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5-Fluorouracil-Loaded PLGA Declined Expression of Pro-Inflammatory Genes IL-9, IL-17A, IL-23 and IFN-γ in the HT-29 Colon Cancer Cell Line

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Abstract

Background: Pro-inflammatory cytokines play critical roles in cancer pathobiology and have been considered potential targets for cancer management and therapy. Understanding the impact of cancer therapeutics such as 5-fluorouracil (5-FU) on their expression might shed light on development of novel combinational therapies. This study aimed to encapsulate 5-FU into PLGA and evaluate their effects on the expression of pro-inflammatory genes *IL-9, IL-17-A, IL-23,* and *IFN-γ* in the HT-29 cells.

Methods: PLGA-5-FU NPs were constructed and characterized by Dynamic Light Scattering (DLS) and Atomic Force Microscopy (AFM). The cytotoxicity was evaluated by MTT test and, the IC_{50} was identified. HT-29 cells were treated with different concentrations of the PLGA-5-FU NPs for 48 hours and, gene expression levels were analyzed by qRT-PCR.

Results: DLS and AFM analysis revealed that the prepared PLGA-5-FU NPs were negatively charged spherical-shaped particles with a mean size of 215.9 ± 43.3 nm. PLGA-5-FU NPs impacted the viability of HT-29 cells in a dose- and time-dependent manner. The qRT-PCR results revealed a dose-dependent decrease in the expression of *IL-9, IL-17A, IL-23* and *IFN-γ* genes, and their expressions were significantly different in both 10 and 20 µg/mL treated groups compared to the control. However, although the treatment of HT-29 cells with 20 µg/mL free 5-FU resulted in decreased expression of the studied genes, the differences were not statistically significant compared to the control group.

Conclusions: PLGA-5-FU NPs significantly suppressed expression of the *IL-9, IL-17A, IL-23* and *IFN-γ* genes, and the encapsulation of 5-FU into PLGA improved considerably impact of the 5-FU on the HT-29 cells.

Keywords: Cancer therapy, Colorectal cancer, Fluorouracil, Polylactic Acid-Polyglycolic Acid Copolymer **(**PLGA), Pro-inflammatory cytokine.

Introduction

Inflammation acts as an innate immune system involving different immune cells and soluble factors such as chemokines and cytokines (1). Tumor transcriptome analysis and several other studies have linked inflammation to tumors by indicating a discrete expression pattern of inflammatory cytokines in different tumors (2, 3). Pro-inflammatory cytokines, as the main mediators of inflammatory responses, may

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involve in the pathophysiology of cancers (4), and in-deep understanding of their role in cancer may help discovery of novel potential treatments. Interlukine-9 (IL-9), as a pro-inflammatory cytokine, plays a critical role in immunity and the pathogenesis of cancer (5). In solid tumors such as breast cancer and melanoma, IL-9 acts as an anti-tumor factor, but, in hematologic cancers such as Hodgkin's lymphoma, chronic lymphocytic leukemia, and diffuse large B lymphoma, it functions as a lymphocyte growth factor and promotes progression of the tumor (6). Similarly, although interferon-γ (IFN-γ) serves as a critical factor in promotion of cell immunity and activation of immune-response against tumor, but, based on its ability on the induction of apoptosis and repression of cell growth, it could be considered a possible adjuvant for cancer immunotherapy (7). Interlukine-17A (IL-17A) may promote tumor development directly or indirectly (8). It suppresses anti-tumor immunity while enhancing tumor cell proliferation and metastasis through apoptosis inhibition (9). Also, studies have recently revealed that the pro-inflammatory cytokine interlukine-23 (IL-23) plays fundamental role in the pathogenesis of some diseases such as inflammatory bowel diseases and colitis-associated colon cancer (10).

Colorectal Cancer (CRC) is one of the most common cancers that significantly impacts human health, and it has a complex genetic and environmental etiology (11-13). Although CRC mortality has been steadily dropping since 1990, but, it remains the third largest cause of cancer death in women in the United States(14). Clinical treatment approaches for CRC are mainly radiotherapy, chemotherapy, and surgery (15). However, effectiveness of these therapeutic strategies is restricted. Surgery has a high risk of cancer recurrence and metastasis rate (16). Patients experiencing chemotherapy mainly suffer from a higher toxic effect, resistance to multiple drugs and a weak bioavailability of the therapeutics, leading to significant side effects and reduced efficacy (15). Therefore, the development of safer and effective strategies for the treatment of colorectal cancer is of utmost importance.

