

# MicroRNA-21 inhibitor (miR-21i) and Simvastatin-loaded poly (D,L-lactide-co-glycolide)/polyethylenimine (PLGA/PEI) nanoparticles for synergistic anticancer effect in Gastric Cancers

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## Original article

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# Abstract

In this study, we have successfully developed a simvastatin (SMV) and miR-21i-loaded poly (D,L-lactide-co-glycolide)/polyethylenimine (PLGA/PEI) nanoparticles (NP) to enhance the therapeutic efficacy in gastric cancers. The nanoparticles were characterized for in vitro physicochemical and biological assays and pharmacokinetic study was performed in rats. CLSM/FACS results clearly showed the ability of SMV/miR-21i-loaded PLGA/PEI NP (PPN-S21i) to deliver the combinational therapeutics of SMV and miR-21i to the cancer cells. Combination of SMV+miR-21i showed significantly lower cell viability compared to that of free SMV. Our results clearly highlight the importance of simultaneous interaction of SMV+miR-21i and that it could significantly decrease the cell proliferation in BGC-823, SGC-7901 and HGC-27 gastric cancer cells while it was significantly less cytotoxic to normal gastric mucosa cells (GES-1). Cell apoptosis of SMV+miR-21i was significantly higher compared to that of individual drug or miRNA. Finally, pharmacokinetic analysis revealed that PPN-S21i significantly prolonged the blood circulation time of SMV compared to that of free SMV indicating the potential of carrier system. Overall, results clearly indicate that the combination of SMV+miR-21i (gene + drug therapy) might provide a valuable strategy for the clinical management of gastric cancers.

## Introduction

Gastric cancer is one of the most lethal cancers with high rate of mortality across the world. The severity of gastric cancer could be estimated from that fact that completes surgical resection of this tumor remains the primary curative option. However, 5-year overall survival is limited in patients undergoing surgery alone (Haq et al., 2012; Siegel et al., 2013). According to an estimation, ~40,000 Americans are diagnosed every year with this cancer and equal amount of people are dying each year with gastric cancers making it fourth largest cancer-related death overall (Pennathur et al., 2013). The delay in diagnosis, early reoccurrence, local relapse and distant metastasis are the common cause of the high death rate. Number of treatment strategies have been tried to improve the chemotherapy efficacy including multiple genetic, transcriptional and microenvironmental changes, however, no improved in survival rate of gastric patient was observed (Orditura et al., 2014; Sener et al., 1999). Besides, most patients have no chance of surgical resection in advance stage or metastasis cancers. This kind of grim scenario has highlighted the importance of new therapeutic approaches (Bailey et al., 2008). Therefore, development of effective therapeutic approaches for the effective management of gastric cancer is required.

In this regard, microRNA (miRNA) has garnered increasing attention in the cancer research and treatment of gastric cancers (Passetti et al., 2009). The miRNAs are group of small gene regulating RNAs consisting of 18-24 nucleotides and controls the overexpression in multiple malignancies including gastric cancers (Ramasamy et al., 2019). The miRNA function by base-pairing with the complementary sequences with the messenger mRNAs and inhibit the protein synthesis. The miRNAs regulate the proliferation, progression, metastasis of cancer cells and thereby controls its apoptosis and cell survival (Blower et al., 2008). The inhibition of overexpressed oncogenic miRNAs or restitution of under-expressed miRNAs

could result in the improvement of therapeutic efficacy. Four of the overexpressed miRNAs include miR-155, miR-21, miR-221 and miR-222 and among all, miR-21 is reported to have the highest overexpression in gastric cancers and plays a central role in the cancer cell proliferation and metastasis (Feng et al., 2017). The molecular pathway of miR-21 includes the change in the apoptosis signaling, phosphorylation of AKT pathway and increase in the gene involved in the cell invasion. For example, overexpression of miR-21 downregulates the tumor suppressors phosphatase and tensin homologue (PTEN) and phosphorylates the AKT kinase making it less susceptible to drugs like Gemcitabine. We expect that when miR-21 is inhibited by miRNA-21 inhibitor (miR-21i), cancer cell proliferation will be inhibited (Li et al., 2015). The inhibition of miR-21 will also increase the sensitivity of chemotherapeutic drug and thereby increased apoptosis and cancer cell death could be observed (Hong et al., 2013). However, single agent therapy based on miRNA alone would be ineffective for the higher therapeutic effect.

Simvastatin (SMV) – coenzyme A reductase inhibitors has been indicated in the modulation of inflammation (Cho et al., 2008). Recently several statins have been reported to have cytotoxic effects in cancer cells including colon, ovarian, squamous cell carcinoma, and pancreatic cells like BxPc-3 and MiaPaCa-2 cells (Stine et al., 2016). However, it has also been reported that statins can elevate the risk of cancer incidence. The controversial findings might be attributed to the type of statin, lipophilic and hydrophilic statins used. The lipophilic statin (SMV) has been known to produce the anticancer effect by inhibiting the cell proliferation and promoting the cell apoptosis (Chang et al., 2011). In one instance, it has been reported that SMV induces the anticancer effect by the inhibition of nuclear factor-kB signal pathway. It must be noted that SMV is a highly hydrophobic drug with high first pass metabolism and low systemic availability and low aqueous solubility (Rosenson, 2004; Muzykantov, 2013). Similarly, naked miRNA are subjected to enzymatic degradation in the systemic circulation and leads to instability following the systemic administration, necessitating the need for a stable nanocarrier system. The implementation of nanomedicine has done wonders in improving the therapeutic efficacy of encapsulated components (Ramasamy et al., 2017). In this study, we have utilized poly (D,L-lactide-co-glycolide) (PLGA)-based polymeric nanoparticle system to enhance the delivery and therapeutic efficacy of SMV and miR-21i. PLGA is a US-FDA approved biodegradable and biocompatible polymers that are wide accepted in the clinical applications (Danhier et al., 2012). Specifically, we have introduced Polyethylenimine (PEI) in order to encapsulate the miRNA on the surface of nanoparticles (Langer, Tirrell, 2004).

