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Research Article
5-Fluorouracil and Simvastatin Loaded Solid Lipid Nanoparticles for Effective Treatment of Colorectal Cancer Cells

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Abstract

Background and Objective: Colorectal cancer (CRC) is the most influential cause of cancer death worldwide. Despite its effectiveness in CRC therapy, its clinical applications are restricted because of its short half-life, resistance and severe side effects. The present study was directed to formulate nanoparticles of 5-Fluorouracil (5-FU) in the presence of Simvastatin (SMV) in an attempt to enhance the therapeutic efficacy of 5-FU. **Materials and Methods:** Formulation of Solid Lipid Nanoparticles (SLN) has been performed and cytotoxic activity was tested in human colorectal cancer cell line (HCT-116) the IC_{50} was investigated to evaluate the cytotoxicity, apoptosis induction, cell cycle distribution and the intercellular Reactive Oxygen Species (ROS) after treatment with SLN 5-FU and/or SMV compared with raw drug. **Results:** The particles size was 107-117 nM and stability of formula between -5.53 and -14 mV, Entrapment Efficiency was 80-97.5% for 5-FU and SMV, respectively. Raw 5-FU had IC_{50} 12.69 μM while cells treated with 5-FU SLN, IC_{50} dropped significantly and addition of 5 μM SMV SLN, IC_{50} was significantly reduced. Also, treatment with SLN 5-FU alone and/or SMV significantly accumulated the cells in sub-G1 and dramatically increased the percentage of late apoptotic cells significantly in comparison to raw 5-FU. Moreover, SMV SLN with 5-FU had increased intracellular ROS accumulation. **Conclusion:** The SLN formulation for both 5-FU and SMV showed a significant cytotoxic potentiating effect against the growth of HCT-116 cells.

Key words: 5-FU, simvastatin, cytotoxicity, colorectal cancer, nanoparticle

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Colorectal cancer is the third most commonly diagnosed cancer (10.2% of the total cases) and the second leading cause of cancer death (9.2% of the total cancer deaths). Colorectal cancer (CRC) has been beginning with a high rate of morbidity and mortality worldwide[1]. Mortality from CRC remains high and 40-50% of patients eventually die because of their disease[2]. Cancer treatment modalities usually include surgery, chemotherapy and radiotherapy. Moreover, the most appropriate therapy to target both localized and the metastatic cancer cell is chemotherapy[3]. Due to the failure of traditional anticancer agents to specify and target cancer cells, several attempts were done to improve the selectivity and uptake of anticancer agents into cancer cells with the consequent improvement in cytotoxicity[4-6] and reduce the systemic toxicity[7,8]. In this regard, several approaches have been tried to enhance the efficacy and reduce the toxicity in CRC therapy including the use nanotechnology as a drug delivery system[9]. Solid Lipid Nanoparticles (SLNs) are colloidal carriers that had been utilized as an alternative system to the existing traditional carriers (emulsions, liposomes and polymeric nanoparticles). They offer unique properties such as small size, large surface area, high drug loading and the interaction of phases at the interfaces with the consequent improvement of the performance of pharmaceuticals, nutraceuticals and other materials[10,11].

In the treatment of CRC, 5-Fluorouracil (5-FU) is one of the earliest and still most commonly and continues utilized treatment option for several cancer disorders. Nonetheless, despite its effectiveness in CRC therapy, its clinical applications are narrowed because of its short half-life, disease resistance and severe side effects[12]. The overall and total response rate[13] for advanced CRC to 5-FU alone is still only ≈10%.

On the other hand, Simvastatin (SMV) identified as 3-hydroxy 3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, along its ability in controlling cholesterol, experimental and clinical data found a beneficial effect of statins in different cancer type[14,15]. Earlier studies have demonstrated that simvastatin had consistent chemo-preventive effects against colon carcinogenesis[16,17]. Taken together, this study was aimed to prepare 5-FU nanoparticle in the presence of SMV and to compare its efficacy with the conventional 5-FU on human colorectal cancer cells line (HCT-116).

