

## $\beta 3$ Integrin Silencing via siRNA and Regorafenib/DDAB-mPEG-PCL Nanoparticles in Regorafenib-Resistant SW48 Colon Cancer Cells

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### Abstract

Colorectal cancer (CRC) is the second leading cause of cancer-related mortality worldwide. Regorafenib, a multi-kinase inhibitor, is widely used for treating recurrent CRC; however, resistance mechanisms, such as overexpression of  $\beta 3$  integrin, often lead to chemotherapy failure. Targeting  $\beta 3$  integrin downregulation through siRNA offers a promising strategy to overcome regorafenib resistance. This study developed a regorafenib-resistant SW48 cell line and evaluated the effects of siRNA targeting  $\beta 3$  integrin delivered via (dimethyldioctadecylammonium bromide, DDAB)-methoxy poly(ethylene glycol) (mPEG)-poly- $\epsilon$ -caprolactone (PCL) nanoparticles (NPs) in combination with regorafenib-loaded NPs. Regorafenib resistance was induced by exposing SW48 cells to increasing concentrations (5, 10, 20  $\mu\text{M}$ ). The half-maximal inhibitory concentration ( $\text{IC}_{50}$ ) was determined using the MTT assay in resistant and non-resistant cells. NPs were characterized by dynamic light scattering (DLS) and gel retardation assays. The silencing efficiency of  $\beta 3$  integrin siRNA-loaded NPs combined with regorafenib-loaded NPs was assessed using quantitative real-time PCR.  $\text{IC}_{50}$  values for resistant and non-resistant cells were  $35.12 \pm 3.5 \mu\text{M}$  and  $14.48 \pm 1.2 \mu\text{M}$ , respectively, confirming resistance induction. NPs exhibited a size of  $73.1 \pm 2.5 \text{ nm}$  and a  $28.9 \pm 2.5 \text{ mV}$  charge.  $\beta 3$  integrin expression was significantly higher in resistant cells than non-resistant cells ( $p < 0.05$ ).

This study demonstrates that combination therapy with siRNA and regorafenib-loaded NPs effectively reduces  $\beta 3$  integrin expression and induces apoptosis in colon cancer cells, thereby overcoming regorafenib resistance.

**Keywords:** Colorectal cancer, regorafenib resistance,  $\beta 3$  integrin, siRNA, DDAB-*m*PEG-PCL nanoparticles

**List of Abbreviations:** CRC: Colorectal cancer; siRNA : Small interfering RNA; DDAB-*m*PEG-PCL: dimethyldioctadecylammonium bromide-methoxy poly(ethylene glycol)-poly- $\epsilon$ -caprolactone; NPs: nanoparticles;  $IC_{50}$ : half-maximal inhibitory concentration; DLS: dynamic light scattering; EMT: epithelial-mesenchymal transition; CSCs: cancer stem cells; Reg: Regorafenib; Reg/NPs: regorafenib-loaded nanoparticles; siRNA-NPs: siRNA- loaded nanoparticles; NPD: Drug-loaded nanoparticles; D: Drug

## Introduction

Colorectal cancer (CRC) ranks as the third most common cancer and the second leading cause of cancer-related mortality globally, following lung cancer, in 2022 [1]. The etiology of CRC involves both environmental and hereditary factors, with environmental factors accounting for approximately 65% of CRC cases. Familial associations contribute to 35% of cases, while only 5% are attributed to hereditary cancer syndromes [2].

Current treatment modalities for CRC include surgery, radiotherapy, and chemotherapy. Despite recent advancements in therapeutic approaches, these treatments remain ineffective against metastatic tumors and chemotherapy-resistant cancers [3]. Drug resistance has emerged as a significant obstacle to successful CRC treatment, driven by intrinsic and acquired mechanisms. Key pathways contributing to drug resistance include drug efflux, drug inactivation, epithelial-mesenchymal transition (EMT), and cancer stem cells (CSCs) [4].

Targeted therapy has gained prominence as an innovative strategy to overcome drug resistance by focusing on oncogenes implicated in malignant phenotypes. Oncogenic proteins such as vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR) are frequently overexpressed in various cancers, including colon and breast cancer. Regorafenib, a multi-kinase inhibitor targeting VEGFRs, fibroblast growth factor receptor (FGFR), and proto-oncogene receptor tyrosine kinase (RAF), has been approved by the United States Food and Drug Administration (FDA) for treating metastatic CRC, gastrointestinal stromal tumors, and hepatocellular carcinoma [5, 6].

Recent studies have identified integrins, a heterodimeric surface receptor with a transmembrane loop, critical players in developing drug resistance in cancer cells. Both intrinsic and acquired resistance pathways are associated with integrins. Specifically,  $\beta 3$  integrin is upregulated in cancer cells, particularly in CSCs, and serves as a marker of EMT, playing an essential role in tumor progression via the activation of the Notch pathway [7-9].

Gene knockdown strategies targeting drug resistance-associated genes have shown great promise in overcoming resistance in cancer cells. Small interfering RNA (siRNA) is a potent tool for silencing specific genes implicated in diseases such as CRC [10]. Previous studies have demonstrated that siRNA-mediated gene silencing inhibits cell proliferation, angiogenesis, and metastasis in cancerous cells [11]. Various delivery systems have been developed to enhance siRNA delivery to target cells and improve stability, including hybrid nanoparticles (NPs). Hybrid NPs have garnered attention due to their superior stability, safety, and dual polymer-lipid properties. In this study, biodegradable hybrid NPs composed of dimethyldioctadecylammonium bromide (DDAB)-methoxy poly(ethylene glycol) (mPEG)-poly- $\epsilon$ -caprolactone (PCL) were synthesized. The cationic lipid DDAB facilitates siRNA adhesion by neutralizing negative charges while incorporating PEG enhances nanoparticle stability in biological fluids [12-14].