Overall, main aim of study was to enhance the delivery and therapeutic efficacy of SMV and miR-21i (combination agents) in gastric cancers using PLGA/PEI nanoparticles. The in vitro anticancer effect of single and combinational drugs was tested in BGC-823 cells (cell viability assay, apoptosis assay, and live/dead assay). We have performed the in vitro experiments of cell viability and apoptosis in more than one cancer cells (BGC-823, SGC-7901 and HGC-27 cells) in order to demonstrate its efficacy in wide panel of cancer cells. Besides, we have performed the in vitro experiments of cell viability on normal gastric mucosal cell line (GES-1) to evaluate its effect in non-cancerous cell from the safety perspective. The pharmacokinetic performance of nanoparticle was studied in SD rats.

# Materials And Methods

## Preparation of SMV and miR-21i-loaded PLGA/PEI nanoparticles

The poly (D,L-lactide-co-glycolide) (PLGA, 50:50) and Polyethylenimine (PEI) were purchased from Sigma-Aldrich, China. The PLGA (30 mg) and PEI (0.1 mg) was dissolved in 1ml of chloroform. The simvastatin (SMV, 10% w/w) was dissolved in the organic phase and stirred for 15 min. Followed by a mixture of bovine serum albumin (BSA, 5 mg) and miRNA-21i/miRNA21i-FAM' (Shanghai GenePharma Co., Ltd., Shanghai, China) (300 µg) in 200 µl of EDTA buffer was added to the organic phase slowly and immediately vortexed for 2 min. The mixture was probe-sonicated for 6 min (60% Amplitude) in an ice-bath resulting in the formation of water-in-oil (W/O) emulsions. The so-formed W/O emulsion was added to 5 ml of 2.5% polyvinyl alcohol solution and again sonicated for 4 min in the ice-bath. The emulsion was stirred for 15h at room temperature to allow evaporating all of the organic solvents. The SMV/miRNA-loaded nanoparticles were collected by ultracentrifugation at 12000 rpm for 10 min using a sophisticated centrifuge. The nanoparticles were washed twice and stored at 4°C until further use.

## Gel retardation analysis

Gel retardation assay was performed in 2% Agarose gel pre-stained with 0.5 mg/mL of ethidium bromide. Briefly, 2% Agarose gel was prepared in TRIS-acetate EDTA buffer and condensation ability of different formulations in N/P ratio was evaluated. The experiment was carried out at 80 V for 30 min and the retardation at different N/P ratio was analyzed by gel image analysis system.

## Nanoparticle characterization

The hydrodynamic particle size and zeta potential of nanoparticles were evaluated by laser particle analyzer (Fritsch ANALYSETTE 22, Germany). The experiments were performed at 25°C in triplicate numbers. The morphology of particles was evaluated by transmission electron microscope (TEM) using PHILIPS TECHNICAL-10 (Holland). The TEM was carried out at 100 kV. The diluted samples were placed in a copper grid containing 300-mesh and excess water was removed and in turn stained with 2% uranyl acetate solution.

## In vitro SMV/miRNA release study

The release study was performed by dialysis method. To evaluate the release of miRNA, miR-21i-FAM was used. The nanoparticle dispersion was mixed with 1 mL of release medium (pH 7.4, phosphate buffered saline) and packed in a dialysis membrane (MWCO 3000 Da) which is in turn placed in a rotary shaker (100 rpm) at 37°C. At fixed time interval, 1 mL of release medium was collected and replaced with equal amount of fresh buffer. The release of SMV was determined by HPLC method and miR-21i-FAM was evaluated by measuring the fluorescence intensity of FAM ( $\lambda_{ex}$  488 nm and  $\lambda_{em}$  520 nm) using a microplate reader (Tecan, Durham, USA). WATERS (E2695) HPLC was used with a C18 column. The mobile phase consisted of acetonitrile/water (20:80) at a constant flow rate of 1.2 ml/min at 242 nm.

## **Cellular uptake analysis of PPN-S21i**

The cellular uptake analysis was performed by confocal laser scanning microscopy (CLSM) and flow cytometer. BGC-823 cells (ATCC, USA) were cultured in RPMI-1640 media supplemented with 10% FBS.  $2 \times 10^5$  cells were seeded in each well of 12-well plate and kept aside for 24h. The cells were treated with fresh medium containing the PPN-S21i nanoparticle containing rhodamine-B as a fluorescent tracker. The nanoparticles were incubated for 3h and then washed twice with PBS and then stained with LysoTracker Green for 10 min. The cells were again washed and fixed with 4% paraformaldehyde. The cells were observed under Leica Microsystems, Mannheim, Germany. The cellular uptake was further studied by flow cytometer (BD Biosciences, San Diego, CA). The cells were treated in the same manner as mentioned above and then collected by scrapping and 10,000 events were recorded.

## **Cell viability assay**

The cell viability assay was performed by cell counter kit-8 (CCK8) assay. To begin this assay,  $8 \times 10^3$  BGC-823 cells (ATCC, USA) were seeded in each well of 96 well plates and incubated for 24h in 100  $\mu$ l volume. The cells were then treated with a SMV, SMV+miR-21i and PPN-S20i nanoparticles in a concentration range from 1-100  $\mu$ g/ mL. For all concentration of SMV, miR-21i was fixed at 50  $\mu$ g/ mL. The cells were incubated for 24h. The cells were washed carefully and 10  $\mu$ l of CCK-8 solution was added to each well of 96 well-plate and incubated for 1h at 37°C. The respective absorbance was measured using a microplate reader at 460 nm using BioTek, USA.