MATERIALS AND METHODS

Study area: Part of this study was done in College of Medicine, King Abdulaziz University, Saudi Arabia and the other part was done in National Cancer Institute, Cairo University, Egypt during the period between 2017-2019.

Drug and chemicals: 5-Fluorouracil, simvastatin and other chemicals were purchased from Sigma Aldrich Co. (Saint Louis, Missouri, USA). Dulbecco's Modified Eagle Medium (DMEM), Fetal Calf Serum (FCS), trypsin-EDTA (0.05%) and phosphate buffer (PBS pH 7.4) were purchased from thermo Fisher Scientific Inc (USA). Acridine Orange (AO) (Molecular Probes, Eugene, OR, Cat. No. A1301) and Rhodamine 123 (R8004 Sigma) have been also supplied. The cells cycle kit was purchased from Cayman Chemical Company (USA).

Formulation of drug loaded nanoparticles: Solid Lipid Nanoparticles (SLN) were carried for each of 5-Fluorouracil and simvastatin drugs through several steps according to Abdelbary and Fahmy[18]: First accurately weighed the solid phase (2% of Precinol and 0.5% of Phosphatidylcholine) which is then exposed to water path at highly melting points temperature 65-70°C, then 20 mg of each studied drug was added. The second step by melting the lipid phase which is 2.5% of gelacine with 20 mL sterile water, after ensure melting of materials with drugs, then followed by homogenization step for 3 min at speed 16000 rpm and the final step was ultra-sonication for 5 min, the control of formula as Plain-SLN was prepared from both solid and lipid phase without mains drugs. All the SLN formulation was filtered with 0.02 sterile millipore filter before each experiment. The UV scanning and construction of standard calibration curve of each formula of 5-Fluorouracil and Simvastatin were subjected.

Determination of drug Encapsulation Efficiency (EE): The drug load was calculated using a standard HPLC method[18]. The nanoparticle formulations of each formula of 5-Fluorouracil and simvastatin were evaluated for entrapment efficiency by the following equation:

$$\text{EE (\%)} = \frac{\text{Amount of drug entrapped}}{\text{Original amount of drug used}} \times 100$$

***In vitro* release study:** Dialysis method was used to determine the *in vitro* release profile. Loaded nanoparticles were fixed into the dialysis bag and were carried out using the USP dissolution apparatus[18].

Cells and cell culture: The HCT-116 human CRC were obtained from Pharmacology Unit, National Cancer Institute, Cairo University Egypt. Cells were grown as monolayer in DMEM, supplemented with penicillin G and streptomycin antibiotics and 10% FCS and cultured at 37°C in a humidified incubator containing 5% CO_2.

Assessment of cytotoxicity activity: Cytotoxicity was determined using the SRB method as previously described by Skehan *et al.*[19]. Cells were seeded in 96 well microtiter plates at a concentration of 40×10^3 cells/well in DMEM medium. The cells were kept attaching for 24 h at 37°C, then incubated with different concentration of 5-FU and SMV simultaneously for 72 h in the following range 0.1, 0.5, 1, 5 and 50 μM, respectively for 5-FU and 5 μM of SMV (3 wells for each concentration). After 72 h, the cells were fixed by adding 50 μL of the fixative reagent and kept for 1 h at 4°C. The supernatant was discarded and the plates washed 5 times with bidistilled water and air-dried. The plates stained for 30 min at room temperature in dark with 0.4% of SRB which dissolved in 1x dye solution. The unbound dye was removed by washing 3 times with 1x dye wash solution and plate was air dried. The bound stain was solubilized with 100 μL/well SRB solubilization buffer for 10 min. Finally, the optical density was read in absorbance microplate reader (Bio Tek, ELx808, USA) at the wavelength (490-530 nm).

