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ORIGINAL ARTICLE

A mechanistic study of the effect of transferrin conjugation on cytotoxicity of targeted liposomes

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ABSTRACT

This study was performed to prepare 5-fluorouracil (5FU) containing targeted liposomes for the safety and efficacy enhancement. Liposomes were prepared using thin layer method and transferrin (Tf) was employed as the targeting ligand. Morphology of 5FU-loaded liposomes was assessed by transmission electron microscopy (TEM). The *in vitro* cytotoxicity was investigated *via* MTT assay on HT-29, CT26 and fibroblast cells. Mitochondrial membrane and cell death evaluations were also investigated. Resulted showed that the encapsulation efficiency (EE%) and particle size of the liposomes were 40.12% and 130 nm, respectively. TEM image implied that liposomes were spherical in shape. In cancer cells, targeted liposomes triggered the mitochondrial apoptotic pathway by lower production of reactive oxygen species (ROS) (63.58 vs 84.95 fluorescence intensity), reduced mitochondrial membrane potential and releasing of cytochrome c (68.66 vs 51.13 ng/mL). The results of this study indicated that Tf-targeted 5FU liposomes can be employed as promising nanocarrier for the delivery of drugs to cancer cells.

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KEYWORDS
5-Fluorouracil; liposome; transferrin; cancer; apoptosis

1. Introduction

Colon cancer is the third most common neoplasm that causes morbidity in the world. The incidence of colorectal cancer has increased over the last few years so that every year, 1.36 million new patients are recognised with colon cancer (Ali *et al*. 2013a, 2013c, 2013d, 2015; Imran *et al*. 2012; Zhang *et al*. 2016; Banerjee *et al*. 2017a). Various strategies have been developed for treatment of cancer such as chemotherapy, surgery, radiotherapy and stem cell therapy (Zununi Vahed *et al*. 2017). Chemotherapy is the most common approach for colon cancer patients; however, its efficacy is largely limited due to severe side effects of anticancer drugs on normal tissues (Ali 2011; Jin *et al*. 2011; Ali *et al*. 2013b; Zununi Vahed *et al*. 2017). Consequently, it is needed to employ an ideal drug-delivery system that can improved the efficacy of therapeutic agents while decreasing their toxic side effects (Ali *et al*. 2013e, 2014, 2016, 2017; Saleem *et al*. 2013; Zununi Vahed *et al*. 2017). Recently, application of nanoparticles (NPs) as drug-delivery system for improving treatment of cancer is extensively considered (Banerjee *et al*. 2017a). Among the various nanocarriers, liposomes have attracted considerable interest as drug-delivery system due to their ability to entrap both hydrophilic and hydrophobic drugs, non-toxicity, biocompatibility, biodegradability and ability to reduce the side effect of the encapsulated drugs (Guo *et al*. 2015; Pereira *et al*. 2016; Zununi Vahed *et al*. 2017). For improving the selective delivery of drug to specific tissues, targeting approaches with the conjugation of ligands to the surface of liposomes have been widely studied. Transferrin, folate, polysaccharides and growth factor receptors overexpress on the surface of cancer cells compared to normal cells (Banu *et al*. 2015; Wang *et al*. 2017). Transferrin receptor (TfR) overexpressed on cancer cells 100-fold higher than in normal cells due to their higher iron requires for rapid proliferation (Singh *et al*. 2016). Therefore, transferrin has been explored as a targeting ligand for site-specific delivery of drugs into cancer cells.

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Mechanism of action of many chemotherapeutic agents was studied extensively. The major goal of cancer chemotherapy is induction of apoptosis in cancer cells (Siddik 2003) and mitochondrion is the main organelle for apoptosis induction. Employing of targeted drug-delivery systems can change the amount of drug in contact with cancer cells which may improve the efficacy of anticancer drugs. This approach can also affect cell organelles such as mitochondria to trigger cell death in a way different from traditional formulations which can emerge as promising new means for cancer treatment.

5-Fluorouracil (5FU), a pyrimidine analogue, is widely used in the treatment of colon cancer. It interferes with nucleoside metabolism and can be incorporated into DNA and RNA, which causes cytotoxic effect on cells (Zhang *et al*. 2008; Di Martino *et al*. 2016). Although its efficacy is restricted due to short plasma half-life (15–20 min) and its systemic side effects to normal tissues (Krishnaiah *et al*. 2003; Sun *et al*. 2008). Encapsulation of 5FU in nanocarrier can potentially improve its effectiveness and reduce its side effects.

The aim of this study was to prepare and characterise 5FU-loaded transferrin-targeted liposome. Moreover, to determine whether targeted liposomes could modify the mechanism by which 5FU triggers, we further investigated the associated mechanism of cell death in cancer cells.

2. Materials and methods

5FU and cholesterol were obtained from Acros, USA and Merck, Germany, respectively. dipalmitoylphosphatidylcholine (DPPC) was purchased from Lipoid, Germany. Tf-DSPE synthesis and preparation were outlined in detail elsewhere (data not shown here). Metronidazole was kindly donated by Pars Darou Pharmaceutical Co., Iran.

Human colorectal adenocarcinoma (HT-29) and fibroblast (Hu02) cells were purchased from Iranian Biological Resource Center (IBRC). Murine colon carcinoma (CT26) was obtained from National Cell Bank of Iran (NCBI), Pastor Institute of Iran.

