Arginyl-glycyl-aspartic acid (RGD) containing nanostructured lipid carrier co-loaded with doxorubicin and sildenafil citrate enhanced anti-cancer effects and overcomes drug resistance

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ABSTRACT

Resistance to anticancer agents is considered as the main cause of chemotherapy failure. This study is aimed to prepare and optimize [Doxorubicin (Dox) + Sildenafil citrate (SC)]-co-loaded Arginyl-glycyl-aspartic acid (RGD)-containing nanostructured lipid carriers (NLC-RGD) to overcome multidrug resistance limitation and improve cancer treatments. Consequently, [DOX + SC]-coloaded NLC-RGD were fabricated by homogenization method and characterized by several techniques. Then, cytotoxicity, cellular uptake, apoptosis, and expression level of some multi-drug resistance related genes were evaluated in [DOX + SC]-coloaded NLC-RGD treated cells. As results, particles with nano-size, narrow size distribution and suitable encapsulation efficiency (∼56% for DOX and ∼81% for SC) were prepared. Our Results also demonstrated that co-delivery of DOX and SC by NLC-RGD promotes uptake and accumulation of drugs by integrin mediated endocytosis and possible ABC transporter inhibition. Cytotoxicity and apoptosis experiments revealed that co-delivery of DOX and SC by NLC-RGD is more effective approach for induction of apoptosis in comparison to individual treatment and delivery. Gene expression experiments revealed that SC reduces expression of ABCC1 and Nrf2. These findings indicated that NLC-RGD can be considered as an appropriate delivery system for co-delivery of DOX and SC to overcome DOX resistance to improve treatment efficacy in cancer.

1. Introduction

cancer is known as second leading cause of death next to cardiovascular diseases [1]. Following surgery, the chemotherapy is considered as the convenient treatment approach for cancer [2]. However, this option is limited by acquired extrinsic or intrinsic resistance to chemotherapy agents [3]. Overexpression of ATP-binding cassette (ABC) transporters, including ABCB1 (P-glycoprotein/MDR1), ABCCs [multidrug resistance–associated proteins (MRP)], and ABCG2 (BCRP/MXR/ABCP) is one of the key players in establishment of chemoresistance [4]. Overexpression of ABC transporters in cancerous cells leads to extrusion of chemotherapeutic agents, thereby lowering intracellular drug concentration and resulting in an attenuated chemotherapeutic effects (2). Therefore, discovery of novel strategies to overcome ATP-transporters-based chemoresistance may provide more efficacious cancer treatment. Recently it has been reported that Sildenafil citrate (SC), an inhibitor of cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase type 5 (PDE5), inhibits the activity of ABC transporters such as ABCB1, ABCC4, ABCCS and ABCG2 [5]. However, poor water solubility of SC is a limitation in therapeutic application [6]. Line of evidence indicate that approximately 71 percent of oral dose of SC has first pass hepatic and intestinal metabolism which results in low bioavailability of SC [7,8]. Moreover, oral administration of SC is concomitant with numerous side effects such as headaches, blood pressure reduction, flushing, and nasal congestion [8]. Therefore, nanoparticulate delivery systems are suggested to reduce side effects of SC and enhance bioavailability. Protection of encapsulated bioactive compounds from degradation, enhanced permeability and retention...
(EPR)-mediated passive targeting and ligands-mediated active tumor targeting are the main benefits of nanoparticles based drug delivery systems [9,10]. Among numerous drug delivery systems, lipid carriers have concerned rising attention due to their high drug-loading capacity, easy preparation, low toxicity and physical stability [11,12]. In addition, nano-carriers are able to co-delivery of anti-cancer agents which has been considered as one of the most promising methods to repel multi-drug resistance (MDR) activity [13]. Co-delivery not only delays the cancer adaptation process, but also reduces drug side effects by decreasing drug doses and achieving synergistic therapeutic efficacy [14,15]. Recently, targeted nanoparticles indicating growing interest regarding their ability to increase therapeutic effectiveness of chemical compounds and reducing possible side effects [16]. Nanostructured lipid carriers (NLC) are classified as Lipid-based colloidal drug delivery systems which are used for efficient co-delivery of antitumor agents [17,18] beside active delivery of drugs through coated ligands for precise conveyance to cancer specific receptors [19]. One of these ligands is Arginylic-glycyl-aspartic acid (RGD) which is the most common peptide motif that is responsible for cell adhesion to the integrins (especially αvβ3 integrin) in extracellular matrix (ECM) [12,20]. Due to the overexpression of integrins on various cancer cell, it is prospected that RGD-containing nanoparticles will penetrate into the cells more efficiently by integrin-mediated endocytosis [21,22]. The aim of the present study was to prepare and optimize RGD-containing NLC (RGD-NLC) co-loaded with DOX and SC to increase SC’s bioavailability and explore its ability in enhancing the cytotoxic and apoptotic effects of doxorubicin (DOX) on human lung cancer cell line. Furthermore, to investigate molecular mechanism by which SC causes overcoming to DOX resistance, expression of MDR related genes were studied by Real time Polymerase chain reaction (RT-PCR).