Calculations:

$$\text{Surviving fraction (\%)} = \frac{\text{Optical density of treated cells}}{\text{Optical density of control cells}} \times 100$$

IC_{50} (Concentration of 5-FU necessary to produce 50% inhibition of cells growth) was calculated from linear regression equation of the survival fraction curve:

$$Y = mX + b$$

Where:

Y = 0.5 (Surviving fraction when there is a 50% inhibition of cell growth
m = Slop
X = Dose of 5-FU induces 50% inhibition
b = y-intercept

Assay of apoptosis: Apoptotic and necrotic cells can be and were analyzed by flow cytometry according to the method of Van Engeland *et al.*[20]. Cells were seeded in 6 well plates at a cell density of 10^5 cells/well in DMEM media (supplemented with 10% FBS and 1% streptomycin), then the wells were incubated for 24 h. Later, the cells were incubated with 5-FU concentration (1 and 5 μM) alone and/or with (5 μM) SMV simultaneously for 72 h (3 wells for each concentration). The cells medium was removed and washed with PBS. Then the cells were harvested with Trypsin/EDTA and washed with cold PBS and suspended in 200 μL annexin V incubation reagent prepared by mixing (binding buffer 10×, PI, annexin V-FITC and deionizes water) for each sample. The solution was incubated at room temperature in the dark for 15 min. About 400 μL of binding buffer were added to each sample and processed by flow cytometry (NAVIOS Beckman Coulter, USA).

Cell cycle analysis: Cells were seeded in 6 wells plates at a cell density of 10^5 cells/well in DMEM medium and cultured in CO_2 incubator at 37°C for at least 24 h before treatment. Then cells were incubated with 5-FU concentration (1 and 5 μM) alone and/or with (5 μM) SMV simultaneously for 72 h (3 wells for each concentration). The medium was removed and the wells washed with PBS and harvested with Trypsin/EDTA. Following trypsinization, cells were washed two times with cold assay buffer and cells pellet was re-suspended to a density of 10^6 cells/mL in assay buffer. One milliliter of fixative agent was added to each sample to fix and permeabilize the cells for at least 2 h prior to PI staining. Fixed cells were centrifuged at 500×g for 5 min and the fixative decanted thoroughly. The cells pellet was suspended in staining solution which prepared by mixing (10 mL of assay buffer with 200 μL RNase reagent A and 200 μL PI dye) for every 20 samples to be stained and then the cells were incubated for 30 min at room temperature in the dark. Cell cycle analysis was performed by using flow cytometry (Becton DICNSON (BD) FACS Caliber, USA)[21].

Assessment of cellular reactive oxygen species: Cells were seeded in black 96-well plate overnight at 4×10^3 cells/100 μL per well. About 10 μL of ROS red stain stock solution was added to 5 mL of assay buffer mixed and 100 μL/well of ROS red working solution was added into the cell plate. The cell plate was then incubated in a 37°C in 5% CO_2 incubator for 1 h. The cells were then treated with 20 μL of 11X from both 5-FU and SMV concentrations. The changes in fluorescence intensity[22] were then monitored (Ex/m = 520/605 nm).

Statistical Analysis: Statistical analysis of the data was carried out using computer program Statistical Package of Social Sciences (SPSS, version 18) and stat graphic computer package (Excel, 2007). Data are expressed as mean±standard deviation (M±SD) and analyzed using one-way Analysis of Variance (ANOVA) followed by Tukey-Kramer multiple comparisons between experimental groups. A student t-test was used for comparison between the mean in raw drug and the corresponding mean in SLN formulation. Differences were considered significant at $p<0.05$.

RESULTS

***In vitro* release study:** Following 24 h release study, faster release appeared with 5-FU solid lipid nanoparticles as shown in Fig. 1a, 90% of 5-FU released during the1st 4 h. While the results of SMV revealed a biphasic sustained release pattern, where 53.86% was the initial amount released after 4 h, which is usually attributed to the fast release of drug entrapped near the surface of the nanoparticles (Fig. 1b). After the initial phase, the SMV released profile as shown in Fig. 1b was characterized by a slow phase about half of drug released after 24 h.