Dulbecco's modified eagle's medium (DMEM) and foetal bovine serum (FBS) were acquired from Gibco, USA. Penicillin–streptomycin and 5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma-Aldrich, Germany. Paraformaldehyde, 5(6)-carboxyfluorescein (CF) and 4, 6-diamidino-2-phenylindole (DAPI) were provided from Sigma-Aldrich, Germany.

2′, 7′-Dichlorofluorescin diacetate (DCFDA) was purchased from Sigma-Aldrich, Germany. MitoLight™ Apoptosis Detection kit was acquired from Merck Millipore, USA. Cytochrome c human ELISA kit was obtained from Abcam, USA and Annexin V-FITC apoptosis detection kit was provided from Sigma Aldrich, USA. Male Wistar rats were obtained from the Pasteur Institute of Iran. Other chemicals and solvents were of analytical grade and purchased from Merck, Germany.

2.1. Preparation of liposomes

Liposomes were prepared using thin-film hydration method. DPPC/cholesterol/Tf-DSPE at different molar ratio of 2:1:0.0006 and 2:2:0.0061 were dissolved in chloroform in a round bottom flask. Organic solvent was removed under rotary evaporation (Heidolph, Germany) to form thin lipid film. The lipid film was rehydrated by phosphate buffer saline (PBS, pH 7.4) containing 5FU (1.5 mg) by sonication in a water bath (Elma, Germany) at 55 °C (above phase transition temperature of DPPC) for 30 min (Thakur *et al*. 2014). The suspension then homogenised (Heidolph, Germany) for 5 min and non-encapsulated 5FU separated by centrifugation at 15,000 rpm for 10 min (MPW-350R, Poland). Ultimately, the liposomal suspension was lyophilised and stored at 4 °C for further analysis.

2.2. Determination of encapsulation efficiency

Determination of 5FU loaded in liposomes was performed using high-performance liquid chromatography (HPLC, Waters, USA). The analysis was carried out on C_{18} column (250 × 4mm i.d., 5 μm) at 30 °C. Mobile phase was 0.02 M phosphate buffer pH 4 and methanol at ratio of 70:30 (V/V) and injection volume was 50 μL. Flow rate of 0.8 ml/min was used and wavelength was set at 260 nm. Metronidazole was used as internal standard. The encapsulation efficiency (EE%) was determined according to the following Equation (1):

$$EE\% = (T - C/T) \times 100 \quad (1)$$

where *T* is the amount of 5FU initially added to the formulation and *C* is the amount of the free drug in the supernatant after centrifugation (Negi *et al*. 2015).

2.3. Morphology study and particle size of liposomes

Morphology of liposomes was observed by transmission electron microscopy (TEM, LEO 906, Zeiss, Germany). A droplet of samples dispersed in deionised water was placed on a carbon-coated copper TEM grid and then dried at room temperature. Particle size of

liposomes was also analysed using a particle sizer (Qudix, Scatteroscope I system, Korea) at 25 °C. Samples were diluted with deionised water and sonicated for 5 min prior to measurement.

2.4. Cellular uptake

CF-loaded-targeted liposomes were prepared to study the cellular uptake of liposomes for the reason that 5FU not fluoresce normally. HT-29 cells were seeded onto six-well plates and incubated for 24 h. The culture medium was replaced with growth medium containing CF and CF-loaded-targeted liposome and incubated for 4 h at 37 °C. Then, the culture medium was removed and cells were washed with cold PBS. Then cells were fixed with 4% (w/v) paraformaldehyde for 15 min and further stained with 4, 6-diamidino-2-phenylindole (DAPI) for 10 min. The intracellular uptake of CF in the cells was observed using fluorescent microscope (Olympus IX71, Japan).

2.5. Cytotoxicity assay

In vitro cytotoxicity activity was analysed using MTT method. The MTT assay is based on the cleavage of a yellow tetrazolium salt by the mitochondrial dehydrogenase of viable cells to purple formazan crystals (Lee and Low 1995; Waheed *et al*. 2013). HT-29 and CT26 (as colon cancer cell lines) and fibroblast (as normal cell) were grown at 37 °C, 5% CO_2 and 95% relative humidity in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) penicillin–streptomycin. Cells were cultured in 96-well plates at a seeding density of 1×10^4. After incubation for 24 h, the medium was replaced with fresh medium containing different concentrations of 5FU, liposomal 5FU and Tf-liposomal 5FU for 48 h. At the end of incubation time, mediums were removed and 20 μL of MTT (5 mg/mL in PBS) solution was added into each well and incubated at 37 °C for 4 h. Then, dimethyl sulfoxide (DMSO) (150 μL) was added in each well and gently shaken for 20 min. The absorbance of each plate was read at 570 nm using ELISA plate reader (BioRad, USA). Cellular viability was determined according to Equation (2) and the half maximal inhibitory concentration (IC_{50}) was also calculated as cytotoxicity index.

$$\text{Cell viability } \% = \left(\text{Abs}_{\text{sample}}/\text{Abs}_{\text{control}}\right) \times 100 \quad (2)$$

It should be mentioned that based on MTT results, no significant differences in cell viability were found between 5FU and liposomal 5FU in cancer cells. Hence, further investigations as mentioned below were only performed for 5FU and Tf-liposomal 5FU at their IC_{50} concentrations.