2. Material and methods

2.1. Materials

DOX, 3- (4, 5- Dimethylthiazol- 2- yl)-2, 5- diphenyltetrazolium bromide (MTT), 4’, 6-diamidino-2-phenylindole (DAPI), penicillin-streptomycin, propidium iodide (PI), RPMI 1640 and poloxamer 407 were purchased from Sigma-Aldrich Company (Steinheim, Germany). Precirol® AT50 (Glyceryl palmito stearate) was prepared from Gattefosse (Saint PeriestCedex, France). Fetal bovine serum (FBS) and 1, 2-dioleoyl-sn-glycerol-3-phosphoethanolamine-N-[amino (polyethylene glycol)-2000] (DSPE-PEG-2000-amine) were purchased from Gibco (Carlsbad, CA, USA) and Avanti Polar Lipids (Alabaster, AL) respectively. A549 cancer cell line was supplied from Pasteur Institute Cell Culture Collection (Tehran, Iran). SYBR green PCR Master Mix and cDNA synthesis kit were obtained from Takara Company (Japan).

2.2. Preparation of RGD-containing NLC co-loaded with DOX and SC

Primarily, DSPE-PEG-amine (2000) and RGD were stirred for 24 h in Dimethyl sulfoxide (DMSO) and the solution was transferred to 1000 Mw cutoff dialysis bag (Sigma-Aldrich, USA) to purify the synthesized polymer. Finally, the modified polymer was obtained after freeze-drying and RGD-PEG-DHPE (yield 73.1%) was achieved. Next, hot homogenization technique used to prepare RGD-containing NLCs formulation. For this purpose, Precirol melted as a solid lipid and let to SC and DOX to dissolve or disperse in Miglyol as a liquid oil. Subsequently, RGD-PEG-DHPE was dissolved in aqueous phase containing Poloxamer 407 and added in drop wise manner to the lipid phase under imposed homogenization with high pressure at 20,000 rpm for 30 min at 70°C (Heidolph, Germany). The temperature of hot oil/water formulation was decreased into ambient or lower temperature in order to re-crystallization of NLC-RGD formulation.

2.3. Characterization of prepared nanoparticles

2.3.1. Particle size, size distribution and zeta potential

The mean diameter, polydispersity index (PDI) and zeta potential of prepared nanoparticles (NPs) were measured by photon correlation spectroscopy (PCS) (Zetasizer ZS, Malvern, UK) and also zeta potential values were determined based on laser Doppler anemometry with the same machine. The samples were put into capillary cells and a minimum of three measurements were provided per sample for zeta potential measurements.

2.3.2. Morphology of nanoparticles

To study the morphology of the particles, the prepared NLCs were diluted with distilled water and visualized by scanning electron microscope (SEM) (KYKY-EM3200, Bio-equip, China) at 26 KV excitation voltage. To provide the gold coated samples, they were placed in droplet-by-droplet-manner on glass lamella and then coated under vacuum by a sputter (SC7620-CF, Quorum Technologies, UK).

