blue and red fluorescence are related to the DAPI and RhoB respectively (magnification 40x). The data of each group compared to lipo/RhodB. Data represent mean \pm SD (n=3). MFI: Mean fluorescence intensity.

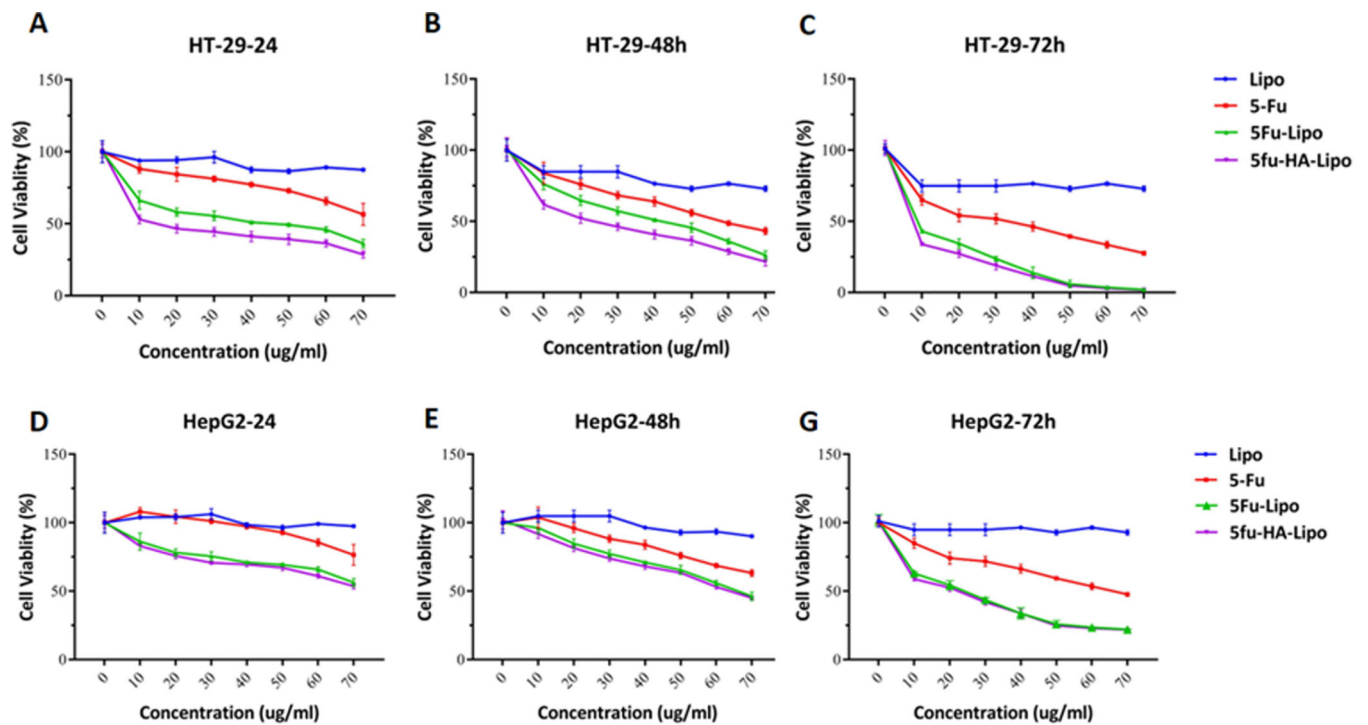


Figure 7. Cell toxicity of 5-FU loaded liposomes in colorectal cancer cells.

Represents *in vitro* cell viability results of blank liposomes, free 5-FU, 5-FU-lipo and 5-FU-lipo-HA in HT-29 and HepG2 cell lines over 24, 48, and 72 h (A-G). The data represent mean \pm SD (n = 3); *P < 0.05, versus blank liposome treated cells.

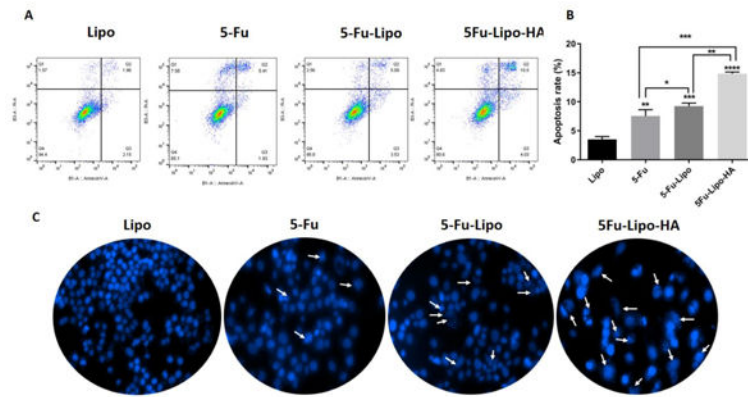


Figure 8. Cell apoptosis induced by free 5-FU, 5-FU-lipo and 5-FU-lipo-HA nanoparticles in colorectal cancer cells.

Annexin V/PI staining in free 5-FU, 5-FU-lipo and 5-FU-lipo-HA over 48 h showed the high rate of apoptosis in 5-FU-lipo-HA group (8A and B). Fluorescent images of treated HT-29 cells stained with DAPI (8C). The data represent mean \pm SD (n = 3); **P < 0.01, ***P < 0.001, and ****P < 0.0001, versus cells with blank liposomes.