In recent years, various nanoparticles (NPs) have been discovered and synthesized. The NPs can be functionalized to target tumor cells specifically, which reduces side effects considerably (17). Various carrier systems such as polymer nanoparticles, liposomes, dendrosomes and solid lipid nanoparticles have been designed for efficient delivery of therapeutics, including proteins, peptides, and chemical drugs (18). Among these carrier systems, the poly (lactic-co-glycolic acid) (PLGA) has attracted more attention. It is a degradable polymer that can be used as a nanoparticle-based delivery system for encapsulation of different drugs and proteins such as allergens (19). 5-Fluorouracil (5-FU), as a fluoropyrimidine analog, is widely used for the treatment of different cancers, but it is associated with non-specific cytotoxicity and adverse effects that restrict its application (20). The encapsulation of chemotherapy drugs into NPs, such as PLGA, may result in the controlled release of the drug and a safer therapeutic profile.

Our aim in this study was to encapsulate the 5-fluorouracil into the PLGA and evaluate its effects on the expression levels of the pro-inflammatory genes IL-9, IL-17A, IL-23, and IFN-γ in the HT-29 colorectal cancer cells.

Materials and Methods

Preparation of PLGA-5-Fu

For encapsulation of the 5-Fu into the PLGA NPs, we followed the W1/O/W2 method (21). Briefly, PLGA (50 mg) was added to 12.5 ml dichloromethane and dissolved in a vial. For obtaining the first emulsion (W1/O), 300 pg 5-Fu was added to the vial and mixed on a magnet mixture. Then, the mixture was placed in a cold-water bath and sonicated for 60 seconds. Then, the primary emulsion was added drop by drop to the polyvinyl alcohol (PVA) while sonicating it in a sonicator for 4 min and the secondary emulsion (W1/O/W2) was obtained. These steps were done in a cold-water bath. The mixture was stirred at room temperature for 2 h to vapor the organic solvent. Finally, the PLGA-5-Fu NPs were

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sedimented by centrifugation at 14,000 rpm for 30 min. This step was repeated three times, and the sedimentation was resuspended in 3 mL ddH2O and lyophilized.

Encapsulation efficiency and loading capacity

In order to measure the encapsulation efficiency (EE%), the amount of the non-encapsulated drug in the supernatant was measured by BCA method. Then, the EE% was calculated as; (amount of encapsulated 5-FU)/ (total amount of 5-FU) × 100. Also, the loading capacity (LC%) was calculated as; (amount of total encapsulated 5-FU)/ (total PLGA-5-FU NPs weight) × 100.

Dynamic light scattering analysis

To measure size and zeta potential, we first dissolved one mg of the lyophilized PLGA-5FU NP in one ml of deionized H2O. It was then homogenized for 15 min in a sonicator bath. The solution was analyzed by dynamic light scattering (DLS) to calculated the intensity, volume, number and zeta potential of the PLGA-5FU NPs.

Atomic Force Microscopy (AFM) analysis

One mg of the lyophilized PLGA-5-FU NP was dissolved in one mL deionized H2O. It was then sonicated for 15 min in a sonicator bath to be uniformed and dispersed. Twenty-five µL of the dispersed sample was placed on a glass slide, air-dried, and examined by AFM.

Cell viability assay

Human colorectal cancer cell line HT-29 was obtained from the Royan Institute Cell Bank, Iran. MTT assay was used for evaluation of the PLGA-5-FU NPs effects on the viability of the HT-29 colorectal cancer cells. For this purpose, the HT-29 cells (104 cells/well) were seeded into 96-well plate and incubated for overnight. A serial concentration of 0.2, 0.5, 5, 10, 20, 40, and 80 µg/mL) of PLGA-5-FU NPs were added to the culture and incubated for 24, 48 and 72 hours. After the mentioned time treatments, the cells in each well were treated with 20 µL MTT for 4 h, and the medium was replaced with 100 µL DMSO. Finally, using an ELIZA reader, the optical density (OD) was assessed at 570 nm.