2.3.3. Drug encapsulation efficiency (EE) and physical stability studies

To examine the entrapment efficiency of DOX and SC, the amount of unloaded drug (DOX or SC) was determined using centrifugal filter tube (Amicon® filter, molecular weight cutoff 100 kDa, Millipore, UK). For this purpose, 1 ml of formulation was diluted with 1 ml of Dimethyl sulfoxide (1% vol/vol) to solve the probable unloaded SC and then centrifuged at 4000 rpm for 8 min to separate the free DOX or SC from the encapsulated drugs. After that, the amount of unloaded drugs were measured by ultraviolet visible spectrophotometer (Ultrascop 2000®; pharmacia biotech, UK) at λmax 480 and 291 nm for DOX and SC, respectively. For this purpose, ultraviolet-visible calibration curve of SC and DOX were prepared and amount of unloaded DOX and SC calculated using acquired absorption from spectrophotometer and equation of calibration curve. Finally the EE of DOX and SC were calculated using the following equations:

\[
EE(\%) = \frac{C_t - C_{ul}}{C_t} \times 100
\]

Where, \(C_t\) was the total added drug concentration, \(C_{ul}\) was the unloaded drug concentration in aqueous phase. To assess the physical stability, the size, PDI and EE of optimum formulation was determined after 8 weeks storage at 2–6°C by mentioned methods.

2.4. Cell viability assay

To study the in-vitro cytotoxicity of nanoformulation, human lung carcinoma A549 cells (1 × 10^4 cells/well) were cultured in RPMI medium with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 5% CO2. Then, the different concentrations of blank NLC-RGD, free SC, free SC + DOX, [SC + DOX]-co-loaded NLC and [SC + DOX]-co-loaded RGD-NLC were used for treatment of A549 cells. After 48 h of incubation, the treated media were removed and substituted with 200 μL of fresh media containing 50 μL of MTT solution (5 mg/ml). Then, the cells incubated for further 3 h at 37°C. After this time, the media were removed and 180 μL of DMSO and 20 μL Sorenson buffer were used to dissolve the formazan crystals. The optical density (OD) values were recorded at 570 nm compared to control cells using a micro-plate reader (Elx808, Biotek, USA).

2.5. Cellular uptake study

To confirm the targetability of NLC-RGD, the cellular uptake of nanoformulation (with and without RGD) were studied by flow cytometry and fluorescence microscopy. Moreover, since DOX is a fluorescent drug, so the permeability of NLC in the cells was explored by encapsulated DOX. For the flow cytometry, the A549 cells were seeded in six-well plates at 3 × 10^5 cells per well and then treated with free
DOX, DOX + SC, [DOX + SC]-coloaded NLC, and [DOX + SC]-co-loaded NLC-RGD for 3 h. Then, a minimum of 1 × 10⁶ cells were collected and washed three times with PBS for analyzing the DOX fluorescence intensity using a FACS caliber flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) for each sample.

2.6 Apoptosis assay

To identify necrosis, early and late apoptosis, Fluorescein isothiocyanate (FITC) labelled annexin V assay was performed. A549 cells were cultured in six-well plates at a density of 5 × 10⁵ cells/well and then treated with 1.26 µM DOX, 1.26 µM DOX + 15 µM SC and equivalent doses [DOX + SC]-coloaded NLC (with and without RGD) for 24 h. Then, the cells harvested, centrifuged and washed twice with cold phosphate-buffered saline (PBS). Next, the cells were re-suspended in 200 µL of binding buffer containing 5 µl FITC-labelled annexin V and incubated for 20 min at ambient temperature in dark room. Finally, the cells were examined using a FACS caliber flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA).