2.6. Measurement of reactive oxygen species

Intracellular reactive oxygen species (ROS) production was recorded by monitoring changes in the 2′, 7′-dichlorofluorescin diacetate (DCFDA). This compound is a non-fluorescent molecule that passively diffuses into cells and cleaved by intracellular esterases. Then, it oxidises by intracellular ROS into fluorescent 2′, 7′-dichlorodihydrofluorescein (DCF) (Wang and Joseph 1999). Cells were seeded into six-well plate at density of 1×10^5 and allowed to attach for 24 h. Then, cells were treated with free 5FU and Tf-liposomal 5FU for 1, 3 and 48 h. After treatment, cells were washed with PBS and exposed with 10 μM DCFDA for 45 min at 37 °C. Fluorescence was monitored at excitation wavelength of 485 nm and emission wavelength of 530 nm using spectrofluorimeter (PerkinElmer, USA).

2.7. Mitochondrial transmembrane potential ($\Delta\Psi_m$) analysis

The $\Delta\Psi_m$ change was analysed using MitoLight™ apoptosis detection kit (Merck Millipore, USA). Briefly, HT-29 cells (1×10^5 cell/well) were seeded into six-well plate for 24 h. Cells were incubated with 5FU and Tf-liposomal 5FU for 48 h. Then, cells were incubated with MitoLight™ solution for 20 min at 37 °C in the dark. Cells were centrifuged and re-suspended in incubation buffer and analysed using flow cytometry (Facs Calibur, BD, USA).

2.8. Release of cytochrome c from mitochondria

Determination of cytochrome c released from the mitochondria to the cytoplasm was performed using cytochrome c human ELISA kit according to the manufacturer's instructions (Abcam, USA). Briefly, cells were treated with 5FU and Tf-liposomal 5FU for 48 h. Then 100 μL of samples was added to the microplates and subsequently biotin conjugated antibody (50 μL) was added to each well and incubated at room temperature. After incubation time, streptavidin-horseradish peroxidase (HRP) (100 μL) was added to all wells and incubated at room temperature for 1 h. Afterward, TMB solution (100 μL) was pipetted into each well. Upon a dark blue colour was observed, the enzyme reaction was stopped by adding stop solution (100 μL). Cytochrome c release was analysed by

measuring absorbance at 450 nm using an ELISA plate reader (BioRad, USA).

2.9. Determination of apoptosis

The rate of Apoptosis in the HT-29 cells was measured using Annexin V-FITC apoptosis detection kit (Sigma Aldrich, USA). Cells at density of 1×10^6 were seeded into six-well plates for 24 h. Cells were exposed to 5FU and Tf-liposomal 5FU for 48 h. Collected cell pellets were washed and re-suspended in 100 µL of binding buffer. Then, 5 µL of Annexin V-FITC and 10 µL of propidium iodide (PI) were added and incubated at room temperature in dark for 20 min. The apoptosis rates were measured using a flow cytometry (Facs Calibur, BD, USA).

2.10. Haemolysis assay

Haemolytic test was carried out to evaluate the compatibility of liposomes with the red blood cells (RBC). RBCs of male Wistar rats were separated from plasma by centrifugation at 1500 rpm for 10 min, washed three times and diluted 1:10 using PBS. Liposomes (0.5 and 1 mg/mL) were added in RBCs suspension and incubated for 3 h at 37 °C. After incubation, the samples were centrifuged at 1500 rpm for 10 min. The supernatant was collected and the amount of haemoglobin released was determined by spectrophotometer (Biochrom WPA biowave II, England) at 540 nm. PBS and H_2O were employed as negative and positive controls, respectively. The haemolysis percentage was calculated according to Equation (3):

$$\text{Hemolysis } (\%): \left(A_s - A_{nc}/A_{pc} - A_{nc}\right) \times 100 \quad (3)$$

where A_s is the absorbance of sample, A_{nc} is the absorbance of negative control and A_{pc} is the absorbance of positive control.

2.11. Statistical analysis

All experiments were carried in triplicate and the results were presented as mean ± SD. Statistical analyses were performed using one-way ANOVA and statistical significance was set at $p < .05$.