## **Flow cytometer-based apoptosis assay**

The apoptosis assay was performed by Annexin-V/PI staining using flow cytometer. To begin this assay,  $2 \times 10^5$  BGC-823 cells were seeded in each well of 12 well plates and incubated for 24h. The cells were then treated with a free SMV, free miR-21i, SMV+miR-21i and PPN-S20i nanoparticles in a fixed concentration. The cells were incubated for 24h and then cells were collected by trypsinization process and pellet was collected by centrifugation process. The cells were resuspended in 200  $\mu$ L of binding buffer and stained with 3  $\mu$ L of Annexin-V and PI solution and incubated for 15 min in dark conditions. The cell apoptosis was evaluated by recording 10,000 events in flow cytometer.

## **Live/Dead assay**

The anticancer effect was further studied by Live/Dead assay. To begin this assay,  $2 \times 10^5$  cells were seeded in each well of 12 well plates and incubated for 24h. The cells were then treated with a free SMV, free miR-21i, SMV+miR-21i and PPN-S20i nanoparticles in a fixed concentration. The cells were incubated for 24h. The cells were washed and stained with Acridine Orange (AO, 5  $\mu$ g/mL) and PI (2.5  $\mu$ g/mL) and incubated for 15 min and then images were captured using a fluorescence microscope (Olympus, USA).

## **Pharmacokinetic analysis in animal**

The Sprague-Dawley (SD) rats were obtained from Animal Facility Center of China-Japan Union Hospital of Jilin University, China. The animal study was approved by Institutional Animal Ethics Committee of China-Japan Union Hospital of Jilin University, Changchun. The experimental protocol for animals was approved by China-Japan Union Hospital of Jilin University Ethical guidelines for Small Animals. The SD rats were given free access to food and water and maintained under ambient conditions with 12h dark/light cycle. The rats were administered with a fixed SMV dose of 7.5 mg/kg of SD rats. The SD rats were divided into two groups with six rats in each group and formulations were administered by tail vein injection. Blood samples were collected in a periodical manner from 0.25-24h. The plasma was separated from whole blood and stored in -80°C until further analysis. At the end of study period, mice were sacrificed with the exposure to CO<sub>2</sub>.

### **Statistical analysis**

Results are expressed as mean  $\pm$  standard deviation (SD). Comparison between two groups was performed using Student's t-test.  $P < 0.05$  was considered statistically significant.

## **Results**

### **Formulation of PPN-S21i nanoparticles**

The preparation of SMV and miR-21i-loaded PLGA/PEI nanoparticles (PPN-S21i) is depicted in the schematic presentation (Figure 1). The optimized PPN-S21i particles have an average size of  $131.5 \pm 1.68$  nm with an effective surface charge of  $19.5 \pm 1.77$  mV. The miRNA binding ability with nanoparticle was assessed by gel retardation assay in 2% Agarose gel at different N/P ratio of miRNA and PEI of PPN-S21i nanoparticles (Figure 2a). As shown, naked miRNA was found at the opposite electrode while retardation of miRNA was gradually increased with the increase in the N/P ratio with no bright miRNA bands were observed at the bottom. A complete retardation of miRNA movement or 100% binding efficiency was observed at N/P ratio of 4.

### **Characterization of PPN-S21i nanoparticles**

PPN-S21i nanoparticles exhibited an entrapment efficiency of  $94.5 \pm 1.12$  % with an active loading efficiency of 8.79% w/w. TEM images revealed a perfect spherical shaped particle spread uniformly on the 300-mesh copper grid (Figure 2b). A dark core with faint surface reveals the surface characteristics of the prepared nanoparticles. The drug release study was performed by dialysis method. Both SMV and miR-21i showed a sustained and controlled release profile on pH 7.4 buffer system. However, small molecule and nucleic acid released in a different pattern, for example, approximately ~45% of SMV released after 24h compared to ~25% of miRNA release during the same time period. After 60h incubation, ~90% of SMV released and ~45% of miRNA released from PPN-S21i nanoparticles (Figure 2c). The release profile definitely points to the fact that nanoparticles allow a controlled released of encapsulated therapeutics. The results clearly advocate the relative stability of nanoparticle system in the

systemic circulation and avoid the unnecessary release of therapeutic load and release the encapsulated drug in the tumor tissues.

### **Cellular internalization in BGC-823 cells**

The cellular uptake of PPN-S21i nanoparticles in BGC-823 cell was first evaluated by confocal laser scanning microscopy (CLSM) (Figure 3a). As shown, a dense red fluorescence was observed in lysosome region of the cancer cells indicating a typical endocytosis-mediated cellular uptake. Merged image clearly showed a perfect merging of lysosome-stained green fluorescence and red fluorescence originated from the PPN-S21i nanoparticles. The uptake was further ascertained by flow cytometer analysis (Figure 3b). FACS analysis showed a typical shift in the histogram towards the right side indicating a definitive cellular uptake. As shown, FACS analysis depicted a typical time-based cellular uptake with increase in the internalization of nanoparticles with the increase in the incubation time.