In this study, we hypothesized that the knockdown of  $\beta 3$  integrin in regorafenib-resistant cells using siRNA in combination with regorafenib-loaded nanoparticles (Reg/NPs) would reverse drug resistance and promote apoptosis. A regorafenib-resistant SW48 colon cancer cell line was established using a gradual exposure method, as previously described for other chemotherapeutic agents. Resistant cells were subsequently treated with a combination of siRNA-loaded NPs and Reg/NPs to evaluate the role of  $\beta 3$  integrin expression in the regorafenib resistance pathway. This combination therapy reduced  $\alpha \beta 3$  integrin expression at both mRNA and protein levels, induced apoptosis, and sensitized resistant colon cancer cells to regorafenib.

## Materials and Methods

### Cell Culture and Compounds

The human colon cancer SW48 cell line (ATCC: CCL-231) was purchased from the Pasteur Institute of Iran (Tehran, Iran). SW48 cells were cultured in RPMI 1640 medium (Gibco, UK) supplemented with 10% fetal bovine serum (FBS, Gibco, UK) and 1% penicillin-streptomycin (Gibco, UK) at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>. To establish regorafenib-resistant cells, SW48 cells were maintained in media containing increasing concentrations of regorafenib. Cells were cultured in 5, 10, and 20  $\mu$ M of regorafenib sequentially for 15 days at each dose, followed by an additional 15 days at the final concentration [15].

mPEG-PCL was provided by the Faculty of Pharmacy at Zanjan University of Medical Sciences [16]. DDAB was purchased from Aldrich (USA), and regorafenib was obtained from Santa Cruz Biotechnology (USA, SC-477163). The  $\beta 3$  integrin siRNA (sense: 5'-GCCCAUGUUUGGCUACAAAAdTdT-3' and antisense: 5'-CGGGUACAAACCGAUGUUUdTdT-3') and control siRNA (sense: 5'-UAGAUUCUCGCGUCAUACdTdT-3' and antisense: 5'-GUAUGACGCGAGAUUAUCUAdTdT-3') were purchased from Microsynth (Switzerland). All other chemicals were of analytical grade and procured locally.

### Preparation of DDAB-mPEG-PCL Nanoparticles

NPs were synthesized using a single-step nanoprecipitation method. A total of 2 mg of mPEG-PCL was dissolved in 800  $\mu$ L of acetonitrile, and 0.2 mg of DDAB was dissolved in distilled water. Subsequently, 7800  $\mu$ L of distilled water was added to the solution in a sonic bath maintained at 50 °C, followed by sonication for 6 minutes. The NPs were purified by centrifugation at 3000 rpm for 30 minutes using a Vivaspin filter (Sartorius Stedim Biotech, Germany) with a molecular weight cutoff of 10 kDa.

### Preparation of Reg/DDAB-mPEG-PCL Nanoparticles

Regorafenib-loaded NPs were prepared using the same nanoprecipitation method. Briefly, 0.0635 mg of regorafenib and 1.85 mg of mPEG-PCL were dissolved in an 800  $\mu$ L mixture of acetonitrile and DMSO (DMSO/acetonitrile ratio: 1:9). Separately, 0.2 mg of DDAB was dissolved in distilled water. After mixing the regorafenib/mPEG-PCL solution with DDAB, 7800  $\mu$ L of distilled water was added, and the entire solution was sonicated for 6 minutes. The NPs were purified using the same centrifugation method described above.

### Characterization of Nanoparticles

The size, surface charge, and polydispersity index (PDI) of the NPs were analyzed at 25 °C using a Malvern Zetasizer Nano ZS90 apparatus (Malvern Instruments, Worcestershire, UK).

### Preparation of siRNA/DDAB-mPEG-PCL Nanoparticles

To determine the optimal positively charged polymer amine nitrogen (N) to negatively charged nucleic acid phosphate (P) ratio (N/P ratio) for siRNA/NP complexes, the following formula was used to calculate the nanoparticle concentration at differ-

ent N/P ratios:

$$\text{Volume of nanoparticles}(\mu\text{L}) = \frac{\mu\text{g of siRNA} \times \text{nmol of phosphate per } \mu\text{g} \times \text{desired N/P ratio}}{\text{nmol of cationic lipid per } \mu\text{L}}$$

A constant siRNA concentration of 1  $\mu\text{M}$  was mixed with NPs at varying N/P ratios (0 to 60). The complexes were incubated at room temperature for 30 minutes. Subsequently, all samples were loaded onto a 2% agarose gel and analyzed by electrophoresis.

### Encapsulation Efficiency (EE) of siRNA

To determine encapsulation efficiency (EE), siRNA was added to NPs at the optimal N/P ratio to form siRNA/NP complexes. The complexes were centrifuged at 5000 rpm for 5 minutes, and the concentration of free siRNA in the supernatant was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). EE was calculated using the following formula:

$$\text{EE (\%)} = \left[ \frac{\text{Total amount of siRNA} - \text{Free siRNA in the supernatant}}{\text{Total amount of siRNA}} \right] \times 100$$

### MTT Assay

Cell viability and  $\text{IC}_{50}$  values were assessed using the MTT assay in SW48-resistant and non-resistant cells. Cells were seeded into 96-well plates at a density of 6000 cells per well and incubated for 24 hours under 5%  $\text{CO}_2$  at 37  $^{\circ}\text{C}$ . After treatment with the groups listed in Table 1, cells were incubated for 48 hours in the enriched medium. After treatment, 20  $\mu\text{L}$  of MTT solution (5 mg/mL in PBS) was added to each well, and the plates were incubated for another 4 hours. The MTT medium was removed, and dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals. Absorbance was measured at 570 nm using a microplate reader (Stat Fax-2100 Microplate Reader, Awareness Technology, USA).

To determine  $\text{IC}_{50}$  (half-maximal inhibitory concentration), various concentrations of regorafenib were used to treat both SW48-resistant and non-resistant cells. The MTT assay was performed as described above.