3. Results and discussion

3.1. Characterisation of the liposomes

In this study, the effects of cholesterol on the EE% were investigated. The efficiency of drug encapsulation in liposomes at different molar ratios of DPPC to cholesterol; 2:1 and 2:2 were 22.55 ± 1.58 and 40.12 ± 0.23, respectively. According to the results, by enhancement of the amount of cholesterol, EE% of liposomes was increased. Cholesterol is a common component of liposomes and can enhance the stability, drug release and encapsulation (Liu *et al*. 2015). The present findings seem to agree with other researches which reported that rotational freedom of the phospholipid hydrocarbon chains reduced by cholesterol and it hinders water diffusion into the hydrocarbon phase in the lipid membrane. On the other hand, its inclusion increases water diffusion in the polar heads of the lipids and may result to improved hydrophilic drug accumulation in the liposomes (Eloy *et al*. 2014). However, our findings were contrary to other studies that implied the EE% of hydrophilic drugs was decreased because of reduction in the internal liposome volume after addition cholesterol (Giulio *et al*. 1991; Glavas-Dodov *et al*. 2005). The morphology of liposomes is shown in Figure 1. They were spherical in shape without any aggregation or fusion and homogenous size distribution. The particle size of liposomes was also 130 nm with polydispersity index (PDI) value of 0.23. Particle size is an important property of NPs which has a significant effect on drug distributions in different organs of the body, mainly cancer cells (Nag *et al*. 2016). 100 to 200 nm NPs can accumulate in solid tumours because of their enhanced permeability and retention (EPR) effects through the defected blood vessels and lymphatic drainage (Shigehiro *et al*. 2014). In this study, the mean particle size of liposomes was around to be 130 nm, which are suitable for internalisation into cancer cells. These findings were in agreement with Jin *et al*. (2016) results which reported that folic-acid-targeted NPs exhibited sizes about 100–200 nm which are able to target cancer cells because of EPR effect (Jin *et al*. 2016).

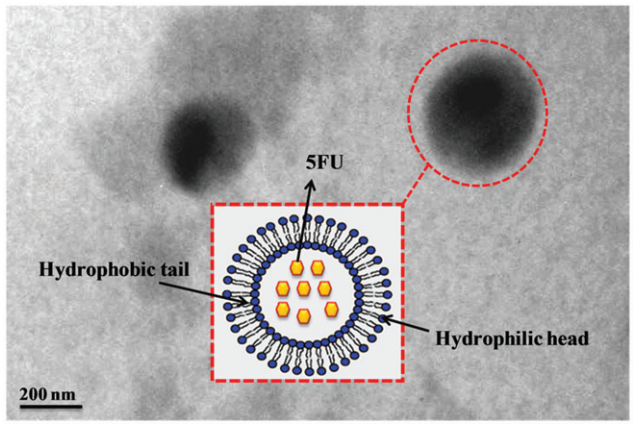

Figure 1. TEM image of liposomes.

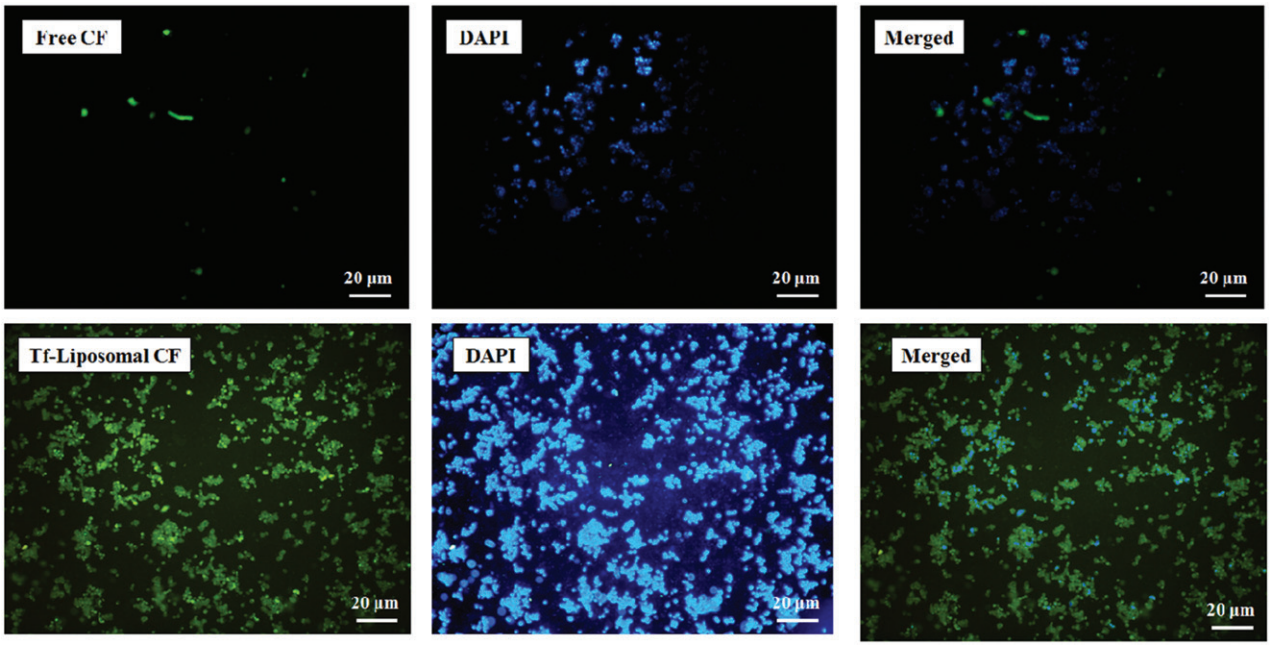

Figure 2. Cellular uptake of (A) free CF and (B) CF-loaded-targeted liposomes (left to right: green fluorescent signals are related to CF that is accumulated in the cytoplasm of the cells, the blue is corresponded to DAPI which is accumulated in the nuclei of cells, and final images at right show the merged CF and DAPI accumulation).