### **Effect of PPN-S21i nanoparticles on cell proliferation**

We have employed three groups to study the effect of single and combinational regiment on the cell viability of three different cancer cells, BGC-823, SGC-7901 and HGC-27 cells (Figure 4a; Figure S1). The three groups include free SMV, SMV+miR-21i, and PPN-S21i. Separately, miR-21i exhibited a concentration-dependent cell killing effect (data not shown). A fixed concentration of 50 ng/ml of miR-21i was used for all the combination dose. As shown, cells treated with combination of SMV+miR-21i showed significantly lower cell viability compared to that of free SMV. In general, all the formulations exhibited a concentration-dependent cytotoxic effect in the BGC-823 cancer cells. Similarly, PPN-S21i showed remarkable antitumor efficacy in SGC-7901 and HGC-27 cells. We have evaluated the effect of individual formulations on normal gastric mucosal cell line, GES-1 cells. Although higher concentrations of free SMV, SMV+miR-21i, and PPN-S21i decreased the cell viability of GES-1 cells, however, it was significantly ( $p < 0.05$ ) less compared to that in cancer cells such as BGC-823 cells.

### **Effect of PPN-S21i nanoparticles on cell apoptosis – flow cytometer**

The combination effect of SMV+miR-21i on BGC-823, SGC-7901 and HGC-27 cells was studied by flow cytometer after staining with Annexin V/PI markers. Annexin V estimates the early apoptosis cells while PI shows positive sign for dead cells or late apoptosis cells. No obvious apoptosis was observed in non-treated cells (Figure 5a). The transfection of miR-21i resulted in ~18% of cell apoptosis while SMV resulted around ~23% apoptosis of cancer cells. As expected, cell apoptosis of SMV+miR-21i was significantly higher compared to that of individual drug or miRNA. Most importantly, 3-fold increase in the cell apoptosis was observed for PPN-S21i treated cancer cells compared to individual formulations.

### **Live/Dead assay**

The anticancer effect of SMV+miR-21i, and PPN-S21i was further confirmed by Live/Dead assay. The cells were exposed to respective formulation and incubated for 24h (Figure 5b). The cells were then stained with acridine orange (AO) and propidium iodide (PI) as a respective live cell (green color) and

dead cell (red color). As shown, compared to individual SMV or miR-21i, combination of SMV+miR-21i yielded a remarkably higher red fluorescence indicating a predominant apoptosis and cell death, simultaneously, green fluorescence decreased. The effect was more pronounced in PPN-S21i treated cells which showed brightest red fluorescence with lowest green fluorescence. The order to red fluorescence was control<SMV<miR-21i<SMV+miR-21i<PPN-S21i, respectively. The results were concordant with the cell viability and apoptosis assay.

### ***In vivo* pharmacokinetic study**

The plasma concentration of SMV against the time profile has been presented in Figure 6. The pharmacokinetic parameters were obtained after the intravenous administration (tail vein) of free SMV and PPN-S21i in SD rats. The free SMV concentration in plasma immediate started decreasing after intravenous administration and quantified until 4h. The pharmacokinetic behavior of SMV was consistent with the profile of any intravenously administered free drug. On the contrary, PPN-S21i significantly prolonged the blood circulation time of SMV indicating the potential of carrier system. The  $AUC_{0-\infty}$  of PPN-S21i ( $3564 \pm 369$  ng.h/ml) was significantly higher compared to that of free SMV ( $498 \pm 114$  ng.h/ml). Moreover, the rate of elimination ( $K_{el}$ ) was markedly lower ( $P < 0.01$ ) for PPN-S21i (0.05h) followed by a high significant increase in  $T_{1/2}$  ( $9.15 \pm 1.25$ h,  $P < 0.001$ ) and MRT ( $P < 0.01$ ) values compared to that of free SMV.

## **Discussion**

PLGA/PEI nanoparticles were prepared by double emulsion method. The inclusion of PEI will introduce the positive surface charge on the nanoparticles that will allow the loading of miRNA based on electrostatic interactions. The SMV is loaded in the core of the nanoparticles which is stabilized by polyvinyl alcohol. The presence of PEG will stabilize the particles in the dispersion and systemic circulations. The optimized PPN-S21i particles have an average size of  $131.5 \pm 1.68$  nm with an effective surface charge of  $19.5 \pm 1.77$  mV. A slight decrease in the surface charge was observed after the loading of miRNA owing to the charge compensation. Overall, a small particle size of nanoparticles will allow the preferential accumulation on the malignant tumors using the well-known enhanced permeation and retention (EPR) effect. A moderate positive charge on the particle surface will allow the favorable interaction with the negatively surface cell membrane and higher cellular internalization.

The cellular uptake of PPN-S21i nanoparticles in BGC-823 cell was first evaluated by confocal laser scanning microscopy (CLSM). The results clearly suggest the ability of PPN-S21i nanoparticles to deliver the combinational therapeutics of SMV and miR-21i in the cancer cells. It could be hypothesized that a positively charged nanoparticles will interact with the negatively charged cell membrane and internalize in the cancer cells. Moreover, a small particle size would be easier to internalize in the cancer cells.

Cells treated with combination of SMV+miR-21i showed significantly lower cell viability compared to that of free SMV. Our results clearly highlight the importance of simultaneous interaction of SMV+miR-21i and

that it could significantly decrease the cell proliferation. Significantly higher cell killing effect was observed with PPN-S21i suggesting the role of delivery carrier that could easily internalize the cancer cells and release the encapsulated components in a controlled manner in specific ratios. A higher cell killing effect of SMV+miR-21i might be attributed to the different mechanism of action of both the therapeutics. It is possible that miR-21i could effectively inhibit the expression of oncogenic miR-21 receptors and thereby sensitizing the action of SMV. Consistently, Wang et al. have reported that a combination of SMV and miR-21i was effective in inhibiting the cell migration/invasion, cell proliferation and induced cell apoptosis in salivary adenoid cystic carcinoma (SACC) (Wang et al., 2018). Similarly, Li et al. reported that miR-21 was the key factor involved in the migration and invasion of pancreatic cancer cells and authors have showed that the knockdown of miR-21 by transfection miR-21i antisense oligonucleotide (ASO) significantly suppressed the migration of cancer cells and significantly inhibited the cell proliferation (Li et al., 2017). Overall, PPN-S21i was effective in inducing a significantly higher anticancer effect than the free drug itself. Similarly, significant difference was observed in cell apoptosis of PPN-S21i treated cells (~70% apoptosis). It has been reported that miR-21i might inhibit the miR-21-based apoptosis pathway by the downregulation of Bcl-2 pathway and upregulation of PTEN, Bax and P53 pathways (Lin et al., 2018). The combination of miR-21i and small molecule drug might act in a synergistic manner for higher apoptosis of gastric cancer cells.