**Table 1:** Treatment groups of Reg, Reg/NPs, and siRNA/NPs

Sub-group	group
A1: siRNA 100 nM	A) siRNA
A2: siRNA 50 nM	
A3: siRNA 25 nM	
B1: siRNA-NP100 nM	B) siRNA-NP
B2: siRNA-NP50 nM	
B3: siRNA-NP25 nM	
C1: siRNA-NP100+Drug20	C) siRNA/NP+ D or NPD
C2: siRNA-NP50+Drug20	
C3: siRNA-NP25+Drug20	
C4: siRNA-NP100+NPD20	
C5: siRNA-NP50+NPD20	
D1: NP 100	D) others
D2: siRNA-NP control	

<b>D3: Drug 20</b>	
<b>D4: NPD20</b>	
<b>D5: NPD10</b>	
<b>E: untreated resistant cells</b>	E) Control

## Treatments

Resistant SW48 cells were treated with Reg, Reg/NPs, and siRNA/NPs. Table 1 provides details of the treatment groups.

## Real-Time Polymerase Chain Reaction (RT-PCR)

Resistant cells ( $5 \times 10^5$ ) were seeded into 6-well plates and incubated for 48 hours. The cells were treated according to the groups listed in Table 1 for 16 hours. The medium was then replaced with an enriched medium and incubated for 20 hours.

Total RNA was extracted using the TrizolEX kit (DNAbiotech, Iran) according to the manufacturer's protocol. The concentration of extracted RNA was measured at 260 nm using a Nanodrop spectrophotometer (Thermo Scientific, UK). A total of 5  $\mu$ g of RNA was reverse-transcribed into complementary DNA (cDNA) using the Yekta Tajhiz Azma cDNA synthesis kit (Catalog No: YT4500).

Subsequently, 1  $\mu$ L of cDNA was used to evaluate  $\beta$ 3 integrin and the housekeeping gene  $\beta$ -actin expression levels using RealQ Plus Master Mix Green (Ampliqon, A325402). RT-PCR was performed using the ABI StepOnePlus system. The following primer sequences were used:

$\beta$ 3 integrin: Forward: 5'-TAATAGCCATCGCTGCAACA-3' Reverse: 3'-ATCTTGCCAAAGTCACTGCT-5'

$\beta$ -actin: Forward: 5'-ACTCTTCCAGCCTTCC TTCC-3' Reverse: 3'-CGTACAGGTCTTTGCGGATG-5'

PCR conditions consisted of an initial denaturation step at 95 °C for 15 minutes, followed by 40 cycles of amplification (95 °C for 15 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds).

The quality of RT-PCR products was confirmed using 2% agarose gel electrophoresis. Bands were visualised with a UV transilluminator after staining with GelRed gel stain.

## Apoptosis Assay

SW48 cells were seeded into 6-well plates at a density of  $5 \times 10^5$  cells per well and incubated for 48 hours. The cells were treated with the groups in Table 1 for 48 hours. After treatment, cells were harvested, and the apoptosis rate was analyzed using flow cytometry according to the protocol provided with the Annexin V apoptosis detection kit (eBioscience, San Diego, CA, USA). Data analysis was performed using FlowJo software.

## Protein Extraction and $\alpha\beta$ 3 Expression in SW48 Cells

The enzyme-linked immunosorbent assay (ELISA) method measured  $\alpha\beta$ 3 protein levels. Cells were seeded into 12-well plates and treated as outlined in Table 1. At 48 hours post-treatment, cells were lysed using a mammalian cell lysis buffer. The total protein concentration in the lysates was quantified using the Bradford assay. The  $\alpha\beta$ 3 ELISA kit (R&D Systems, DYC3050-2) was used to determine protein levels according to the manufacturer's instructions.

Briefly, 96-well plates were coated with a specific mouse monoclonal antibody. Total protein lysates were added to each well

and incubated for 2 hours at room temperature. Wells were washed with wash buffer, and a specific rabbit anti- $\alpha v \beta 3$  detection antibody was added, followed by a 2-hour incubation at room temperature. Subsequently, HRP-conjugated anti-rabbit secondary antibody was added to each well and incubated for 20 minutes. The reaction was developed using a TMB substrate and terminated by adding a stop solution. Absorbance was measured at 450 nm using a microplate reader (Stat Fax-2100 Microplate Reader, Awareness Technology, USA).

Protein levels were calculated using the following formula:

$$\text{Protein level(\%)} = \left( \frac{\text{Mean OD of treated cells}}{\text{Mean OD of control cells}} \right) \times 100$$

## Statistical Analyses

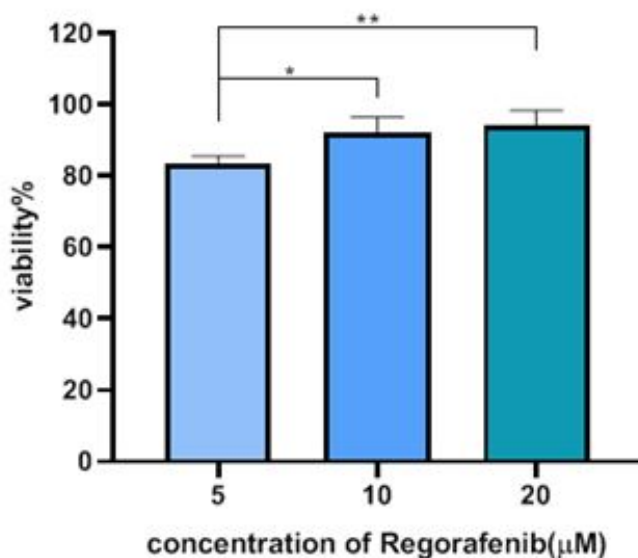
The Kolmogorov-Smirnov test was performed to assess the normality of data distribution. Differences between two variables were analyzed using an independent sample t-test, while differences among treatment subgroups were evaluated using one-way ANOVA followed by a post hoc test. Statistical analyses were performed using IBM SPSS Statistics software (version 24), and graphs were created with GraphPad Prism 8.4. Data were presented as mean  $\pm$  (SD), with p-values less than 0.05 considered statistically significant.