2.7 DAPI staining

To quantify the apoptotic nuclei by DAPI staining, A549 cells (5 × 10⁵ /well) were seeded onto the glass coverslips placed in 6-well plates and incubated for 24 h to reach the 80% confluence. Then, the cells were treated with DOX, DOX + SC, [DOX + SC]-coloaded NLC (with and without RGD). After 24 h, the cells were washed twice with cold PBS and fixed with 4% paraformaldehyde and permeabilized in 0.1% (w/v) Triton X-100. After 15 min, the cells were stained with DAPI for further 30 min. Finally, the morphology of nuclear structure alteration was evaluated by fluorescence microscope to figure out the apoptotic cells.

2.8 RNA extraction, reverse transcription and analysis of gene expression

To examine expression of ABCB1, ABCC1, ABCC2 and nuclear factor-E2-related factor 2 (Nrf2), A549 cells were treated with 25 µM SC and equivalent doses of SC loaded NLC-RGD for 48 h. Subsequently total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer’s procedure and was quantified by nanoDrop (ND-1000, NanoDrop technology, Australia). Complementary DNA (cDNA) synthesized by using First Strand cDNA synthesis Kit (thermo scientific, Schwert, Germany) and Real-time PCR (RT-PCR) was performed by the real time rotary analyzer (Rotor-Gene 6000, Corbet Life Science, Australia) based on SYBR Green chemistry. Sequence of primers are indicated in Table 1. Pfaffl method was used to analyze the data and the cycle threshold (CT) values were standardized regarding to housekeeping gene (GAPDH) expression.

2.9 Statistical analyses

All the analysis were carried out triplicate and obtained values were expressed as the means ± standard deviation (SD) that was calculated by Microsoft Excel 2016. Statistical analyses were performed using Student’s t-test and ANOVA for two groups and multiples comparison respectively. P value less than 0.05 was considered to be statistically significant.

3. Results

3.1 Preparation and characterization of [DOX + SC]-coloaded NLC-RGD

[DOX + SC]-coloaded NLC-RGD were prepared by modified hot homogenizing method using different percentage of Precirol, Miglyol and Poloxamer. In this process approximately 10 different formulations (table. 2) were assessed according to nanoparticle size, polydispersity index and loading capacity for both drugs. Based on mentioned parameters, the optimum formulation was composed of 110 mg Precirol as central substance of nanoparticles, 15 mg Miglyol as stabilizer and 30 mg Poloxamer as surfactant of NLC. DLS graph showed that mean particle size of prepared NPs was 80.5 nm with 0.23 PDI (Fig. 1a). Subsequently these results confirmed by scanning electron microscope imaging (Fig. 1b) which showed spherical morphology with nanoscale size and narrow polydispersity for prepared nanoparticles. Zeta potential analysis showed a negative value of −18.5 mV surface charge at the original pH 7.4 (Fig. 1c).

3.2 Encapsulation efficiency (EE) and stability

Encapsulation efficiency is an important factor for selecting optimum formulation. Fig. 2 shows the ultraviolet-visible calibration curve of SC and DOX which are used for calculation of unloaded drugs and subsequently EE. EE for DOX and SC in different formulation was reported in Table 2. EE for DOX and SC in optimum formulation were 56.04 ± 1.25% and 81.62 ± 3.14% respectively. [DOX + SC]-coloaded NLC-RGD showed no notable alteration in clarity and phase separation when stored in 2–6°C for 8 weeks. After this period, size and PDI of nanoparticles was 86.2 nm and 0.28 which showed that prepared NPs are stable minimum for 8 weeks at 2–6°C (Fig. 1a). Furthermore, after 8 weeks storage at 2–6°C, the unloaded form of DOX and SC was 61.03% and 26.54% respectively which shows that during these 8 weeks, approximately 17% of DOX and 8% of SC were released from nanoparticles.