Cell culture, treatment and total RNA extraction

The HT-29 cells were cultured in six-well plates (10^5 cells/well) and incubated until 80 percent confluency in RPMI medium supplemented with 10% FBS. The cells were treated with different concentrations (0.2, 0.5, 5, 10, and 20 µg/ml in the medium) of the prepared PLGA-5-FU NPs for 72 hours. The cells were detached using trypsin-EDTA and harvested by centrifugation and used for total RNA extraction. For total RNA extraction, we used the TRIzol reagent (Invitrogen) by following the manufacturer's instructions. The concentration and integrity of the total RNA samples were assessed by NanoDrop and gel electrophoresis, respectively.

cDNA synthesis and qRT-PCR

To synthesize cDNA, one µg total RNA was treated with DNase I and applied for revers transcription using the Pars Tous cDNA synthesis kit (Mashad, Iran). Each reaction was included 10 µl buffer, 2 µl reverse transcriptase and about 2 µl total RNA and DEPC-treated water up to final volume (20µl). The vials were placed in the thermal cycler and incubated for 10 min at 25 ºC, 60 min at 47 ºC and 5 min at 85 ºC. The synthesized cDNAs were used in qRT-PCR to quantify the studied genes. Primers were designed by Gene Runner and Primer-blast programs (Table 1). GAPDH was used as the normalizer. The Real-time PCR reactions were done in duplicate using SYBR Green master mix in a Corbett Rotor-Gene 6000 system. The relative expressions of the studied genes were calculated using the $2^{-\Delta Ct}$ method.

Statistical analysis

The statistical analysis was done by Sigma Plot 12 (SYSTAT software, Inc., San Jose, CA, USA). Comparisons between multiple groups were conducted by one-way ANOVA. The results were stated as means ± standard deviation (SD). We considered $p< 0.05$ statistically significant.

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Table 1. The sequence of the primers used for Real-time PCR.

Genes	Accession number	Primer	Sequence (5' → 3')	Amplicon Size (bp)
IL-23	NM_016584	Forward	TCTCTGCTCCCTGATAGCCC	163
		Reverse	TGCGAAGGATTTTGAAGCGG	
IFN-γ	NM_000619	Forward	ACCAGAGCATCCAAAAGAGTGT	176
		Reverse	GCGACAGTTCAGCCATCACT	
IL-9	NM_000590	Forward	AGTCCGCTGTCAAGATGC	120
		Reverse	TGTTGATGAGGAAGTTGATGTC	
IL17A	NM_002190	Forward	CCTCATTGGTGTCACTGCTAC	125
		Reverse	ATCTCTCAGGGTCCTCATTGC	
GAPDH	NM_001357943	Forward	GGAAGGTGAAGGTCGGAGTCA	101
		Reverse	GTCATTGATGGCAACAATATCCAT	

Results

Characterization of the PLGA-5-FU NPs

The efficiency of encapsulation (EE%) of the 5-FU by the PLGA was calculated to be 73±2.15 percent. Also, the capacity of the PLGA nanoparticles for loading of the 5-FU drug (LC%) was calculated to be 1.71±0.21percent. The DLS analysis was used for determination of the size of the PLGA-5-FU NPs. The mean size of the particles was 215.9 ± 43.3 nm. Zeta potential of the PLGA-5-FU NPs was also measured by the zeta sizer. The zeta potential distribution graph and the obtained results. Based on this analysis the mean zeta potential of the PLGA-5-FU NPs was calculated to be -16.8 ± 0.25 mV and their electrophoretic mobility was also calculated to be -.000130 cm^2/Vs. We further analyzed the PLGA-5-FU NPs with the Atomic Force Microscopy (AFM) to understand the shape and the size of the prepared particles (Fig. 1). AFM analysis showed that PLGA-5-FU NPs had spherical shapes with mean size of 249 nm.

Cytotoxic effect of the PLGA-5-FU NPs on the HT29 cells

Impact of the PLGA-5-FU NPs on the viability of the HT29 colorectal cancer cells was assessed by the MTT assay. The cells were treated with a serial concentration of the PLGA-5-FU NPs including 0.0, 2, 4, 8, 16, 32, 64 and 128 µg/mL, for 24-, 48- and 72-hours time points. A dose- and time-dependent cytotoxic effect on the HT29 cells for all three time points was found. The IC_{50} (50% inhibitory concentration) for the 24 hours time point was not reached, however, for 48- and 72 hours time points it was 19.4 µg/mL, and 12.8 µg/mL, respectively (Fig. 2).