The pharmacokinetic behavior of SMV was consistent with the profile of any intravenously administered free drug. On the contrary, PPN-S21i significantly prolonged the blood circulation time of SMV indicating the potential of carrier system. The enhanced pharmacokinetic profile of PPN-S21i was attributed to the prolonged blood circulation property of nanoparticulate system and controlled release of encapsulated drug. The presence of PEG (steric hindrance) on the surface further increased the blood circulation properties of the PPN-S21i by reducing its uptake by the reticuloendothelial system (RES). The prolonged circulation of PPN-S21i allows the greater accumulation potential in the malignant tumors (EPR effect) and enhances the therapeutic efficacy in gastric cancers (Ruttala et al., 2019).

In summary, we have successfully developed a simvastatin (SMV) and miR-21i-loaded PLGA/PEI nanoparticles to enhance the therapeutic efficacy in gastric cancers. The SMV was loaded with high loading efficiency and miRNA was maximally loaded at an N/P ratio of 4. CLSM/FACS results clearly showed the ability of PPN-S21i nanoparticles to deliver the combinational therapeutics of SMV and miR-21i to the cancer cells. Cells treated with combination of SMV+miR-21i showed significantly lower cell viability compared to that of free SMV. Our results clearly highlight the importance of simultaneous interaction of SMV+miR-21i and that it could significantly decrease the cell proliferation. As expected, cell apoptosis of SMV+miR-21i was significantly higher compared to that of individual drug or miRNA. The anticancer effect was more pronounced in PPN-S21i treated cells which showed brightest red fluorescence with lowest green fluorescence in Live/Dead assay. Finally, pharmacokinetic analysis revealed that PPN-S21i significantly prolonged the blood circulation time of SMV compared to that of free SMV indicating the potential of carrier system. Overall, results clearly indicate that the combination of SMV+miR-21i (gene + drug therapy) might provide a valuable strategy for the clinical management of gastric cancers.

# Abbreviations

SMV - Simvastatin

PLGA/PEI - poly (D,L-lactide-co-glycolide)/polyethylenimine

MiRNA - microRNA

PPN-S21i - SMV and miR-21i-loaded PLGA/PEI nanoparticles

# Declarations

## Author's Contribution

YL, BY, and LZ carried out the main experimental parameters. XC and HL have carried out the cell-based assays and protocols. MY involved in animal experimental. BS has designed and written the entire manuscript. All authors have read and approved the manuscript.

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## Competing interests

The authors report no conflict of interest.