3.3 In vitro cell viability study

MTT assay was performed to determine cytotoxicity of DOX, SC and synergistic effects of combinatorial treatment of DOX and SC in free form and in the form of co-loaded to NLC-RGD on A549 lung cancer cell line. Results showed that SC (up to 80 µM) and blank NLC-RGD has no remarkable cytotoxicity (Fig. 3a). Co-treatment of the cells with 1 µM of DOX and serial of different concentrations of SC showed that, SC causes a notable increase in DOX cytotoxicity in concentration of ≥15 µM (Fig. 3b). Due to numerous side effects of SC, we decided to select minimum effective concentration of SC for rest of experiments. Regarding to encapsulation efficiency of DOX and SC, 15 µM of SC in formulation is equivalent with 1.26 µM of DOX. Data analysis of the cytotoxicity assay also revealed that combination treatment of the cells with [15 µM SC + 1.26 µM free DOX], [15 µM SC + 1.26 µM DOX co-loaded NLC] and [15 µM SC + 1.26 µM DOX co loaded NLC-RGD] results in 70.62%, 65.47%, 51.36% cell viability, which shows that [SC + DOX]-coloaded NLC-RGD has 19.26% and 14.11% more

Table 1 Primers sequences.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5'-3' direction)</th>
<th>Reverse Primer (5'-3' direction)</th>
<th>Products size (bp)</th>
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<tr>
<td>ABCB1</td>
<td>TGACAGCTACCGCAGCGGAAG</td>
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<tr>
<td>Nrf2</td>
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<td>83</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GACAGTCAGCGCCATCTTCT</td>
<td>TTAAGACGCCCCTGGTGAC</td>
<td>127</td>
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Table 2
Composition and characteristics of prepared nanoparticles.

<table>
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<tr>
<th>Formulation Code</th>
<th>Precirol (mg)</th>
<th>Miglyol (mg)</th>
<th>RGD-PEG-DSPE (mg)</th>
<th>Poloxamer (mg)</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>EE for DOX (%)</th>
<th>EE for SC (%)</th>
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</thead>
<tbody>
<tr>
<td>F1</td>
<td>200</td>
<td>10</td>
<td>–</td>
<td>100</td>
<td>225.0 ± 6.5</td>
<td>0.54</td>
<td>73.4 ± 2.3</td>
<td>92.2 ± 1.3</td>
</tr>
<tr>
<td>F2</td>
<td>200</td>
<td>15</td>
<td>–</td>
<td>120</td>
<td>236.1 ± 4.3</td>
<td>0.51</td>
<td>72.7 ± 4.1</td>
<td>93.9 ± 2.7</td>
</tr>
<tr>
<td>F3</td>
<td>200</td>
<td>10</td>
<td>–</td>
<td>150</td>
<td>280.7 ± 3.9</td>
<td>0.54</td>
<td>76.5 ± 2.6</td>
<td>93.1 ± 3.5</td>
</tr>
<tr>
<td>F4</td>
<td>150</td>
<td>15</td>
<td>–</td>
<td>100</td>
<td>205.6 ± 3.7</td>
<td>0.40</td>
<td>68.8 ± 3.9</td>
<td>87.2 ± 2.9</td>
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<tr>
<td>F5</td>
<td>200</td>
<td>20</td>
<td>–</td>
<td>80</td>
<td>220.4 ± 4.1</td>
<td>0.53</td>
<td>70.2 ± 3.3</td>
<td>90.4 ± 3.1</td>
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<tr>
<td>F6</td>
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<td>15</td>
<td>–</td>
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<tr>
<td>F7</td>
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<td>2</td>
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<td>52.4 ± 3.1</td>
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<tr>
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<td>80</td>
<td>15</td>
<td>2</td>
<td>20</td>
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<td>0.27</td>
<td>35.1 ± 3.0</td>
<td>69.9 ± 4.1</td>
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<td>100</td>
<td>15</td>
<td>5</td>
<td>30</td>
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<td>0.24</td>
<td>43.1 ± 1.5</td>
<td>77.1 ± 19</td>
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<tr>
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<td>110</td>
<td>15</td>
<td>5</td>
<td>30</td>
<td>80.5 ± 4.0</td>
<td>0.23</td>
<td>56.0 ± 1.2</td>
<td>81.6 ± 3.1</td>
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</table>


Fig. 1. Characteristic of [DOX + SC]-coloaded NLC-RGD. a) Size and polydispersity index (PDI) of nanoparticles, (I) after preparation, (II) after 8 weeks of storage at 2–6 °C. b) Scanning electron microscopy (SEM) image of prepared nanoparticles. c) Zeta potential distribution of [DOX + SC]-coloaded NLC-RGD. (DOX, Doxorubicin; SC, Sildenafil Citrate; NLC-RGD, Arginyl-glycyl-aspartic acid containing nanostructured lipid carriers).
cytotoxicity in comparison to [SC + DOX] and [SC + DOX co-loaded NLC] respectively (Fig. 3c–e). These differences are statistically significant (p < 0.05).