3.2. *In vitro* cellular uptake of NPs

To evaluate the intracellular uptake of liposomes, CF-loaded-targeted liposomes were prepared. Along with the green colour of liposomes containing CF, nuclei were stained with DAPI which appear in blue. CF was selected based on the similarity of the physicochemical properties with drug. According to Figure 2, CF-loaded-targeted liposomes exhibited higher fluorescence intensity than free CF which is indicating that more dye was taken up by cancer cells. These findings confirmed that targeted liposomes improved delivery of CF into cancer cells. These results are in parallel with cytotoxicity data (Figure 3). As a hydrophilic molecule, CF delivery like other hydrophilic molecules remains a challenge due to low intracellular absorption (Eloy *et al*. 2014). Moreover, because of low intracellular absorption, determination of the efficacy of hydrophilic drugs may be restricted *in vivo* (Eloy *et al*. 2014). Liposomes as nanocarrier can release encapsulated molecules in the cells and improve the low cellular uptake of hydrophilic drugs (Sadhukha and Prabha 2014). The present findings were in agreement with Sadhukha and Prabha's (2014) results, which reported that carboplatin, a hydrophilic drug encapsulated in NPs was taken up by the cells efficiently more than free drug (Sadhukha and Prabha 2014). The enhanced internalisation of CF-loaded-targeted liposomes may prove the hypothesis that transferrin-targeted liposomes are taken up by endocytosis. It is well established that endocytosis is one of the main mechanisms for the uptake of NPs (Zeng *et al*. 2014). Therefore, according to the present results, it can be concluded that higher cellular uptake of transferrin-targeted liposomes can be attributed to the binding of Tf to the TfR overexpressed on the cancer cell which facilitate the entry of therapeutic agents in the cells by receptor-mediated endocytosis (Wang *et al*. 2016). These findings support previous research of Soni *et al* (2008). They found that the brain uptake of Tf-liposomal 5FU was approximately 17 and 10 times higher than free drug and non-targeted liposomes, respectively. They also suggested that the higher uptake of targeted liposome was related to the presence of the higher number of TfR, which results in a greater access of targeted liposomes across the blood brain barrier (BBB) *via* receptor-mediated endocytosis (Soni *et al*. 2008). On the other hand, our findings confirmed the biocompatibility of liposomes as drug-delivery systems because efficient cellular uptake was demonstrated. As a result, it can be deduced that Tf-targeted liposomes could be employed as a drug delivery for the transport of drug molecules across cancer cells.

3.3. Evaluation of cytotoxic activity

The *in vitro* cytotoxicity assay was carried out on HT-29 and CT26 cells as colon cancer cells and fibroblast cells also were used as normal cells to evaluate

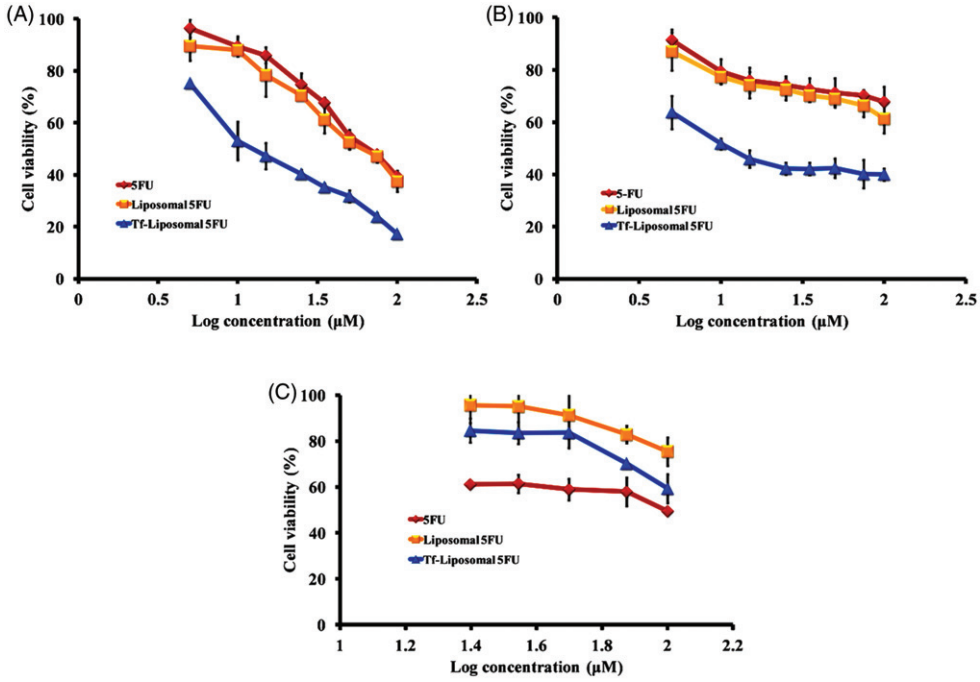

Figure 3. Cytotoxicity of different formulations of 5FU on (A) HT-29, (B) CT26 and (C) fibroblast by MTT method in DMEM medium at 37 °C for 48 h. Data were given as mean ±SD ($n = 3$).

Table 1. IC_{50} values (μM) for 5FU, liposomal 5FU and Tf-liposomal 5FU on various cells ($n = 3$).