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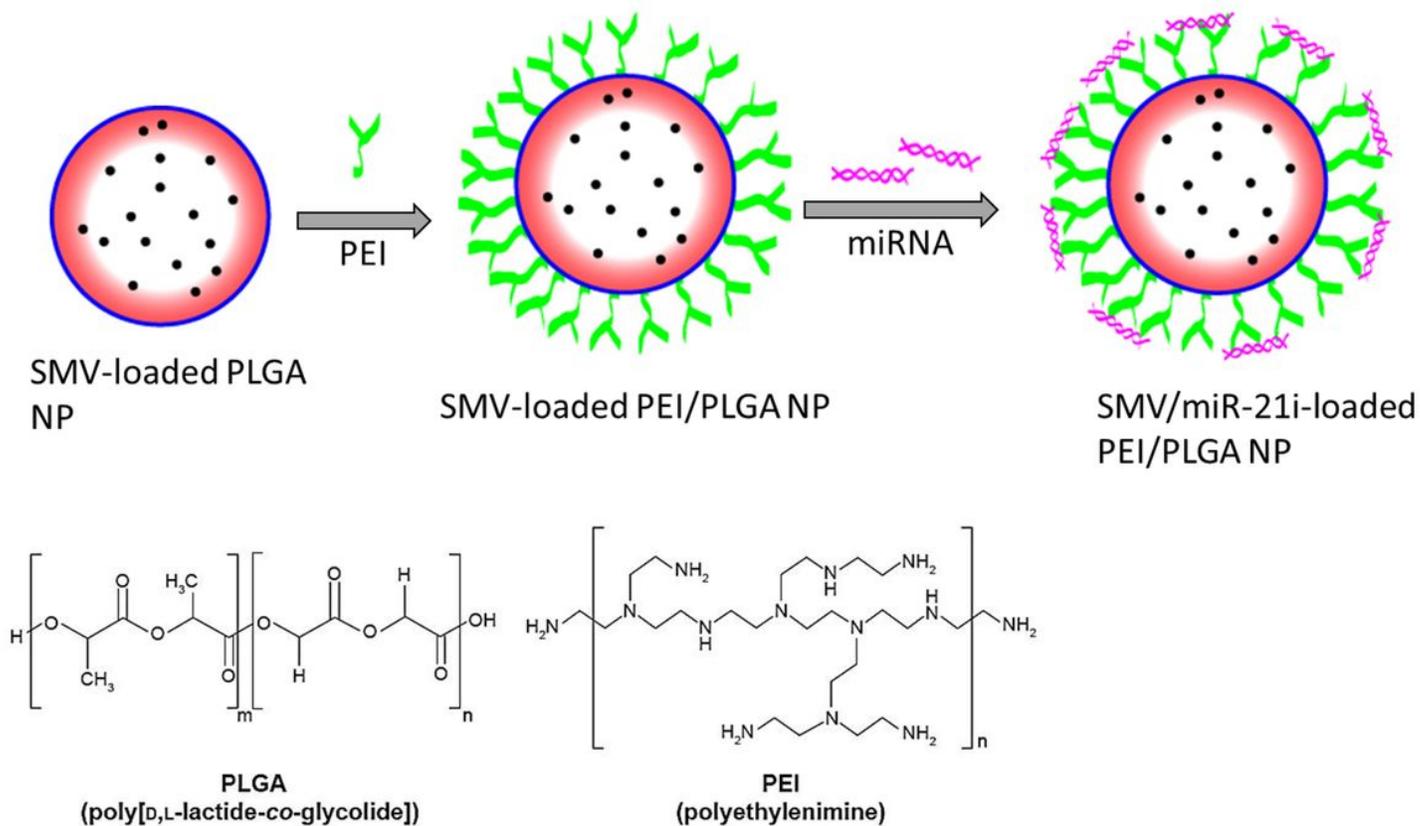
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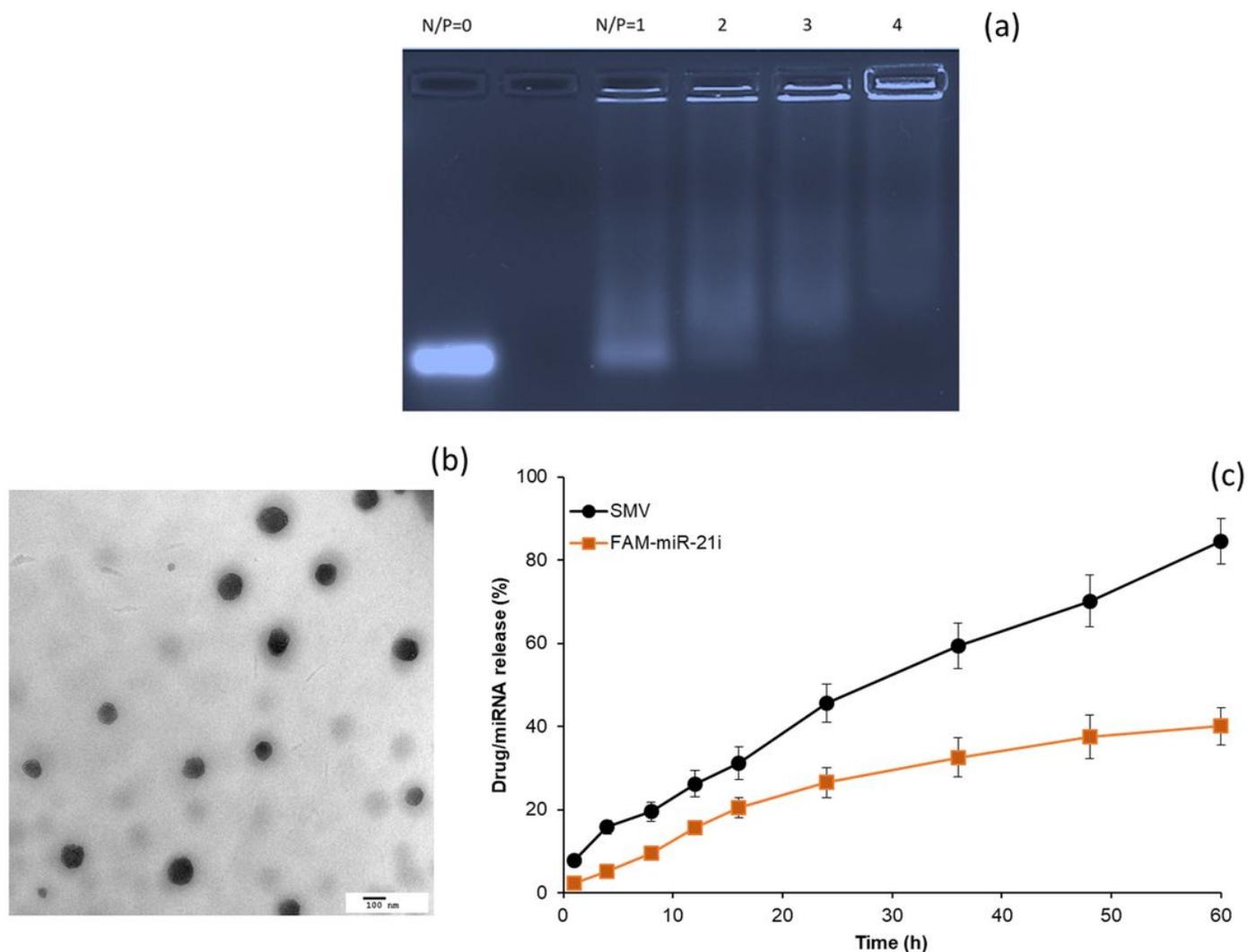
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## Figures