## Results

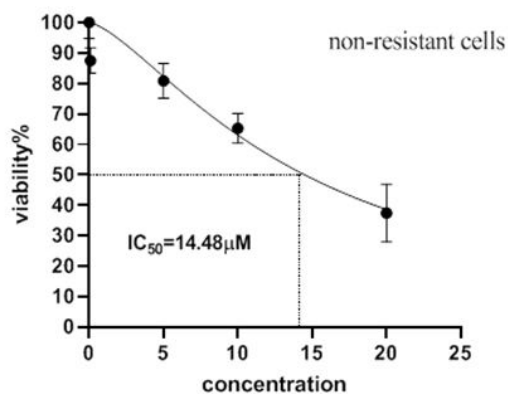
### Establishment of a Resistant SW48 Cell Line

At the end of the 15th day of regorafenib treatment, the cell viability percentages were  $83.3 \pm 1.1\%$  for 5  $\mu\text{M}$ ,  $92 \pm 2.3\%$  for 10  $\mu\text{M}$ , and  $94.1 \pm 2.2\%$  for 20  $\mu\text{M}$  regorafenib (Figure 1). A significant increase in cell viability was observed from the initial to the final dose, indicating cell adaptation to regorafenib.

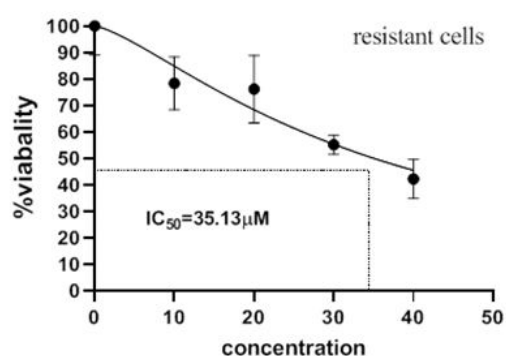
The half-maximal inhibitory concentration ( $\text{IC}_{50}$ ) values for regorafenib were  $14.48 \pm 1.2 \mu\text{M}$  in non-resistant cells and  $35.13 \pm 3.5 \mu\text{M}$  in resistant cells (Figures 2 and 3). These findings demonstrate that the  $\text{IC}_{50}$  in resistant cells increased due to adaptation and resistance development.



**Figure 1:** Cell survival percentage in the resistant cells at the enhancement concentration of regorafenib.



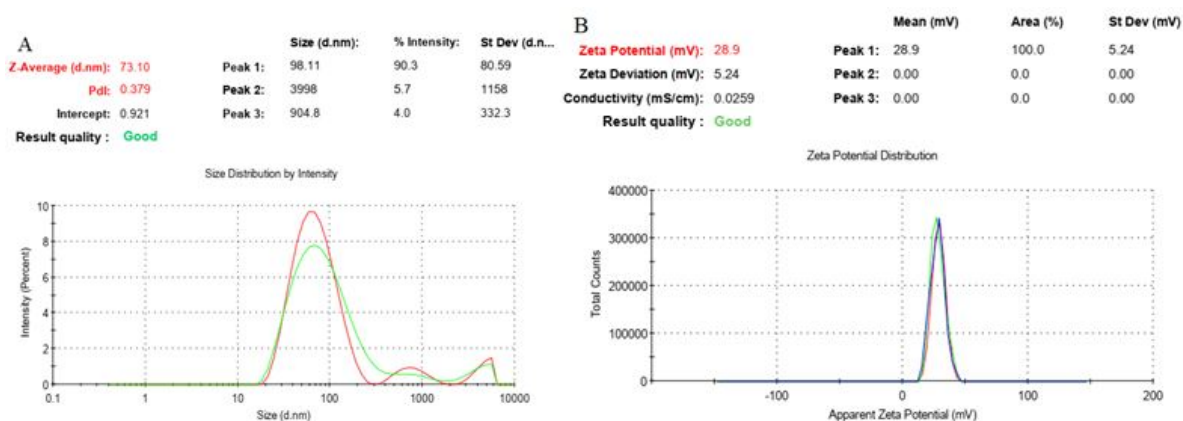
**Figure 2:** The IC<sub>50</sub> values of the regorafenib in the non-resistant SW48 cells.



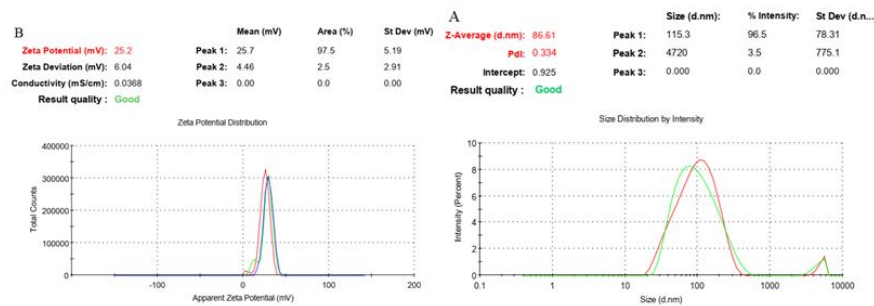
**Figure 3:** The IC<sub>50</sub> values of the regorafenib in the resistant SW48 cells.

## Characterization of Nanoparticles

The Z-average size, PDI, and zeta potential of DDAB-mPEG-PCL NPs were  $73.1 \pm 2.5$  nm,  $0.379 \pm 0.07$ , and  $+28.9 \pm 2.5$  mV, respectively (Figures 4A and 4B). Similarly, the Z-average size, PDI, and zeta potential of Reg/DDAB-mPEG-PCL NPs were  $86.61 \pm 3$  nm,  $0.334 \pm 0.03$ , and  $+25.2 \pm 2.1$  mV, respectively (Figures 5A and 5B).