3.4. DOX uptake into cells

Cellular uptake studies by flow cytometry and fluorescence microscopy were performed to evaluate the ability of NLC and NLC-RGD in enhancing accumulation of DOX into the A549 lung cancer cells. As shown in Fig. 4a, SC causes more accumulation of DOX into cell during 3 h incubation, suggesting that SC may decrease efflux of drug by inhibiting drug extrusion pumps. Results also showed that the cellular uptake of [DOX + SC]-coloaded NLC-RGD was significantly higher than those of [DOX + SC]-coloaded NLC in cancerous cells (Fig. 3a), suggesting that NPs were absorbed into the tumor cells more efficiently using RGD mediated endocytosis. This results confirmed by fluorescence microscopy imaging which shows that cells treated with NLC-RGD display more strength red fluorescence in their cytoplasm, in comparison with cells treated with NLC and free DOX (Fig. 4b).

3.5. Cell apoptosis

In order to compare the strength of apoptotic effect of DOX, DOX + SC and [DOX + SC]-coloaded NPs, flow cytometry analysis of Annexin V/PI double staining was performed. In normal viable cells phosphatidyl serine (PS) is located on the cytoplasmic surface of the cell membrane. Upon induction of apoptosis, rapid alterations in the organization of phospholipids occurs leading to exposure of PS on the cell surface. In vitro detection of externalized PS can be achieved through interaction with the anticoagulant annexin V. In the presence of calcium, rapid high affinity binding of annexin V to PS occurs. Propidium iodide is used to distinguish between viable, early apoptotic, and necrotic or late apoptotic cells. Propidium iodide will be excluded from viable (FITC negative) and early apoptotic (FITC positive) cells. Results of flow cytometry are displayed in Fig. 5a. Viable cells are shown in bottom left quadrant, early stage apoptotic cells bind primarily to Annexin V are shown bottom right quadrant, the late apoptotic cells binds to both of Annexin V and PI, are shown in top left quadrant and necrotic cells are shown in top left quadrant. As seen in Fig. 5a. more than 91% of cells in control group are viable. Treating cells with DOX, DOX + SC, [DOX + SC]-coloaded NLC and [DOX + SC]-coloaded NLC-RGD induced apoptosis in 29.87%, 34.69%, 38.37% and 44.32% of cells respectively. These results confirmed higher programmed cell death by combination therapy with DOX and SC compared to pure DOX. Furthermore, obtained result concludes that co-delivery with NLC-RGD is highly competent compared to NLC. In other word, co-treatment of cells with DOX and SC intensify apoptotic effects of DOX, suggesting Inhibition of ABC transporters. Co-delivery of DOX and SC by NLC-RGD may enhances drugs uptake through integrin mediated endocytosis which lead to more apoptotic effects of DOX in compared with free DOX and SC.

Fig. 2. Ultraviolet-visible calibration curve of Doxorubicin (DOX) and Sildenafil Citrate (SC).