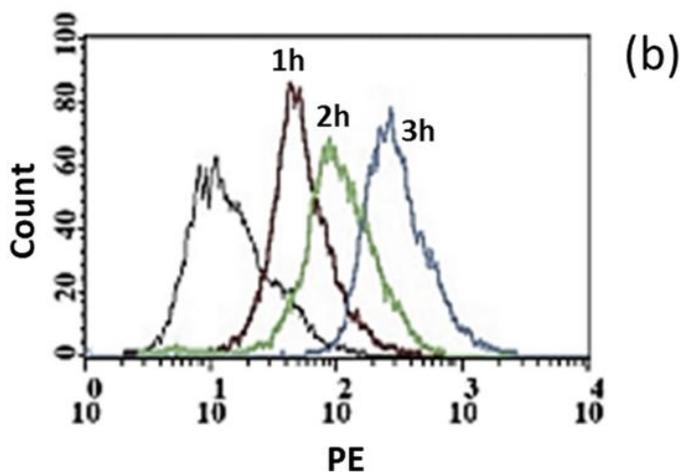
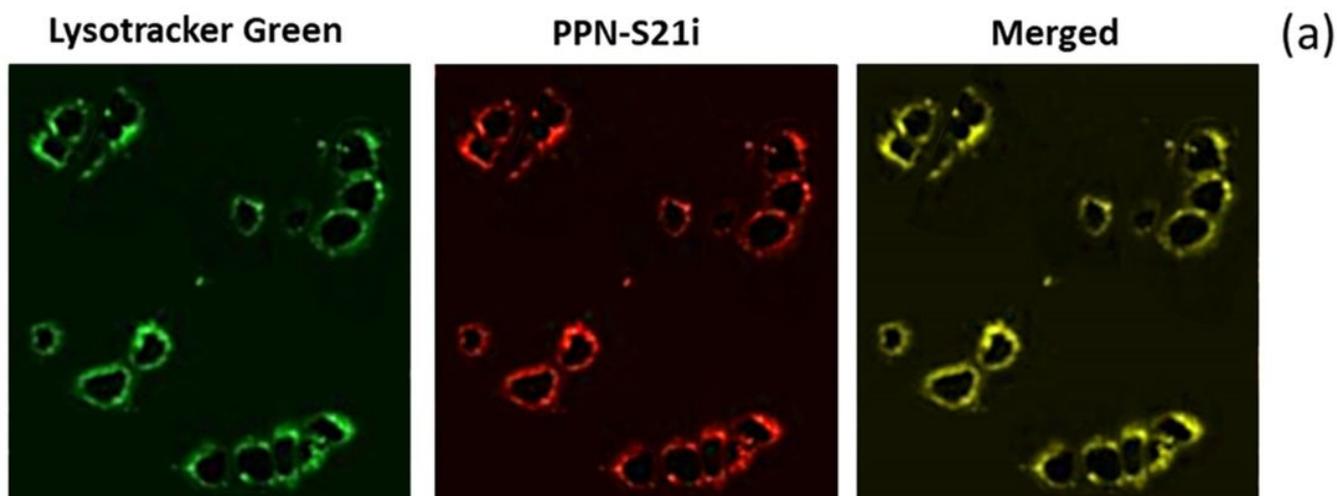
**Figure 1**

Graphical presentation depicting the preparation of simvastatin and miRNA-21i-loaded PLGA/PEI nanoparticles. The PEI-grafted polymeric nanoparticles were prepared by double emulsion method and miRNA was surface loaded onto the carrier system making a SMV/miR-21i-loaded PEI/PLGA NP.



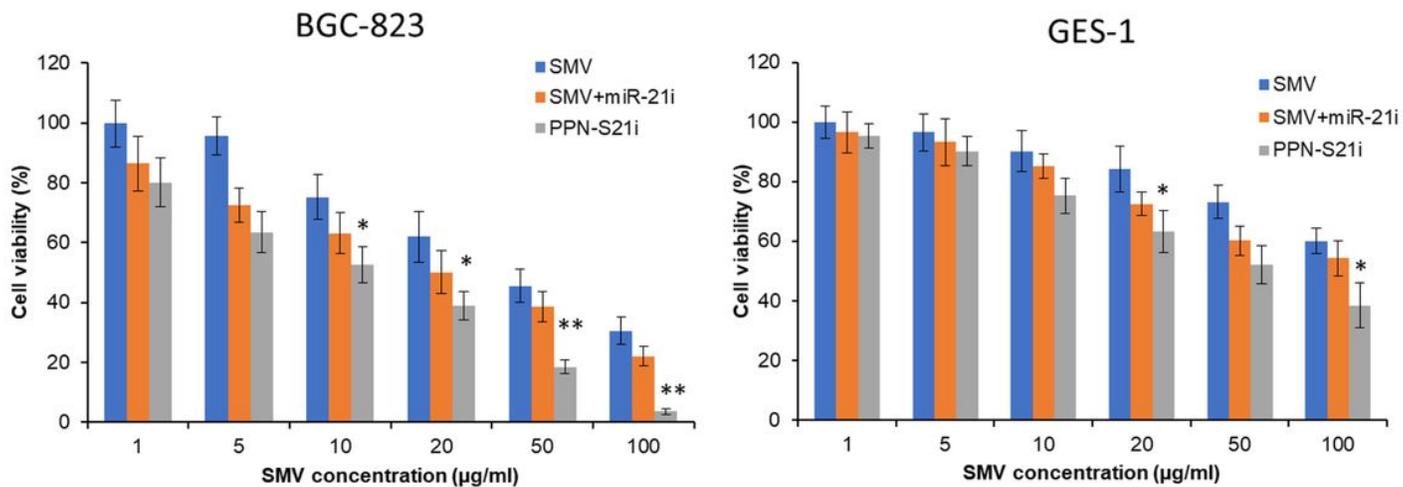
**Figure 2**

(a) Gel retardation assay of PPN-S21i and miR-21i at different N/P ratio (1-4). Naked miRNA completely moved to the opposite electrode and 100% binding efficiency was observed at N/P ratio of 4; (b) Morphology characterization of PPN-S21i by means of transmission electron microscopy (TEM) at 100 kV; (c) In vitro release kinetics of SMV and miR-21i from PPN-S21i in pH 7.4 phosphate buffer saline (PBS) for 60h (n=3). The drug release was performed through dialysis method and release rate of SMV was determined by HPLC method and miR-21i-FAM was evaluated by microplate reader.