**Figure 4:** (A): Z-average (size) and (B): zeta potential of DDAB-mPEG-PCL nanoparticles



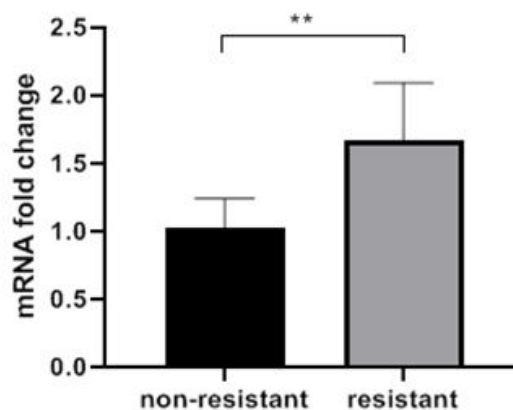
**Figure 5:** (A): Z-average (size) and (B): zeta potential of Reg/DDAB-mPEG-PCL nanoparticles.

### Preparation of siRNA/NP Complexes

The gel retardation assay was employed to determine the optimal N/P ratio for siRNA/NP complexes. Based on the gel retardation results in previous studies [17, 18], an N/P ratio of 20 was selected. The EE of the siRNA/DDAB-mPEG-PCL NPs was  $98.07 \pm 2.11\%$ .

### $\beta 3$ Integrin Expression in Resistant and Non-Resistant SW48 Cell Lines

Figure 6 illustrates the RT-PCR results for  $\beta 3$  integrin gene expression levels in non-resistant and resistant SW48 cells. The  $\beta 3$  integrin mRNA expression significantly differed between non-resistant and resistant cells ( $p < 0.005$ ). Notably,  $\beta 3$  integrin mRNA levels increased 1.63-fold in resistant cells compared to non-resistant ones.