Effect of 5-FU and/or simvastatin on the growth of HCT-116 cells: Table 1 Showed the cytotoxic activity of 5-FU either raw or SLN alone or with (5 μM) SMV against the proliferation of human HCT-116 cells. A concentration-dependent cytotoxicity curves were established and the IC_{50} were 12.69 μM for 5-FU raw drug. Treatment with SLN 5-FU alone caused IC_{50} to decrease significantly to its half value 5.98 μM. Co-treatment of HCT-116 cells with SLN 5-FU/SMV simultaneously dramatically increased the cytotoxic effect of 5-FU manifested as marked significant decrease in IC_{50} which decreased to 1.58 μM.

Effect of 5-FU and/or simvastatin on induction of apoptosis: The effects of 5-FU treatment with or without SMV on the induction of apoptosis in HCT-116 cells are shown in Table 2. The percentage of late apoptotic cells showed a significant increase after treatment with 5-FU or 5-FU plus SMV, compared to control cells. Treatment with 5 μM SMV and 1 μM 5-FU resulted in a significant 30.66 and 71.90% increase of late apoptotic cells, respectively. While combination of 1 μM 5-FU plus 5 μM SMV obviously increased percentage of late apoptotic. Similarly, treatment with 5 μM 5-FU showed 78.37% of late apoptotic cells. While combination of 5 μM 5-FU+5 μM SMV showed 81.94% of late apoptotic cells. Comparing the percentage of late apoptosis between SLN form and raw drugs treatment in HCT-116 cells, the percentages of late apoptosis were significantly higher when cells were treated by SLN 5-FU alone either with 1 or 5 μM. In the same manner, the sensitivity of cells to SLN SMV increased the percentage of late apoptotic when combined with 5-FU especially with 5 μM treatment.

Table 1: Effect of raw 5-FU and/or SMV 5-FU alone or in formulation of SLN on the growth of HCT-116 cells

Treatments	IC_{50} (μM)
5-FU (raw)	12.69±0.39
5-FU (raw) and SMV (5 μM) supplied simultaneously	6.11±0.054[a]
5-FU (SLN)	5.98±0.16[a]
5-FU (SLN) and SMV (5 μM) supplied simultaneously	1.58±0.054[a,b]

Data are expressed as Mean±SD of 3 separate experiments, each performed in triplicate, IC_{50}: Concentration of 5-FU necessary to produce 50% inhibition in the growth of cells, Statistical analysis was determined by one-way ANOVA followed by the Tukey-Kramer multiple comparison test, [a]Significant different from raw 5-FU, [b]Significantly different from SLN 5-FU at $p<0.05$, 5-FU: 5-Fluorouracil, SLN: Solid Lipid Nanoparticles