Impact of the PLGA-5-FU NPs on the expression of pro-inflammatory cytokines

The HT-29 cells were treated with the different concentrations of the prepared NPs including 0.0, 0.2, 0.5, 5, 10, and 20 µg/mL for 48 h, and then expression of the pro-inflammatory genes *IL-9, IL-17A, IL-23* and *IFN-γ* were quantified by q-RT-PCR. We observed a dose-dependent decrease in the *IL-23* expression (Fig. 3). However, only the differences between the cells treated with 10 and 20 µg/mL in comparison to the control cells were statistically significant ($p=0.0431$ and $p< 0.0001$ respectively). The difference between the cells treated with 20 µg/mL of the free 5-FU and PLGA-5-FU was

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not also statistically significant (p> 0.9999). This dose-dependent impact on the reduction of expression was also observed for the other three genes *IL-9, IL-17A* and *IFN-γ*. Additionally, a decrease in *IL-9*, *IL-17A*, and *IFN-γ* genes expression was observed in cells treated with PLGA-5FU 20 compared to the free 5-FU 20, but the differences were not statistically significant. None of the studied genes showed a significant difference in expression level between the cells treated with 20 mg/mL free 5-FU and controls (Fig. 3).

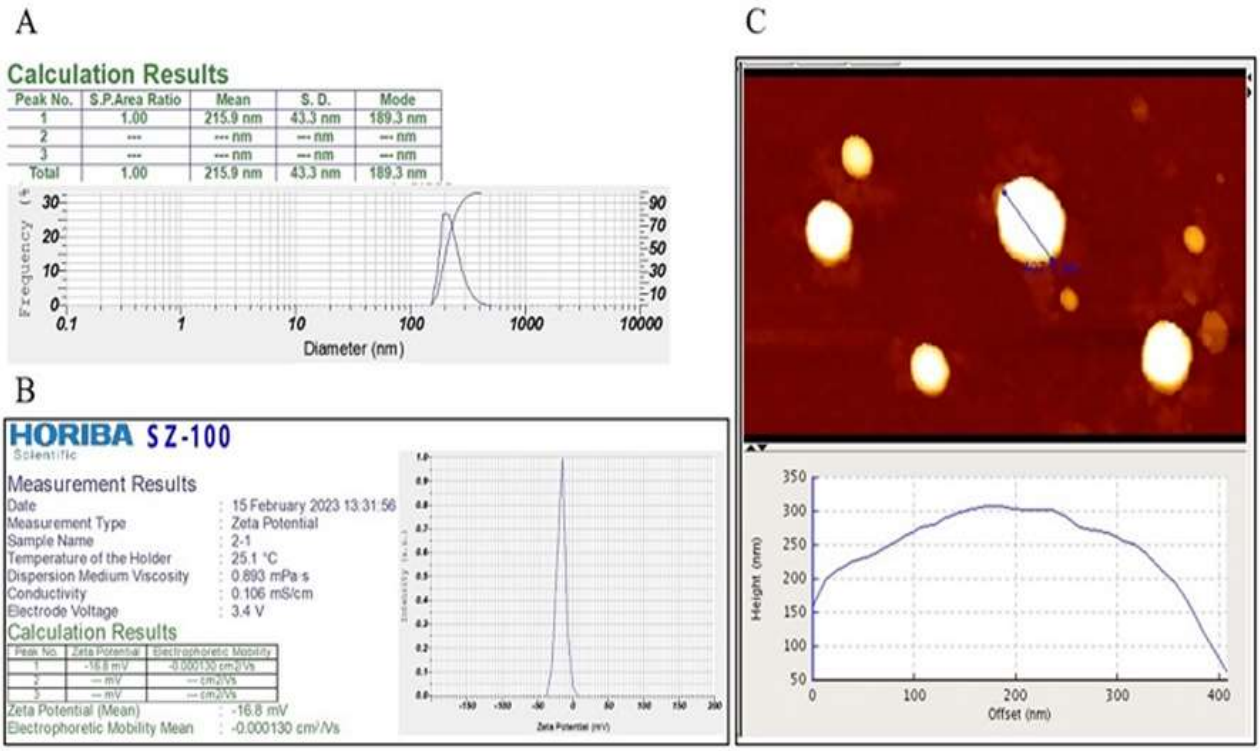

Fig. 1. Characterization of the PLGA-5-FU NPs by dynamic light scattering (DLS) and atomic force microscopy (AFM) analysis. A) The number distribution graph representing the size of the PLGA-5-FU NPs. **B)** Zeta potential distribution graph representing the surface charge of the PLGA-5-FU NPs. **C)** Size distribution graph and a field picture captured by AFM.