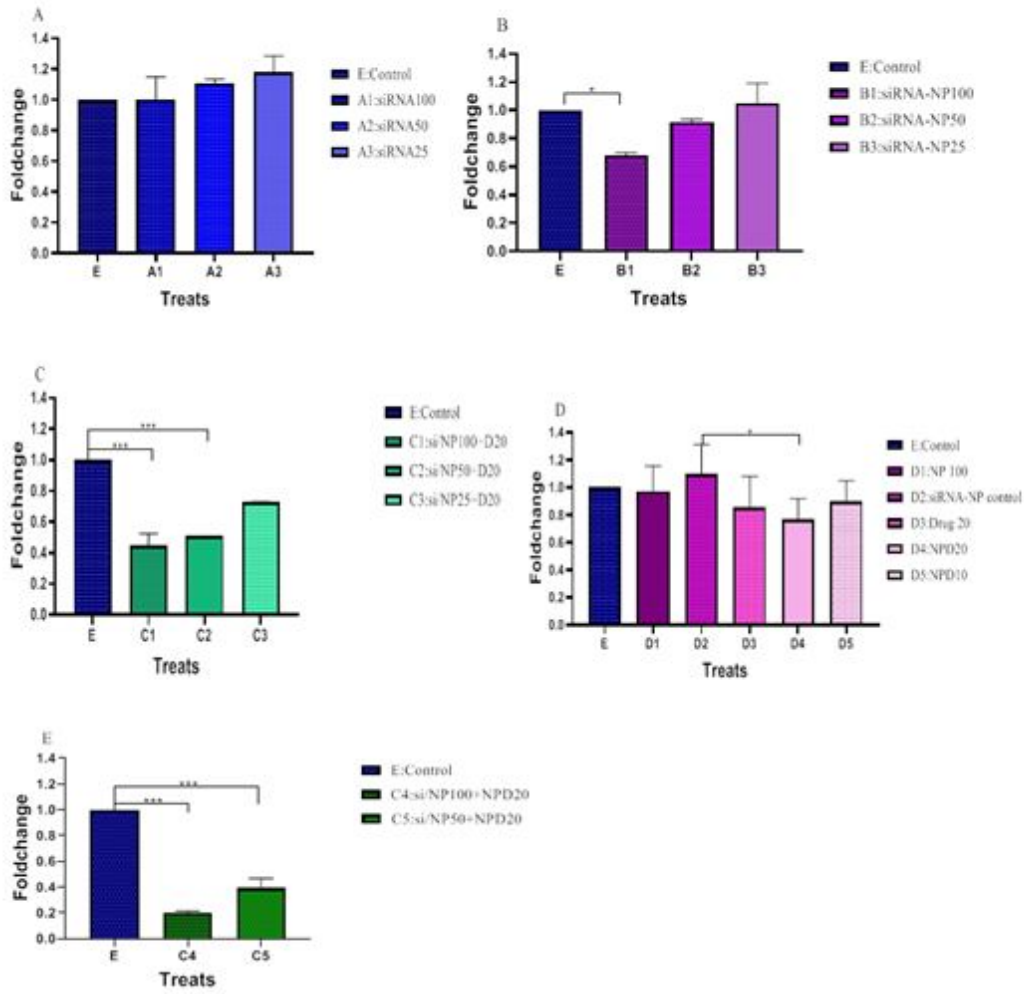


**Figure 6:** Comparing  $\beta 3$  integrin mRNA level in the resistant and non-resistant SW48 cell line

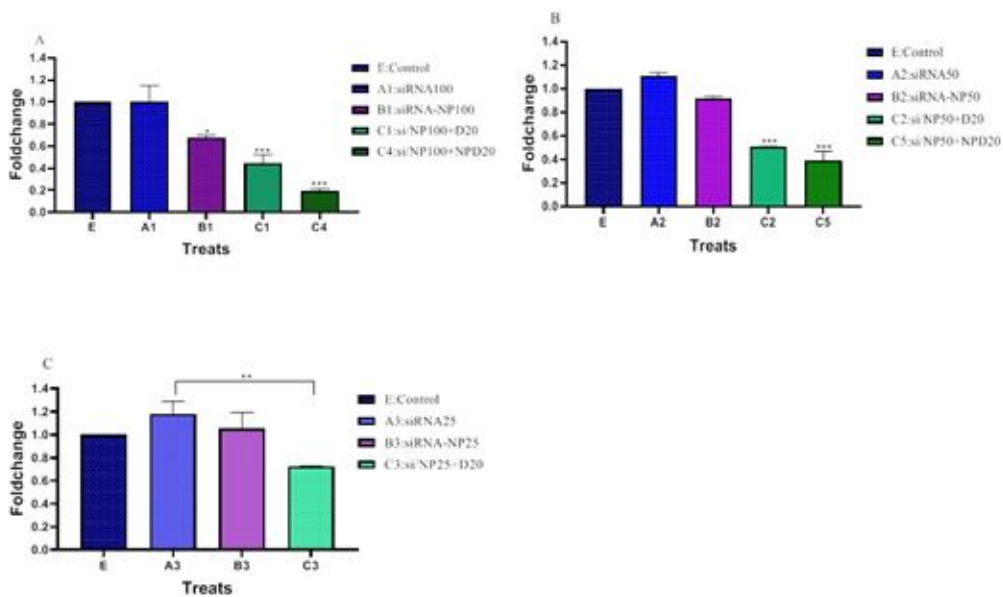
### Combination of siRNA/NPs and Reg/NPs Mediated Downregulation of $\beta 3$ Integrin Expression in Resistant SW48 Cells

RT-PCR analysis revealed that  $\beta 3$  integrin expression was unchanged in the D1 (NP100), D2 (siRNA-NP control), and A (free siRNA) groups. Among the B group, only B1 (siRNA-NP100) exhibited a significant reduction in  $\beta 3$  integrin mRNA levels compared to the control group ( $p < 0.05$ ).

Combination treatments involving regorafenib, siRNA-NPs, and Reg-NPs demonstrated significant downregulation of  $\beta 3$  integrin expression in the C1 (D20+siRNA/NP100), C2 (D20+siRNA/NP50), C4 (NPD20+siRNA/NP100), and C5 (NPD20+siRNA/NP50) groups ( $p < 0.001$ ). The analyses indicate that the best treatment for  $\beta 3$  integrin downregulation was the combination of Reg20/DDAB-mPEG-PCL with siRNA100/DDAB-mPEG-PCL (Figures 7 and 8).



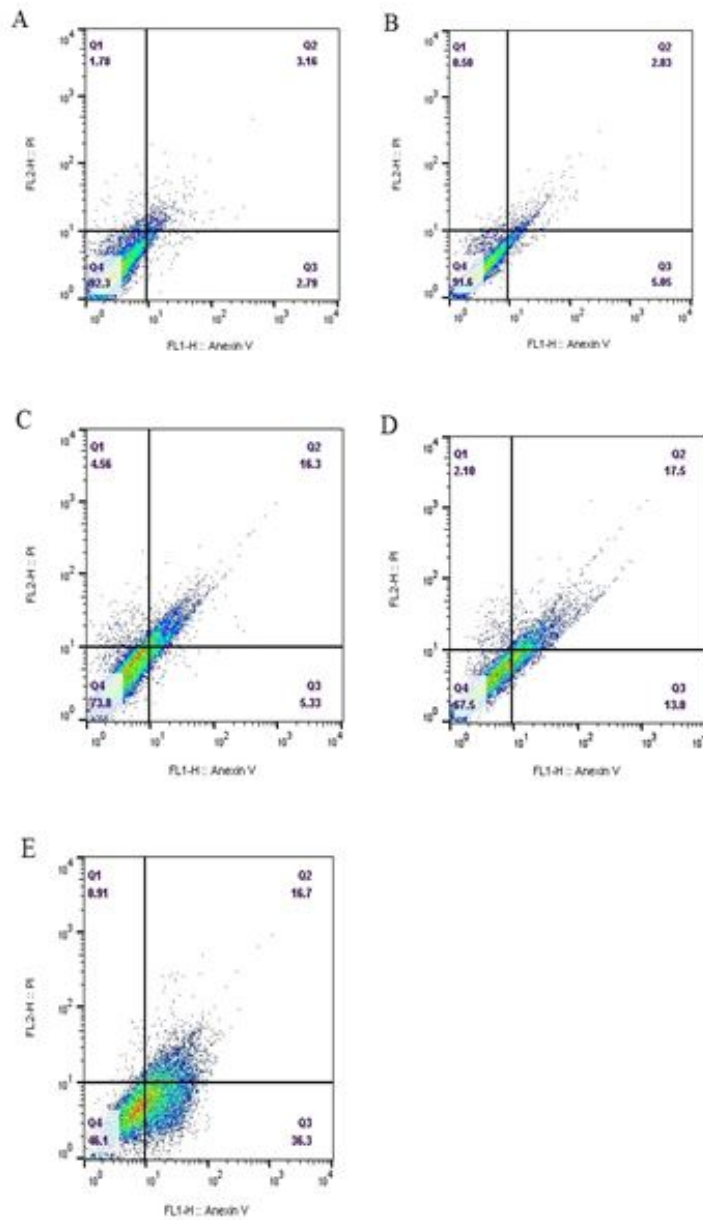
**Figure 7:** Intergroup comparison of the  $\beta 3$  gene expression level in treatment groups in the resistant cells. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$



**Figure 8:** Intragroup comparison of the  $\beta 3$  gene expression in resistant cells. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

### Apoptosis Induction in Resistant SW48 Cells Treated with siRNA/NPs and Reg/NPs

No significant apoptosis was observed in the A1 (free siRNA) group. However, the apoptosis rate increased significantly in B1 (siRNA-NP100) to  $19.6 \pm 2.8\%$ . Among the combination treatment groups, the percentage of apoptotic cells was significantly higher in C1 (D20+siRNA/NP100) and C4 (NPD20+siRNA/NP100), reaching  $28.25 \pm 3.17\%$  and  $52.58 \pm 0.59\%$ , respectively ( $p < 0.001$ , Figures 9 and 10).