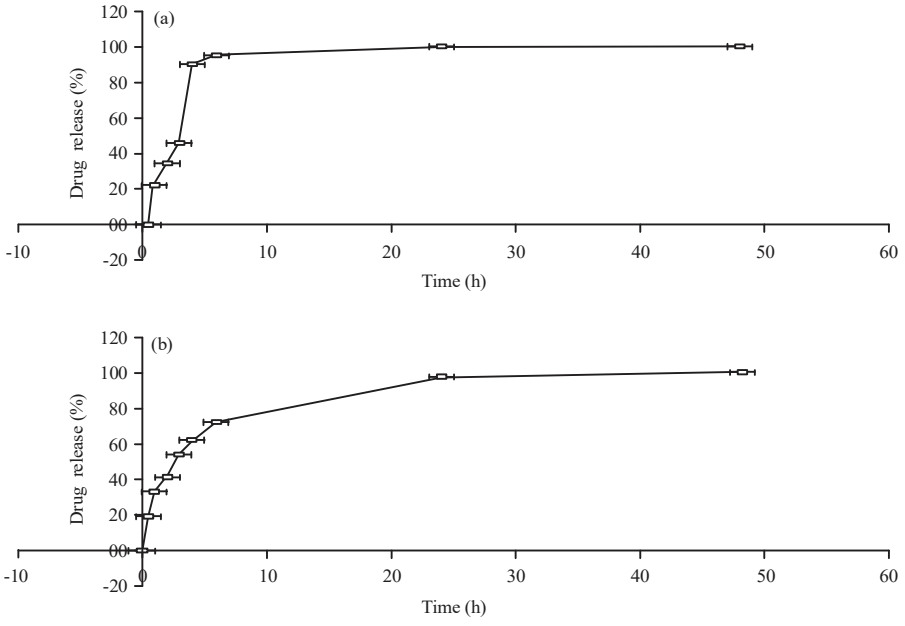

Fig. 1(a-b): Drug release profile of (a) 5-FU SLN and (b) SMV SLN

Table 2: Effect of 5-FU and/or SMV treatment on induction of late apoptosis following treatment by SLN formulation and raw drug in HCT-116 cells

	Late apoptosis (%)	
Treatments	Raw drug	SLN formulation
Control	0.28±0.04	02.90±0.63[e]
SMV 5 μM	30.66±0.12[a]	57.93±0.16[a,c,e]
5-FU 1 μM	71.90±0.15[a]	74.20±0.20[a,c,e]
5-FU 1 μM+5 μM SMV	79.66±0.05[a,b]	81.65±0.13[a,b,c,e]
5-FU 5 μM	78.37±0.09[a,b]	84.51±0.10[a,b,e]
5-FU 5 μM+5 μM SMV	81.94±0.04[a,c,d]	86.30±0.31[a,c,d,e]

Apoptosis was analyzed when the cells exposed to drugs for 48 h then staining with annexin-FITC and PI, Data are expressed as Mean±SD of 3 separate experiments each performed in triplicate. Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer multiple comparison between means within raw drug, Means of SLN formulation, A student t-test was used for comparison between the mean in raw drug and the corresponding mean in SLN formulation, [a]Significant change from control, [b]Significant change from 5-FU 1 μM alone, [c]Significant change from 5-FU 5 μM alone, [d]Significantly change from 5-FU 1 μM+SMV 5 μM, [e]significant change for SLN formulation versus corresponding raw drug at p<0.05, 5-FU: 5-Fluorouracil, SLN: Solid Lipid Nanoparticles

Table 3: Effect of 5-FU and /or SMV treatment in SLN formulation and pure drug on cell cycle phase distribution of HCT-116 cells

	Sub G_1 (%)	
Treatments	Raw drug	SLN formulation
Control	03.3±0.05	03.6±0.05
SMV 5 μM	37.4±0.05[a]	48.5±0.03[a,e]
5-FU 1 μM	03.6±0.08	10.8±0.03[a,e]
5-FU 1 μM+SMV 5 μM	22.2±0.03[a,b]	50.3±0.05[a,b,e]
5-FU 5 μM	10.9±0.03[a,b]	14.0±0.03[a,b,e]
5-FU 5 μM+SMV 5 μM	43.6±0.05[a,b,c,d]	56.2±0.05[a,b,c,d,e]

Data are expressed as Mean±SD of 3 separate experiments each performed in triplicate, Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer multiple comparison between means within raw drug and the means of SLN formulation, A student t-test was used for comparison between the mean in raw drug and the corresponding mean in SLN formulation, [a]Significant change from control, [b]Significant change from 5-FU 1 μM alone, [c]Significant change from 5-FU 5 μM alone, [d]Significant change from 5-FU 1 μM+SMV 5 μM, [e]Significant change for SLN formulation versus corresponding raw drug at p<0.05, 5-FU: 5-Fluorouracil, SLN: Solid Lipid Nanoparticles