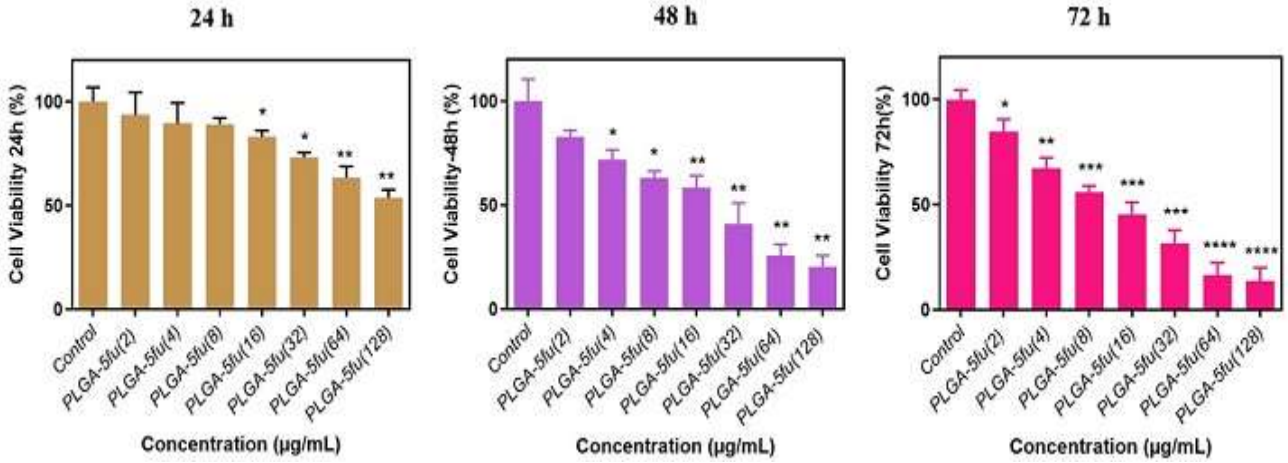

Fig. 2. Effect of PLGA-5-FU NPs on the viability of the HT29 cells. The HT29 cells were treated with 0.0, 2, 4, 8, 16, 32, 64, and 128 µg/mL concentrations of the PLGA-5-FU NPs for 24-, 48-, and 72 hours time points and the viability of the cells were measured by MTT assay.