Formulation	HT-29	CT26	Fibroblast
5FU	79.78 ± 1.3	–	141.25 ± 1.27
Liposomal 5FU	61.66 ± 1.12	562.34 ± 2.39	660.70 ± 3.66
Tf- liposomal 5FU	15.49 ± 2.20	15.135 ± 2.23	218.78 ± 1.14

whether targeted liposomes have any cytotoxic effect on normal cells. The IC_{50}s of every cell line are shown in Table 1. As Figure 3(A) and (B) shows, Tf-liposomal 5FU exhibited significant cytotoxicity compared to 5FU and liposomal 5FU in cancer cells ($p < .05$). The anti-proliferative activity has also been improved dose dependently. Moreover, as it could be seen from Figure 3(A) and (B), no significant difference was observed between cytotoxicity of 5FU and liposomal 5FU. It means that Tf was effective in promoting the internalisation of 5FU containing liposomes to the cancer cells with a rather reduced dose of 5FU (Sun and Sun 2016). It is well established that the effect of 5FU is lost very quickly after the administration due to its short half-life time. Encapsulated 5FU into targeted liposomes can primarily attach on the surface of cells and subsequently resulted in sustain release of the drug facilitate for longer time period activity (Jayaprakasha *et al.* 2016; Wang *et al.* 2015). In addition, it is suggested that Tf incorporation facilitates the entry of liposomes to the cancer cells *via* receptor mediated endocytosis (Nag *et al.* 2016). The effect of Tf-receptor blocking on the performance of Tf-targeted NPs was observed in the study of Mulik *et al.* (2010). With pre-addition of free Tf or in Tf-receptor blocked cells the anti-proliferative activity of Tf-targeted NPs was reduced by 1.5-fold compared to unblocked cells. These results confirmed the uptake of Tf-targeted NPs by Tf-receptor-mediated endocytosis in cancer cells (Mulik *et al.* 2010). The present findings agree with results of Nag *et al.* (2016). They reported that paclitaxel-loaded Tf-targeted NPs showed significant cytotoxicity than free drug and paclitaxel-loaded non-targeted NPs. They also indicated that conjugation of Tf to NPs could be one of the promising ways to transport of drugs to cancer tissue (Nag *et al.* 2016). Li *et al.* (2009) implied that doxorubicin-loaded Tf-targeted liposomes exhibited the most cytotoxic effect on cancer cell compared to free drug. They demonstrated that Tf was effective in promoting the internalisation of drug loaded in liposomes to the target cells (Li *et al.* 2009). The similar results were observed in the study of Sun and Sun (2016) which reported that Tf-targeted NPs displayed higher cytotoxic activity on cancer cells. This higher cytotoxic activity was attributed to the Tf-receptor-mediated cellular uptake which resulting the higher accumulation of NPs and gradual release of drug in cancer cells (Sun and Sun 2016). Zhang *et al.* (2015) indicated that interaction between Tf and TfR was led to rapid uptake of NPs into tumour cells, resulted in increase in artesunate intracellular accumulation in MCF-7 cancer cells (Zhang *et al.* 2015). The findings of Singh *et al.* (2016)

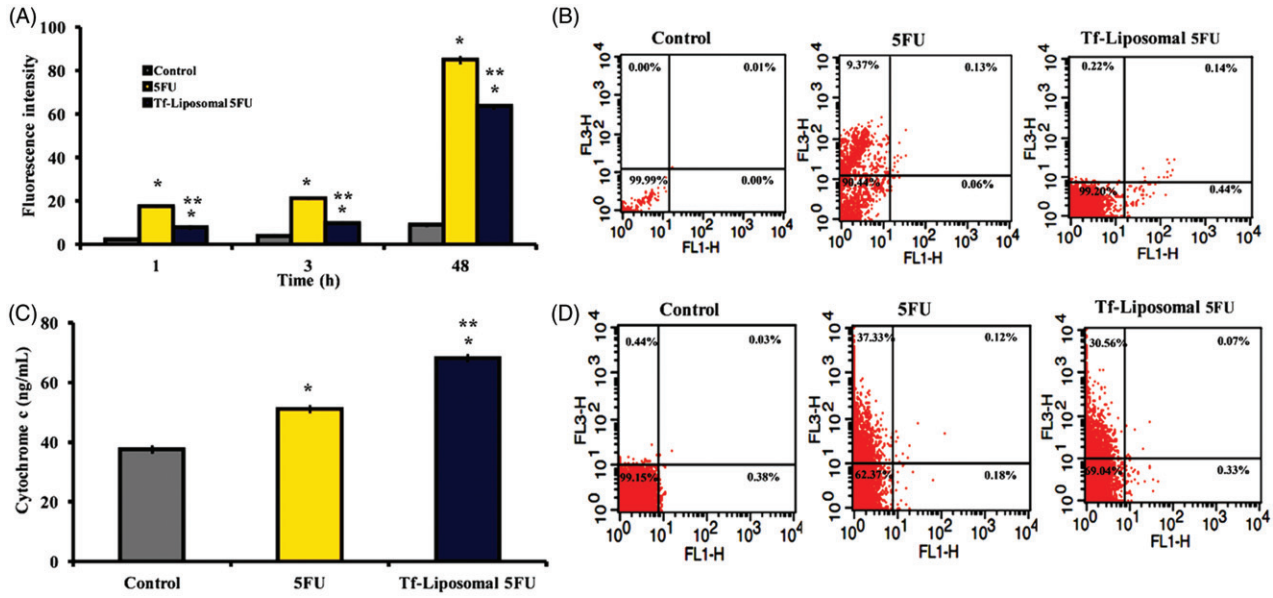

Figure 4. The intracellular effects of free 5FU and Tf-liposomal 5FU in HT-29 cells: (A) ROS production, (B) $\Delta\Psi_m$ collapse, (C) cytochrome c release and (D) apoptosis and necrosis rate. Cells were exposed to the IC_{50} of free 5FU and Tf-liposomal 5FU for 48 h at 37 °C. Data were given as mean ± SD ($n = 3$). *Significant different with control. **Significant different with free drug.