Effect of 5-FU and/or simvastatin on the cell cycle phase progression of HCT-116 cells: Table 3 shows the effect of various SLN treatments formulation of 5-FU and/or SMV on cell cycle distribution. HCT-116 cells were treated with 5-FU concentrations (1 or 5 μM) either alone or in combination with SMV 5 μM. Treatment with either SMV 5 μM or 5-FU 1 μM alone, showed a preferential block in Sub G1 and S phases at the expense of G1/G0 phase cells, where, there were 48.5 and 10.5% accumulations, respectively. On the other hand, combined treatment with 5-FU 1 μM and SVM 5 μM showed a significant increase in the percentage of cells in sub G1 by 50.3% compared with 10.8% in case of 5-FU 1 μM alone. In addition, increasing the concentration of 5-FU to 5 μM in presence of SMV 5 μM showed a significant increase of cells in sub G1 by 56.2% compared to 14% in treatment with corresponding 5-FU 5 μM alone.

Table 4: Effect of raw 5-FU and/or SMV alone or in SLN formulation on induction of ROS content in HCT-116 cells

	ROS content relative fluorescence unit	
Treatments	Raw drug	SLN formulation
Control	100.00±4.80	100.0±4.58
SMV 5 μM	120.76±4.05[a]	125.5±4.25[a]
5-FU 1μM	115.18±2.60[a]	119.9±2.7[a]
5-FU 1 μM+SMV 5 μM	130.91±4.91[a,b]	145.2±3.9[a,b,e]
5-FU 5 μM	125.50±2.2[a,b]	135.6±2.9[a,b,e]
5-FU 5 μM+SMV 5 μM	135.85±2.34[a,b,c]	161.5±2.6[a,b,c,d,e]

ROS content was analyzed when cells stain with ROS red dye then exposed to drugs, Data are expressed as ROS Content Relative fluorescence unit (Mean±SD) of 3 separate experiments each performed in triplicate taking the control values as 100%, Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer multiple comparison between means within raw drug and the means of SLN formulation, A student t-test was used for comparison between the mean in Raw drug and the corresponding mean in SLN formulation, [a]Significant change from control, [b]Significant change from 5-FU 1 μM alone, [c]Significant change from 5-FU 5μM alone, [d]Significant change from 5-FU 1 μM+SMV 5 μM, [e]Significant change for SLN formulation versus corresponding raw drug at p<0.05, 5-FU: 5-Fluorouracil, SLN: Solid Lipid Nanoparticles

Effect of 5-FU and/or SMV treatment in SLN formulation and raw drug on ROS production: To examine whether treatment with 5-FU and/or SMV lead to accumulation of intercellular ROS in HCT-116, the level of intracellular ROS was determined in HCT-116 after treatment with 5-FU and/or SMV in raw and SLN formulation. As shown in Table 4, intracellular accumulation of ROS is significantly increased when cells treated with 5 μM SMV alone. When cells treated by 5-FU 1 μM alone as compared to control. Combined treatment with SMV 5 μM plus 5-FU 1 μM resulted in a significant increase in ROS accumulation in HCT-116 cells. Moreover, when the concentration of 5-FU was increased to 5 μM, the results showed that intercellular ROS content increased when given alone or combined with 5 μM SMV, respectively. The SLN formulation of 5-FU increased ROS accumulation by about 10% compared to 5-FU raw, while in combination with SMV in SLN form ROS accumulation increased by about 61.5% in compare to control and 25.65% compare to combination of raw.

DISCUSSION

Nanotechnology is one of the most popular areas of scientific research, particularly used in developing a new generation of drug delivery systems with greater targeting selectivity and better delivery efficiency[23,24]. The 5-FU is the most widely used cytotoxic drug in the treatment of many kinds of tumors, especially CRC, either alone or in combination with other cytocidal drugs. However, its clinical uses are limited by its adverse effects, short half-life and disease

resistance[25]. Recent studies have discovered new protocols, compounds, enzymes and molecular alteration that reduced the side effects of anticancer drugs and enhanced their cytotoxic effects[5,6,8,24,26-28].