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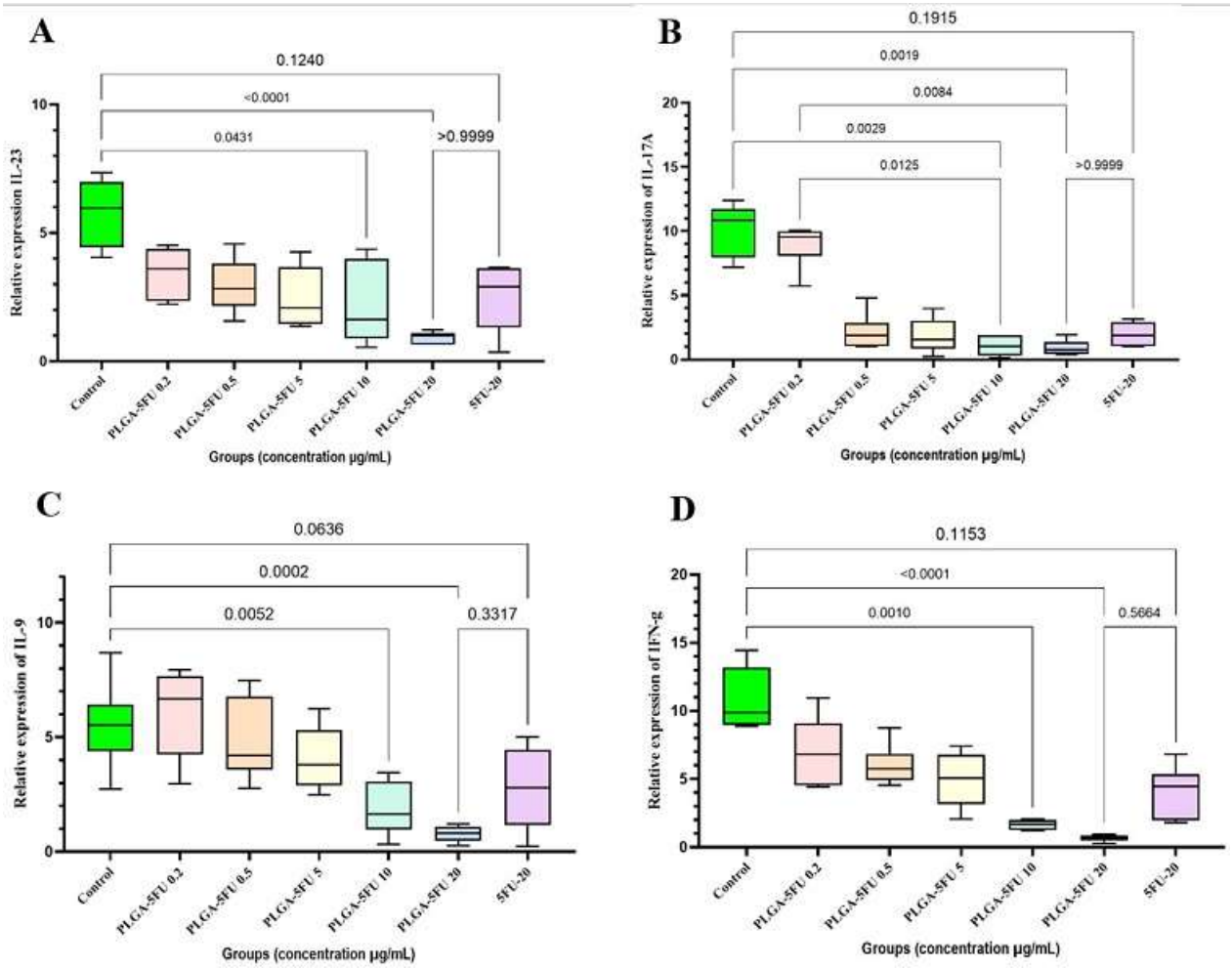

Fig. 3. Impact of PLGA-5-FU NPs on the expression of the pro-inflammatory genes. HT-29 cells were treated with different concentrations of the PLGA-5-FU NPs and free 5-FU (20µg/mL) for 48 hours and the expression levels of **A)** *IL-23*, **B)** *IL-17A*, **C)** *IL-9*, and **D)** *IFN-γ* genes were quantified and compared between the groups.

Discussion

This study showed that encapsulation of 5-FU into PLGA particles efficiently suppressed expression of pro-inflammatory genes. We encapsulated the 5-FU into the PLGA particles to prepare the PLGA-5-FU NPs. The HT-29 colorectal cancer cells were treated with the PLGA-5-FU NPs to find out its effect on the expression of pro-inflammatory genes *IL-23*, *IL-17A*, *Il-9* and *IFN-γ*. Characterization of the PLGA-5-FU NPs by DLS analysis showed a mean particle size of 215.9 ± 43.3 nm, which was suitable and in agreement with other reports (22, 23). Based on the AFM analysis, the particles were spherical shaped (Fig. 1). The mean zeta potential of the prepared nanodrug was - 16.8 ± 0.25 mV indicating a negative surface charge for the particles, which is in line with the previous reports (22-24). It was reported that the negative surface charge of the PLGA-5-FU NPs might be resulted from the uncapped carboxylic groups in the surface of the PLGA particles (22).

To obtain the IC_{50} and accordingly the approximate concentrations for treatment of the HT-29 cells for expression analysis of the pro-inflammatory genes, the MTT test was used. The results showed a dose- and time-dependent impact on the HT-29 cells viability (Fig. 2). Based on the results, subsequent treatments for gene expression assessment were implemented with concentrations lower than IC_{50} for 48 hours. Expression analysis of

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pro-inflammatory genes revealed that PLGA-5-FU NPs impact *IL-23, IL-17A, Il-9* and *IFN-γ* genes expressions in a concentration-dependent manner (Fig. 3). In fact, the PLGA-5-FU NPs significantly reduced expressions of all four studied genes in concentrations higher than 10 µg/mL while treatments with ever 20 µg/mL of free 5-FU could not result in significant decreases in the expression of studied genes compared with the control group. These results confirmed that encapsulation of 5-FU into PLGA could efficiently enhance 5-FU effectiveness on HT-29 cells.