Fig. 3. Cell viability of non-small cell lung cancer (A549) after 48 h incubation with (a) SC and SC loaded nanoparticles, (b) Different concentration of doxorubicin, (c) 1 μM DOX + different concentration of SC, and (d) combination of 1.26 μM doxorubicin and 15 μM SC in free form and in the form of loaded NLC and NLC-RGD. e) phase-contrast image of A549 cells when treated with DOX and SC in free form and in the form of loaded NPs. The figure illustrates that [DOX + SC]-coloaded NLC-RGD are more anti-proliferative when compared with free form of DOX + SC (p < 0.05). Data is presented as mean ± standard deviation (n=3), * Showing Significant Differences. (DOX, Doxorubicin; SC, Sildenafil Citrate; NLC-RGD, Arginyl-glycyl-aspartic acid containing nanostructured lipid carriers; NPs, Nanoparticles).
Fig. 4. Doxorubicin uptake into cells. DOX fluorescence intensity graphs (a) and fluorescence microscope images (b) when cells were treated with DOX and SC in the free from and in the form of loaded NLC and NLC-RGD. (DOX, Doxorubicin; SC, Sildenafil Citrate; NLC-RGD, Arginyl-glycyl-aspartic acid containing nanostructured lipid carriers).

Fig. 5. The apoptosis induction flow cytometry graphs (a) and fluorescent images of DAPI stained cancerous cells following a 48 h treatment of cells with DOX and SC in the free from and in the form of loaded into NLC and NLC-RGD. (DOX, Doxorubicin; SC, Sildenafil Citrate; NLC-RGD, Arginyl-glycyl-aspartic acid containing nanostructured lipid carriers).
3.6. DAPI staining

In order to compare the influence of DOX, DOX + SC and [DOX + SC]-coloaded NPs on morphologic feature of the nucleus, as a simple factor for the determination of healthy and apoptotic cells, DAPI staining was performed. Based on the obtained results, the frequency of DAPI staining in SC treated cells was more than free DOX, suggesting synergistic effect of SC and DOX in inducing apoptosis (Fig. 5b). Furthermore, the cells treated with [DOX + SC]-coloaded NLC-RGD showed nucleus condensation or chromatin degradation more noticeably than the cells grown in the presence of [DOX + SC]-coloaded NLC which suggest more absorbance of [DOX + SC]-coloaded NLC-RGD in compared with [DOX + SC]-coloaded NLC (Fig. 5b).

3.7. Quantitative real-time PCR

In order to understand the molecular mechanism of SC in enhancement of apoptosis ability of DOX, the expression level of important MDR resistance genes were evaluated in SC treated A549 lung cancer cell line. The mRNA level of ABCB1, ABCC1, ABCC2 and Nr2 were normalized to mRNA level of the uniformly expressed reference gene, GAPDH, within each sample. Results revealed that treatment of A549 cells with 25μM SC or SC loaded NLC-RGD cause’s considerable decrease in the expression level of ABCC1 and Nr2 (Fig. 6). However, there was no significant alteration in the expression of ABCB1 and ABCC2 genes under influence of SC or SC loaded NLC-RGD. The RT-PCR data, also demonstrated that downregulation of ABCC1 and Nr2 expression will intensify when SC is loaded into NLC-RGD suggesting capability of NLC-RGD in the more precise delivery of SC into cells than free SC. In other word, downregulation of ABCC1 and Nr2 in SC-loaded NLC-RGD treated cells are more than those who were treated with SC, suggesting that NLC-RGD can be considered as an appropriate delivery system for SC to overcome its therapeutic limitation and its specific delivery into cancerous cells.

4. Discussion

Most of ABC transporter Inhibitors were found to lack significant efficacy in late-phase clinical trials [5]. Therefore, it is clear that there is still a need to develop and test efficacious inhibitors.