Simvastatin is one of the most frequently prescribed drugs due to its effectiveness when used to treat hyperlipidemia[29]. Previously, statins were identified to reduce proliferation and induce apoptosis in several cancer cells[30]. In an attempt to increase the cytotoxic activity of 5-FU and minimizing its toxicity, SLN combined form of 5-FU and SMV as drug delivery vehicles that form a nanosized solution as new strategy to increase its effectiveness, therefore reducing the dose of antitumor and reducing the toxicity. Therefore, the current study focused on investigating whether SLN combined form would enhance the cytotoxic effects of 5-FU against growth of HCT-116 human cell line. In the current study, treatment of human HCT-116 cells lines with different concentrations of 5-FU SLN significantly enhanced the cytotoxic activity of 5-FU, as evidenced by 6.1-fold decrease of IC_{50} against the growth of HCT-116 cells for 5-FU SLN (5.98 μM) compared to free 5-FU (12.69 μM) (Table 1). Daumar *et al.*[31] reported that due to high rate of metabolism of 5-FU in the liver and blood, maintenance of a therapeutic plasma concentration requires the continuous administration of high doses which may lead to severe toxicity if the drug concentration exceeds a critical limit. So that, by encapsulating 5-FU in a nanocarrier, the dihydropyrimidine dehydrogenase attack on 5-FU could be reduced or avoided and efficacy of 5-FU could significantly be improved while its associated toxicity would be greatly reduced.

On the same line, Udofot *et al.*[32] reported that, nanocarriers as anticancer drug delivery systems are generally designed to improve, for example high drug loading capacity and prolonged systemic circulation.

In the current study, treatment of HCT-116 cells with different 5-FU SLN concentrations showed more cytotoxic activity to the tumor cells. The cytotoxic effect of 5-FU SLN has been confirmed by significant increase in the percentages of late apoptosis and arrest of the cells at sub G1 phase compared with control and 5-FU treated cells (Table 3). In agreement with the above results, Udofot *et al.*[32] reported that the treatment with 5-FU liposomal nanoparticles resulted in significant cytotoxic effect compared to pure 5-FU against HT-29 and HCT-116 cancer cells. The increase in the 5-FU cytotoxicity in cells treated with 5-FU SLN could be explained by the unique pathophysiologic characteristics of tumor vessels that enable macromolecules, including nanoparticles, to selectively accumulate in tumor[33]. These features are called Enhanced Permeability and Retention (EPR), which represent a crucial mechanism by which nanoparticles can selectively accumulate in the tumor tissues[34]. As a result of EPR, 5-FU SLN targeted more inside the tumor tissues which may contribute to more cell death. Furthermore, the SLN retained all the advantages associated with nano-scale drug carriers such as prolonged release of drug, enhanced drug permeability and retention, tumor targeting and reducing the toxicity of the incorporated drug[35]. Statins exhibit a number of effects on cancer cells[36] including inhibition of cancer cell growth, metastasis and invasion, angiogenesis and the induction of apoptosis. By inhibiting the mevalonic acid pathway, statins reduce the levels of the isoprenoid intermediates Farnesyl Diphosphate (FPP) and Geranylgeranyl Diphosphate (GGPP) which are critical for post-translational modification of the intracellular G-proteins[37]. These proteins, in turn, are essential for the gene transcription involved in cell proliferation, differentiation and cells apoptosis[36]. Therefore, it could be stated that SMV is an ideal candidate through its ability to modulate a number of signaling molecules, chemo-sensitizing CRC cells to the cytotoxic effect of 5-FU.