It was reported that the expression of pro-inflammatory cytokines impacts tumor development by affecting the proliferation and apoptosis of tumor cells, and/or promoting anti-tumor immunity. IL-9 may have positive or negative function in different tumors development. It can promote thymic lymphomas cells proliferation and involve in some T cell malignancies (25), and play vital role in the growth of Hodgkin's lymphoma (26), suggesting an oncogenic function for IL-9. However, IL-9 may function as an antitumor cytokine directly by killing the tumor cells or indirectly by inducing tumor-specific immune responses. Consistent with other reports (26-27), we detected a higher expression of IL-9 in HT-29 cells which was suppressed by PLGA-5-FU NPs treatments. Another pro-inflammatory cytokine IL-17A which is known as IL-17 was observed to be central in patients with gastric ulcer caused by Helicobacter pylori and the Helicobacter pylori-associated diseases (1). It was also reported that Th-17, a pro-inflammatory infiltrative cell, produce IL-17 pro-inflammatory signature including IL-17A (9). IL-17A plays vital role in breast cancer (9, 28, 29) and esophageal adenocarcinoma (30) development, invasiveness and metastasis via upregulation of MMP-2 and MMP-9, and activating the MEK1/2 and NF-kB signaling pathways (9, 30). All these findings together with our observation in this study highlight IL-17A as an astonishing target for cancer therapy. Notably, anti IL17A monoclonal antibody, secukinumab, that is used primarily for treatment of psoriasis (31), might be considered as a potential anticancer therapeutic especially in combination with chemotherapeutics.

We also observed that the expression level of IL-23 was significantly decreased in the HT-29 cells in response to the PLGA-5-FU NPs 10 and 20 µg/mL concentrations. Ljujic B. et al. observed that serum IL-23 levels were significantly higher in colorectal cancer patients vs. controls and its levels were strongly associated with the VEGF overexpression (32). IL-23 expression was also higher in breast tumor tissues and was correlated with the tumor size, TNM stage and metastasis (33). Conversely, IL-23 has shown anti-tumor function in skin photocarcinogenesis (34). IL-23 that is secreted from monocytes stimulates natural killer cells to produce IFN-γ (35). IFN-γ is a pro-inflammatory cytokine that primarily acts as an antiviral and antitumor factor, and shows immunomodulatory functions (7). However, it was demonstrated that IFN-γ may play as a pro-tumorigenic factor. In prostate cancer, IFN-γ by activating the JAK/STAT1 signaling and induction of IFIT5 promoted the epithelial-to-mesenchymal transition state (36). Upregulation of IFN-γ was also reported in metastatic renal cell carcinoma and pancreatic cells (37, 38). In the present study we also observed that expression of IFN-γ was significantly decreased in response to the PLGA-5-FU NPs treatment. It was also reported that encapsulation of doxorubicin, a common chemotherapeutics, into the PLGA nanoparticles could suppress pro-inflammatory genes expression including IL-6, iNOS, IL-1β and TNF-α (39).

The chemotherapy drug 5-FU is currently utilized for therapy of various malignancies, including CRC (40). However, use of 5-FU for cancer treatment in clinical settings is restricted because of its short half-life, serious side effects, the need for frequent doses to maintain therapeutic levels, and therefore, monotherapy with 5-FU is often insufficient for colon cancer treatment (41). Combination

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of chemotherapeutics with new technologies like plasma-activated medium (PAM) and herbal medicines might be considered as an alternative cancer therapy method (42, 43). Also, the encapsulation of 5-FU into nanoparticles like PLGA prolongs its presence in the body and advances the drug uptake by the tumor cells (44). In line with these reports, our findings clearly showed that the encapsulation of 5-FU into the PLGA nanoparticles could reduce the effective dose of 5-FU which is needed for cancer treatment, and thereby preventing some adverse effects associated with the 5-FU chemotherapy.

In conclusion, The PLGA-5-FU NP was significantly decreased expression of the pro-inflammatory genes *IL-23, IL-17A, Il-9* and *IFN-γ* in the HT-29 cells. Entrapment of 5-FU into the PLGA nanoparticles was intentionally improved effectiveness of the drug on the suppression of the studied genes.

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Conflict of interest

None.

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