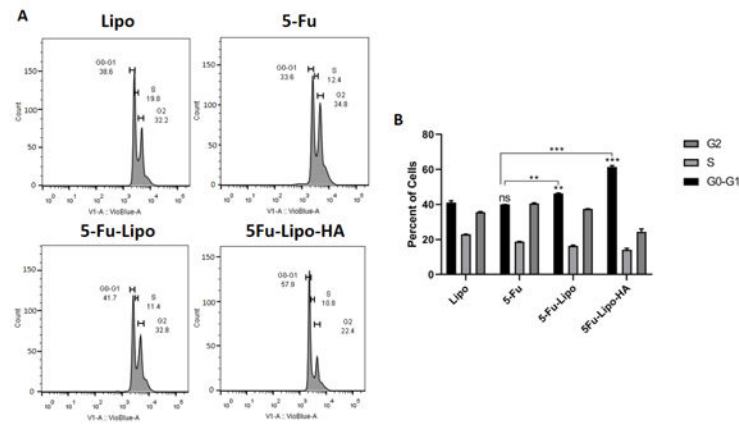


Figure 9. 5-FU-lipo-HA could arrest the cell cycle in the G0/G1 phase in colorectal cancer cells. Blank liposomes, free 5-FU, and 5-FU-lipo and 5-FU-lipo-HA treated cells were prepared and stained with the DAPI. The percentage of the cell population in the blank liposomes, free 5-FU, 5-FU-lipo and 5-FU-lipo-HA groups were evaluated in each cell cycle phase. The data represent mean \pm SD (n = 3); **P < 0.01, and ***P < 0.001 versus blank liposome treated cells.

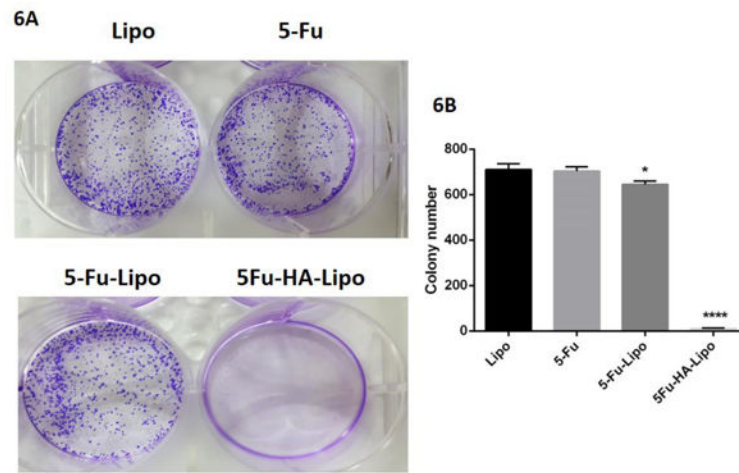


Figure 10. 5-FU loaded liposome suppressed the clonogenicity in HT-29 colorectal cancer cells. Free 5-FU and 5-FU-lipo and 5-FU-lipo-HA decreased the formation of the colorectal cancer cell colonies (5A and B). Data are presented as means \pm SD. (n=3); * $p < 0.05$, and **** $p < 0.0001$ versus blank liposome treated cells.

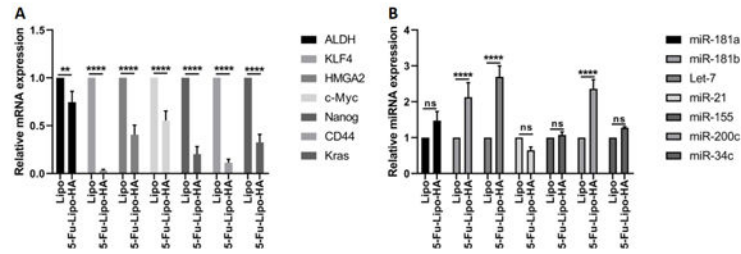


Figure 11. 5-FU-lipo-HA changes the expression of mRNAs and miRNAs in colorectal cancer cells.

Relative ALDH, KLF4, HMGA2, cMyc, Nanog, CD44, Kras mRNAs, miR-181a, miR-181b, Let-7, miR-21, miR-155, mir-200c and miR-34c were evaluated by the qRT-PCR assay. Data are presented as means \pm SD. (n=3); ** p<0.01, and **** p<0.0001 versus blank liposome treated cells.

Table 1.

Primer sequences

Primers	Sequences (5'–3')
Beta-actin	F TCCCTGGAGAAGAGCTACG
	R GTAGTTTCGTGGATGCCACA
ALDH	F AGATGTGGACAAGGCAGTGAA
	R ATCCACCAGGTAGGAGATGAC
KLF4	F ACCTTCTCACCCCTAGAGC
	R CCCAGTCACAGTGGTAAGGT
HMGA2	F TGGGAGGAGCGAAATCTAAA
	R TCCCTGGAGAAGAGCTACG
c-Myc	F CACATCAGCACAACACTACGCA
	R GCTCCAAGACGTTGTGTGT
Nanog	F CAATGGTGTGACGCAGGGAT
	R TGCACCAGGTCTGAGTGTTC
CD44	F CAAGCCACTCCAGGACAAGG
	R ATCCAAGTGAGGGACTACAACAG
Kras	F CGTAGGCAAGATGCCTTGA
	R CCTCTTGACCTGCTGTGTCG
miRNA	Target Sequence
miR-181a	AACAUUCAACGCUGUCGGUGAGU
miR-181b	AACAUUCAUUGCUGUCGGUGGGU
Let-7	UGAGGUAGUAGGUUGUAUAGUU
miR-21	UAGCUUAUCAGACUGAUGUUGA
miR-155	UUA AUGCUAAUCGUGAUAGGGGUU
miR-200c	CGUCUUACCCAGCAGUGUUUGG
miR-34c	AGGCAGUGUAGUUAGCUGAUUGC