**Figure 9:** The apoptotic rate of resistant SW48 cells in (A) control, (B) siRNA 100, (C) siRNA/NP 100, (D) siRNA/NP 100+ Drug 20, and (E) siRNA/NP 100+ NPD20.

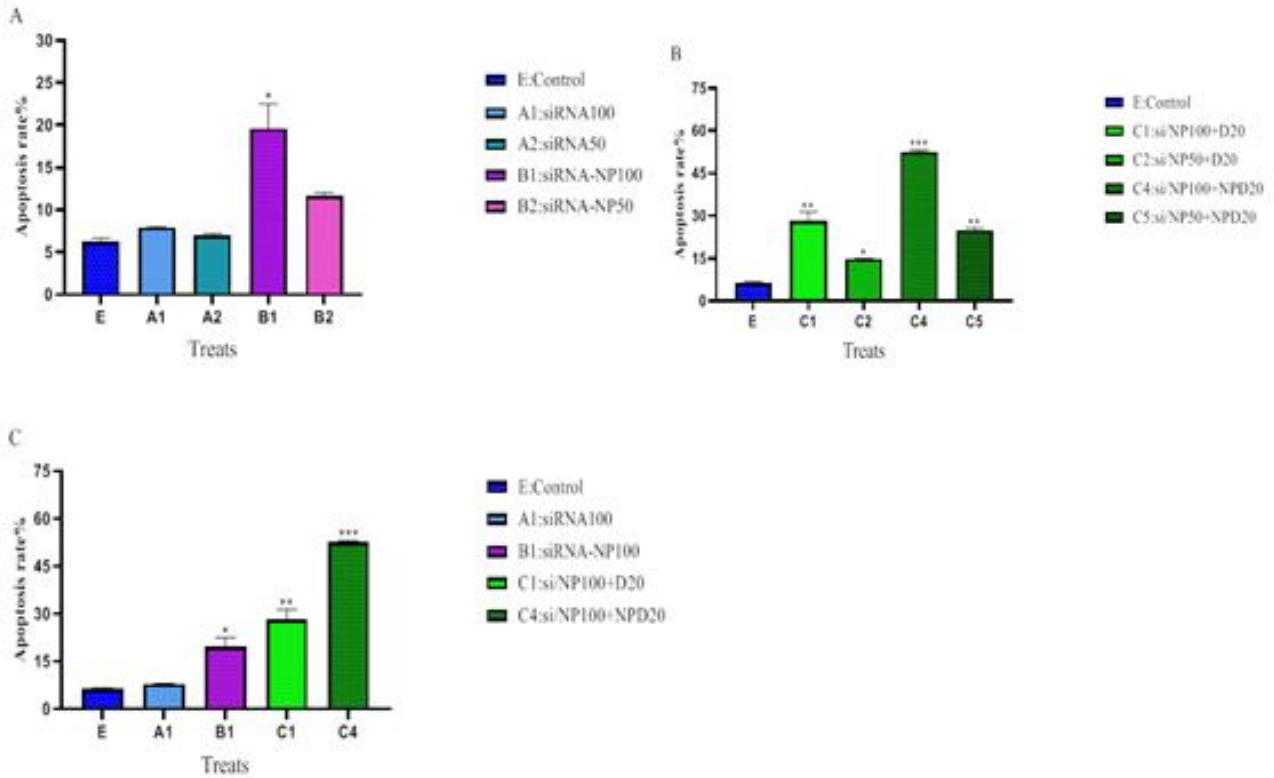


Figure 10: Comparison of the apoptotic rate of resistant cells in different treatment groups.

### Integrin $\alpha v\beta 3$ Protein Level in Resistant SW48 Cells

The protein levels of  $\alpha v\beta 3$  integrin were measured in the treatment groups using ELISA. Results demonstrated a significant increase in  $\alpha v\beta 3$  integrin protein levels in resistant cells compared to non-resistant cells ( $1.14 \pm 0.07$  ng/mL vs.  $1.01 \pm 0.02$  ng/mL,  $p < 0.001$ ).

Treatment with siRNA-NPs and Reg-NPs significantly reduced the  $\alpha v\beta 3$  integrin protein levels in the B1 (siRNA-NP100), C1 (D20+siRNA/NP100), C2 (D20+siRNA/NP50), C4 (NPD20+siRNA/NP100), and C5 (NPD20+siRNA/NP50) groups to  $0.95 \pm 0.07$ ,  $0.91 \pm 0.01$ ,  $0.97 \pm 0.03$ ,  $0.87 \pm 0.01$ , and  $0.93 \pm 0.01$  ng/mL, respectively ( $p < 0.001$ , Figure 11).

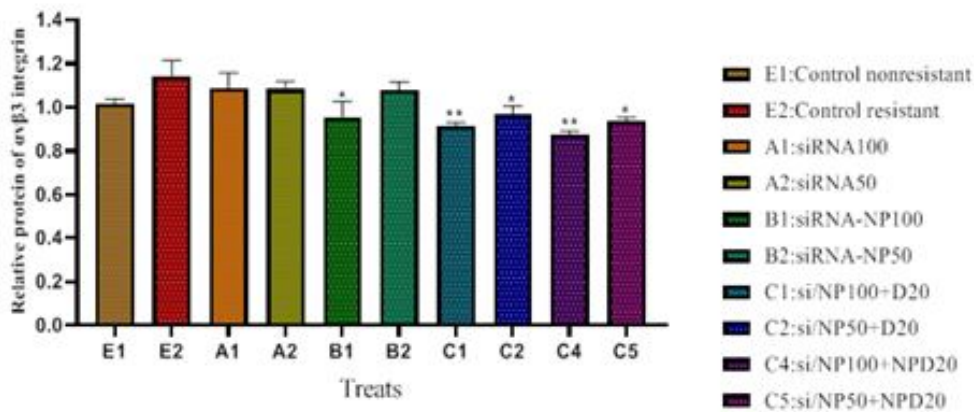


Figure 11:  $\alpha v\beta 3$  protein expression in the resistant SW48 cell line in different treatment groups.