are also accordant with our results. They expressed that the higher cytotoxicity effect of Tf-targeted docetaxel-loaded NPs on cancer cells is related to the Tf receptor-mediated endocytosis (Singh *et al*. 2016).

The most interesting finding was that liposomal 5FU and Tf-liposomal 5FU exhibited lower toxicity than free drug on fibroblast cells (Figure 3C), presumably due to cancer cells more expression of TfR than normal cell lines (Bao *et al*. 2015; Kawamoto *et al*. 2011). This result was in agreement with Huang *et al*.'s (2013) results that toxicity of Tf-targeted selenium NPs on cancer cells was much more than on human normal cells (Huang *et al*. 2013). Previous study of Kim *et al*. (2017) in this field was also accordant with our finding. They observed that Tf-targeted NPs prevented the cellular uptake of doxorubicin on L929 cells as normal cells. However, Tf-targeted NPs enhanced the anticancer activity of the drug towards KB and HCT 116 cells as cancer cells (Kim *et al*. 2017).

Since chemotherapy often leads to severe side effects, recent attempts have been focussed on cancer therapies that promote toxicity towards cancer cells without damaging normal cells (Chiu *et al*. 2014). Considering these results, it seems that TfR could be an appropriate target for targeted therapies against tumours.

3.4. Cytotoxicity pathway evaluation

Apoptosis and necrosis are the most important mechanisms of the anticancer agents to remove cancer cells (Eltayeb *et al*. 2016). As displayed in Figure 4(A), after treatment of cancer cells to 5FU and Tf-liposomal 5FU, 5FU exhibited a higher intracellular ROS generation. This finding was accordant with previous researches which reported that 5FU significantly elevated ROS levels in cancer cells (Liu *et al*. 2016). Mitochondrial electron-transport chain proposed to be the major site for ROS production (Stowe and Camara 2009), then anticancer agents may damage electron transport chain and result in leakage of ROS in cancer cells. The mitochondrial membrane potential ($\Delta\psi_m$) drops followed by ROS generation; here, the loss of mitochondrial membrane potential was measured by MitoLight dye. In cells with normal $\Delta\psi_m$, this dye aggregates and produces red fluorescence, while in cells with collapsed mitochondrial $\Delta\psi_m$ the dye turns in monomers and emits green fluorescence (Śliwka *et al*. 2016). The $\Delta\Psi_m$ of cancer cells treated with free drug and Tf-liposome 5FU was 0.06 and 0.44%, respectively (Figure 4B), indicating that following the applying of targeted liposomes, cells had the biggest dissipation of $\Delta\Psi_m$. This disruption leads to the release of cytochrome c from mitochondria to cytosol which is a key initiator for triggering apoptosis pathway (Shen *et al*. 2013). A significant release of cytochrome c was observed in the cells that treated with targeted drug liposomes (Figure 4C). These data further confirmed that the apoptotic pathway was activated. In addition and according to the previous researches, low and high levels of ROS regulate apoptotic and necrosis pathway, respectively (Higuchi *et al*. 1998). Since necrosis induces inflammation, apoptosis is considered more favourable than necrosis in induction of cell death in cancer therapy (Iba *et al*. 2013). As

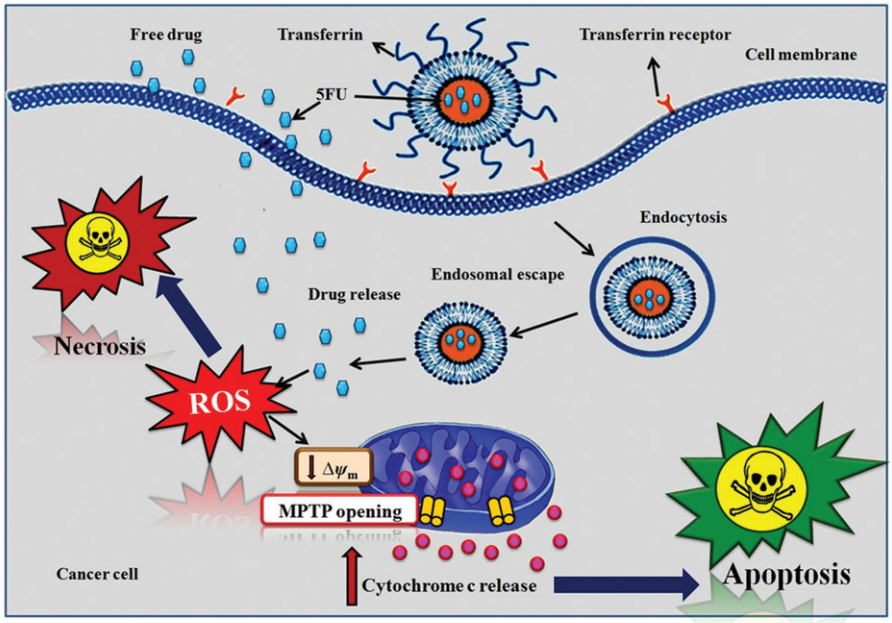

Figure 5. Schematic illustration of mechanism of action of 5FU and Tf-liposomal 5FU.