In the current study, IC_{50} for treatment of HCT-116 cells was significantly decreased by 3.5-folds, after simultaneous addition of SMV SLN with 5-FU SLN (from 5.98-1.58 μm) (Table 1). These results have been confirmed by induction of percentages of apoptosis when SMV SLN was added to the 5-FU SLN. The percentage of cells in late apoptosis was significantly increased after combination treatment compared to control and corresponding pure 5-FU treated cells. These results agree with that reported by Buranrat *et al.*[38], who investigated the role of SMV in modulating doxorubicin cytotoxicity by enhancing the growth inhibition and anticancer activity of doxorubicin in human breast cancer cell line (MCF-7). This could be explained based on the ability of SMV to inhibit ras-related C3 botulinum toxin substrate 1 (Rac1) pathway and induce caspase and cytochrome c-dependent apoptosis in a process involving oxidative stress. Recently it has been shown that the SMV liposomal form is much more effective than its free form in *in vivo* treatment of colon carcinoma[39]. Kang *et al.*[40] have developed self-micro emulsifying drug delivery system for SMV. The authors have conducted bioavailability studies in beagle dogs and were successful in achieving 1.5-fold increase in oral bioavailability. Similar studies are also recently reported by Zhang *et al.*[41] where a 2-fold increase in oral bioavailability was observed for SMV SLNs. the current results showed that the HCT-116 cells, treated with low concentration of 5-FU SLN, showed a significant increase in percentages of arrested cells at late apoptosis (Table 2), compared to cells treated with free 5-FU.

The increase in cytotoxic and apoptotic effect of 5-FU when SMV was simultaneously added have been further confirmed by observed increase in arrested cells in Sub G_1 (Table 3). This could be explained by the ability of SMV to decreased cell viability and induced cell apoptosis in HCT116 cells through the modulation of p21 (cip/Waf1) and survivin. Moreover, SMV caused an increase in p53 phosphorylation and acetylation[42]. These results could be attributed to the nano sized solution as a result of EPR effect and also related to the underlying mechanism for SLN internalization to cells rather than merely a simple passive permeation, thus reducing excretion of 5-FU from the cells. This suggests the long presence of 5-FU inside the tumors due to the SLN formulation, it can effectively activate apoptosis by inhibiting DNA replication[43]. It's well-known that ROS are involved in a variety of physiological and pathological processes intracellularly[27,44]. In addition to programmed cell death, necrotic cell death is observed when the levels of ROS in cancer cells exceed those necessary for inducing apoptosis and autophagy and this has been reported in multiple myeloma, prostate cancer, hepatoma and leukemia cells[45]. In the current study, treatment with 5-FU and/or SMV led to accumulation of intercellular ROS in HCT-116. Moreover, the results showed that intercellular ROS content increase by 1.9-folds when given combined with 5 μM SMV than 5-FU alone (Table 4). Qi *et al.*[46] reported exposure of CT26 colon carcinoma cells to SMV caused significant apoptotic cell death and perturbations in parameters indicative of oxidative stress. The signaling pathway induced by SMV disturbed the antioxidant defense system by suppressing the expression of ROS scavengers, thereby inducing oxidative stress and apoptotic cell death. In the present study, SMV SLN alone increased intercellular ROS by 25% from control (Table 4).

CONCLUSION

Collectively, these results demonstrated that simvastatin induces colon cancer cell death at least in part by increasing intracellular oxidative stress and inducing apoptosis. It seems that SLN improved the cytotoxic activity of 5-FU and may reduce their systemic toxicity as a result of physical and chemical characteristic of nano-size itself and the unique pathophysiologic characteristics of tumor cells which passively accumulate 5-FU SLN inside them.

SIGNIFICANCE STATEMENT

This study discovered the importance of formulation of the nano particle of both 5-FU and simvastatin. It proved that nano particle more cytotoxic that the raw drugs. This can be beneficial for treatment of colorectal cancer with little toxicity of the anticancer 5-FU. This study will help the researcher and oncologist to uncover the critical areas of toxicity of the anticancer agents that many researchers were not able to explore. Thus a new theory on the use of nano particle in cancer therapy may be arrived.

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