## Discussion

This study successfully established a regorafenib-resistant SW48 colon cancer cell line using a gradual dose-escalation method to investigate the potential role of  $\beta 3$  integrin in developing drug resistance.

The  $IC_{50}$  value of regorafenib for resistant SW48 cells was 35.13  $\mu M$ . Comparatively, Fang Wei et al. reported  $IC_{50}$  values of 19.41  $\mu M$  and 11.57  $\mu M$  for regorafenib-resistant SW480 and HCT116 colon cancer cell lines, respectively [19]. Similarly, the  $IC_{50}$  for regorafenib in the MHCC-97H hepatocellular carcinoma-resistant cell line was reported as 13.78  $\mu M$  [20]. Variations in  $IC_{50}$  values among studies can be attributed to factors such as differences in cell lines, drug concentrations, origins of cells, and passage numbers.

The current study utilised DDAB-mPEG-PCL and Reg/DDAB-mPEG-PCL NPs for siRNA delivery and drug encapsulation. The Z-average size and zeta potential of DDAB-mPEG-PCL NPs were 73.1 nm and +28.9 mV, respectively, while those of Reg/DDAB-mPEG-PCL NPs were 86.61 nm and +25.2 mV. These characteristics are suitable for siRNA delivery and efficient encapsulation. An N/P ratio of 20 was selected for siRNA/NP complexes based on gel retardation assays.

Comparatively, Stefano Colombo et al. used DOTAP-PLGA NPs with an average size of 222.5 nm, a zeta potential of +43.5 mV, and an N/P ratio of 10 to deliver siRNA into human H1299 non-small lung carcinoma cells, resulting in a significant decrease in target gene expression [21]. Another study by Shin-Yu Lee demonstrated that PDMA-b-PCL NPs with an average size of 237.7 nm, a zeta potential of +34.2 mV, and an N/P ratio of 8 effectively delivered VEGF siRNA into LS174T human colon adenocarcinoma cells, reducing VEGF gene expression and tumor size [22]. The cationic lipid composition is critical in determining N/P ratio, zeta potential, and siRNA loading efficiency [23].

The  $\beta 3$  integrin is overexpressed in several cancers, including breast, melanoma, pancreatic, and lung, and plays a crucial role in resistance, angiogenesis, metastasis, and tumor progression [24, 25]. In this study,  $\beta 3$  integrin expression increased 1.63-fold in resistant SW48 colon cancer cells compared to non-resistant cells. This aligns with findings from Boz Er et al., who observed a 4-fold and 2-fold upregulation of  $\beta 3$  integrin in trastuzumab-resistant SKBR3 and HCC1954 breast cancer cells, respectively [26].

The role of  $\beta 3$  integrin in cancer progression is well established, influencing cancer cells' proliferation, migration, metastasis, and apoptosis. Targeting  $\beta 3$  integrin has been suggested as a therapeutic strategy for breast cancer and skeletal metastasis [27, 28]. Madhumathy G. Nair demonstrated that  $\beta 3$  integrin overexpression prevents apoptosis in MDA-MB-231 breast cancer cells resistant to epirubicin by suppressing pro-apoptotic proteins and stimulating anti-apoptotic pathways, thereby contributing to resistance [29].

The current study treated resistant cells with various groups to identify the most effective strategy for reducing  $\beta 3$  integrin gene expression. Combination therapy with siRNA/NPs and Reg/NPs was the most effective approach, significantly decreasing  $\beta 3$  integrin expression and inducing apoptosis in resistant cells.

To further investigate the role of the  $\beta 3$  integrin subunit in the resistance process, the total  $\alpha v\beta 3$  integrin protein levels were measured. Protein levels were elevated in resistant cells but significantly decreased after combination treatment with siRNA/NPs and Reg/NPs. Similar results were reported by Javadi, who demonstrated increased  $\alpha v\beta 3$  integrin protein levels in erlotinib-resistant colon cancer cells, which were effectively reduced by combination therapy with curcumin and erlotinib [15].

Resistance in cancer cells is induced by multiple mechanisms, including  $\beta 3$  integrin overexpression. The knockdown of  $\beta 3$  integrin by siRNA in combination with chemotherapy offers a promising strategy to reduce drug concentrations, enhance therapeutic

tic efficacy, and mitigate the adverse effects of chemotherapeutic agents.

## Conclusion

This study demonstrated that combination therapy with siRNA/DDAB-mPEG-PCL NPs and Reg/DDAB-mPEG-PCL NPs effectively downregulated  $\beta 3$  integrin gene expression, induced apoptosis, and reduced  $\alpha \beta 3$  integrin protein levels in regorafenib-resistant SW48 colon cancer cells. These findings suggest that this combination therapy may modulate drug resistance signaling pathways, offering a promising strategy to overcome chemoresistance in CRC.

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## Author Contributions

Mir Ali Mousavi: wrote and edited the manuscript, carried out the experiments, data collection, and analysis data. Mina Zhiani: performed the experiments, data collection, editing of the manuscript. Kobra Rostamizadeh: designed the project, analysis data. Reza Pirizadeh: performed the experiments, data collection. Afsaneh Mennati: performed the experiments, data collection, editing of the manuscript. Mojtaba Fathi: designed the project, analysis data, editing of the manuscript.

## Conflicts of Interest

The authors declare no conflicts of interest.

## Data Availability Statement

The information in the current study are available from the corresponding author upon logical request.

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