shown in Figure 4(D), the cancer cells showed more apoptosis following treatment with Tf-targeted liposome. It should be mentioned that targeted liposomes revealed higher apoptosis with lower IC_{50} (15.49 μM) in comparison with free drug (79.78 μM). 5FU is a first-line chemotherapeutic agent for colon cancer, but its application is limited due to toxicity at high doses. The results of this study confirmed that targeting liposomal 5FU with Tf improved its efficacy and trigger apoptosis pathway in cancer cells at lower concentration which lead to reduced side effects. Our findings confirmed by findings from other research groups. Similar results were observed in the study of Dilnawaz *et al.* (2012) which reported that Tf-targeted NPs reduced $\Delta\psi_m$ more than nano-targeted NPs and free drug. They also indicated that higher cellular uptake of NPs was due to Tf-conjugated drug-delivery system (Dilnawaz *et al.* 2012). Previous study of Huang *et al.* (2013) in this field is also accordant with our results. They indicated that Tf-targeted NPs induced apoptosis pathway in cancer cells following over production of ROS (Huang *et al.* 2013). Szwed *et al.* (2014) also expressed that Tf-targeted doxorubicin promoted apoptosis more than free drug (Szwed *et al.* 2014). The mechanism of action of 5FU and Tf-liposomal 5FU is illustrated schematically in Figure 5.

3.5. Haemolysis assay

Haemo-compatibility is one of the crucial properties for *in vivo* applications of NPs. Therefore, in the study, haemolysis analysis was performed to evaluate the blood compatibility of liposomes. The haemolysis percentage and image of RBCs treated with H_2O (as positive control), PBS (as negative control) and liposomes at concentration of 0.5 and 1 mg/mL are shown in Figure 6(A) and (B). The haemolysis percentages of liposomes at different concentrations were lower than 5%. The acceptable limit of haemolysis for intravenous applications should be below 5% (Banerjee *et al.* 2017b). In addition, as displayed in Figure 6(B), the results were similar to the PBS. However, the RBCs exposed to H_2O exhibited a huge haemolysis. This findings support the results of Kuznetsova *et al.* (2012) and Banerjee *et al.* (2017), which reported that liposomes revealed good haemo-tolerance (Kuznetsova *et al.* 2012; Banerjee *et al.* 2017b). Consequently, according to the results, low haemolysis induced by the liposomes ensured their good haemo-compatibility and these nanocarriers would be suitable for intravenous injection.

4. Conclusion

In vitro cytotoxicity showed that the Tf-liposomal 5FU exhibited significant cytotoxicity compared to 5FU and liposomal 5FU in cancer cells. Results of toxicity evaluations also revealed that in cancer cells, targeted liposomes induced cell death mainly through the apoptosis. Enhanced apoptosis in cancer cells through mitochondrial signalling pathways was evidenced by lower production of ROS, decreased $\Delta\Psi_m$, and higher

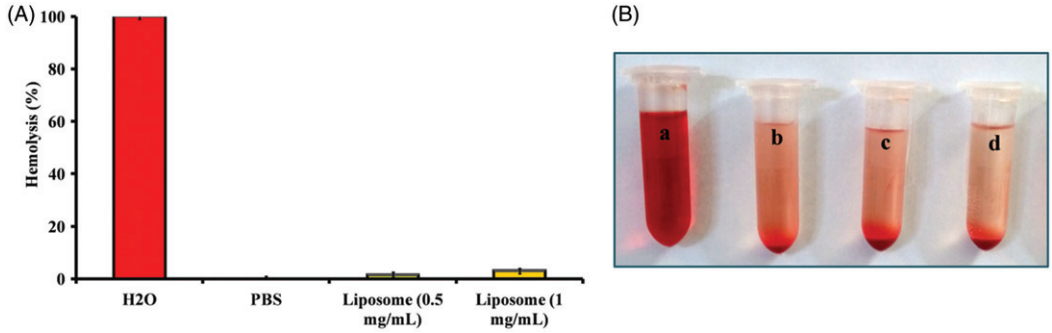

Figure 6. Haemolysis assay: (A) haemolysis (%) and (B) image of RBCs treated with (a) H_2O, (b) PBS, (c) liposome (0.5 mg/mL) and (d) liposome (1 mg/mL). Data were given as mean ± SD ($n = 3$).

release of cytochrome c at fairly lower concentration than free 5FU. According to the obtained results, Tf-targeted liposomal 5FU can be extensively employed as a promising potent and safe drug-delivery system for colon cancer therapy.

Disclosure statement

No potential conflict of interest was reported by the authors.

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