Present study recognized a targeted nano-carrier system capable of delivering DOX simultaneously with SC as a MDR inhibitor. Characteristic of prepared nanoparticles (suitable Size, ZP, High EE and long physical stability) confirmed appropriateness of optimum formula. Prepared NPs were smaller than 200 nm to escape from reticuloendothelial system [23] and have reasonable ZP which not only causes repulsive forces among nanoparticles to stop it from aggregation, but also causes better permeability across cell membranes. In this study, prepared NPs contain RGD as a ligand of integrin’s for efficient delivery of drug into the cells. Regarding to elevated expression of certain patterns of integrins in cancerous cells and their central role in cellular adhesion and cancer development, targeting with RGD not only enhances drug penetration into the cells through integrin related endocytosis, but also hinders cell invasion and metastasis by blocking of integrins. In this study, highly expressed αvβ3 and αvβ6 integrin [24], A549 non-small cell lung cancer (NSCLC) cell line was used to compare the penetration rate of NLC and NLC-RGD. As shown in Fig. 4, accumulation of drug loaded NLC-RGD into cell are noticeably more than NLC which confirmed that RGD have a high binding affinity to cancerous cells and is able to enhance endocytosis. In in-vivo systems, in addition to the active targeting, NLC-RGD may benefit passive targeting through enhanced permeability and retention (EPR) which indicates that NPs leak favorably into tumor tissue through permeable tumor vessels and then retained in the tumor tissue due to poor lymphatic drainage [25]. Furthermore, absorbance of NPs occurs through lymphatic system by Peyer’s patches in the small intestine [26,27] which leads to bypassing the liver and consequently reduce first pass metabolism [28]. The main aim of this study was to overcome MDR by co-delivery DOX and SC. Data of MTT assay showed that A549 cells co-treatment with DOX and SC resulted in significantly increased cytoxicity compared to DOX. In accordance to these data, Greish et al. [29] showed that combination therapy with DOX and SC results in statistically significant 4.7-fold reduction in tumor size compared to DOX. Enhanced cytotoxicity of DOX may be due to decreasing activity of ABC transporter by SC. Furthermore, Das et al. [30] showed that combination therapy with DOX and SC increased DOX-induced apoptosis in cancer cells by upregulation of caspase-3 and caspase-9 activities, downregulation of Bcl-xl, and phosphorylation of Bad. As seen in Fig. 5, co-delivery of DOX and SC by NLC-RGD intensify apoptotic effects of DOX, suggesting that Inhibition of ABC transporters would lead to sensitization of MDR cancer cells to chemotherapeutic agents. In accordance to these data, Shi et al. [31] showed that Sildenafil interacts with transmembrane regions of ABCB1- and ABCG2 transporter and decreases ABCB1- and ABCG2-mediated drug resistance by directly obstructing the transport function of ABCB1 and ABCG2. They also confirmed that Sildenafil itself may be a substrate of ABC transporter and may act as a competitive inhibitor of ABCB1 and ABCG2 by competing with other substrates. The results of this study also showed that loading SC into NPs enhances its adjuvant effects, which may benefit from increased uptake by cells and overcoming its low bioavailability. In addition to synergistic effects of SC beside DOX, several studies also revealed that SC may attenuate the DOX-induced cardiotoxicity [32,33]. Inhibition of multidrug resistance, may make low dose of anticancer drugs more effective, whereby could reduce the side effects of these agents [34]. Improved targeting of SC can render DOX active at much lower concentrations in cancer treatment which in turn cause decreasing the side effects in the patient. Regarding to enhanced expression of MDR related genes in NSCLC [35,36], In this study influence of SC on mRNA level of some of these genes were evaluated. Results revealed that, SC decreases the transcription of Nr2 and ABC1. Nr2 is an important transcription factor which its aberrant expression is related to poor outcomes in the treatment of NSCLC [37]. In accordance to our results, Ji et al. [38] showed that knocking down of Nr2 expression lead to down regulation of ABC1 in H69AR cells, which in turn increase intracellular concentrations of anticancer drugs. Therefore, in addition to direct inhibition of ABC transporter, downregulation of Nr2 and ABC1 are other probable mechanism, through which SC
enhance sensitivity of chemotherapeutic agents.

5. Conclusion

Co-delivery of DOX and SC by NLC-RGD is an appropriate approach to overcome MDR problems of DOX. Results of this study suggested that loading of SC and DOX into NLC-RGD prepare them more effective in accumulation into cancerous cells due to specialized endocytosis which mediated by RGD adhesion to integrins. This study also revealed that SC enhances DOX induced apoptosis by reversing ABC-transporter–mediated MDR and downregulation of Nr2f and ABCCl. Therefore, co-delivery of SC and chemotherapeutic agents by NLC-RGD can be considered as a significant method to overcome MDR in cancer treatment.

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References