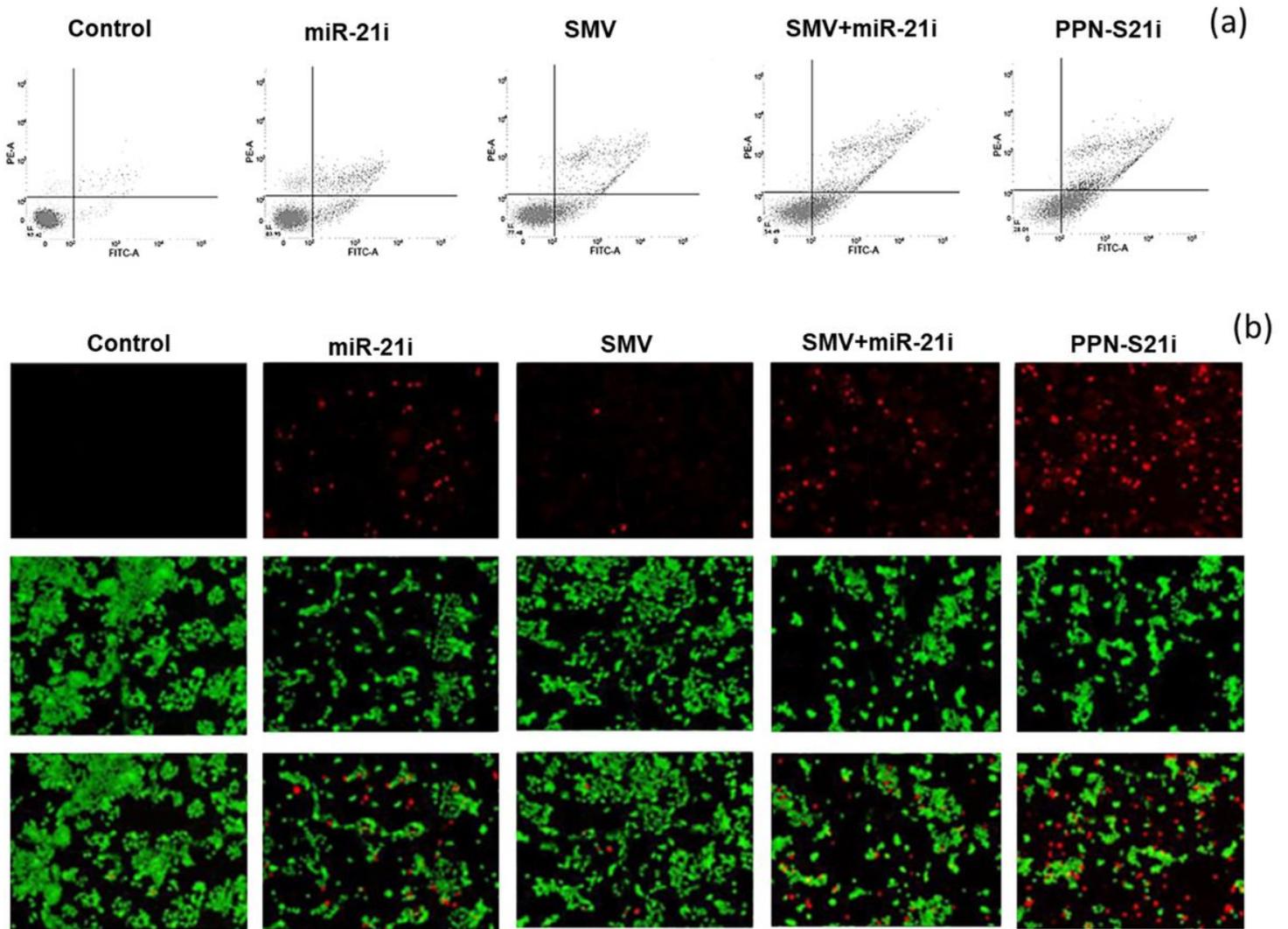
**Figure 3**

(a) Cellular uptake of PPN-S21i in BGC-823 cells using confocal laser scanning microscopy (CLSM). Rhodamine B was loaded in the nanoparticle as a fluorescent tracker (10  $\mu\text{g}/\text{mL}$ ). The lysosome of cancer cell was stained with Lysotracker Green<sup>TM</sup>; (b) Flow cytometer-based cellular uptake analysis of PPN-S21i after incubation for 1-3h. Time dependent increase in cellular uptake was observed for PPN-S21i.



**Figure 4**

Cell viability analysis of BGC-823 cancer cells and normal gastric mucosal cells (GES-1) upon treatment with SMV, SMV+miR-21i, and PPN-S21i through CCK-8 assay. Cell viability decreased with the increase in the concentration of formulations (1-100 µg/ml) after 24h incubation. PPN-S21i showed significantly higher cell killing effect compared to that of SMV+miR-21i (\* $p < 0.05$  and \*\* $p < 0.001$ ,  $n = 4$ ). Non-treated control was considered control.



**Figure 5**

a) Flow cytometer apoptosis analysis of BGC-823 cells after staining with Annexin-V and PI. Effect of single and combination dose on the apoptosis of BGC-823 cells. The cells were treated with 10  $\mu\text{g/ml}$  equivalent of SMV and 50 ng/ml of miR-21i. Remarkably higher proportions of apoptosis were observed for PPN-S21i compared to that of SMV; (b) Live/Dead assay of BGC-823 cells by staining with Acridine orange and PI after treatment with respective formulations and observed using fluorescence microscopy. The live cell is stained with green dye and dead cells are stained with red dye. Higher proportions of red cells were observed for PPN-S21i compared to that of individual drugs.

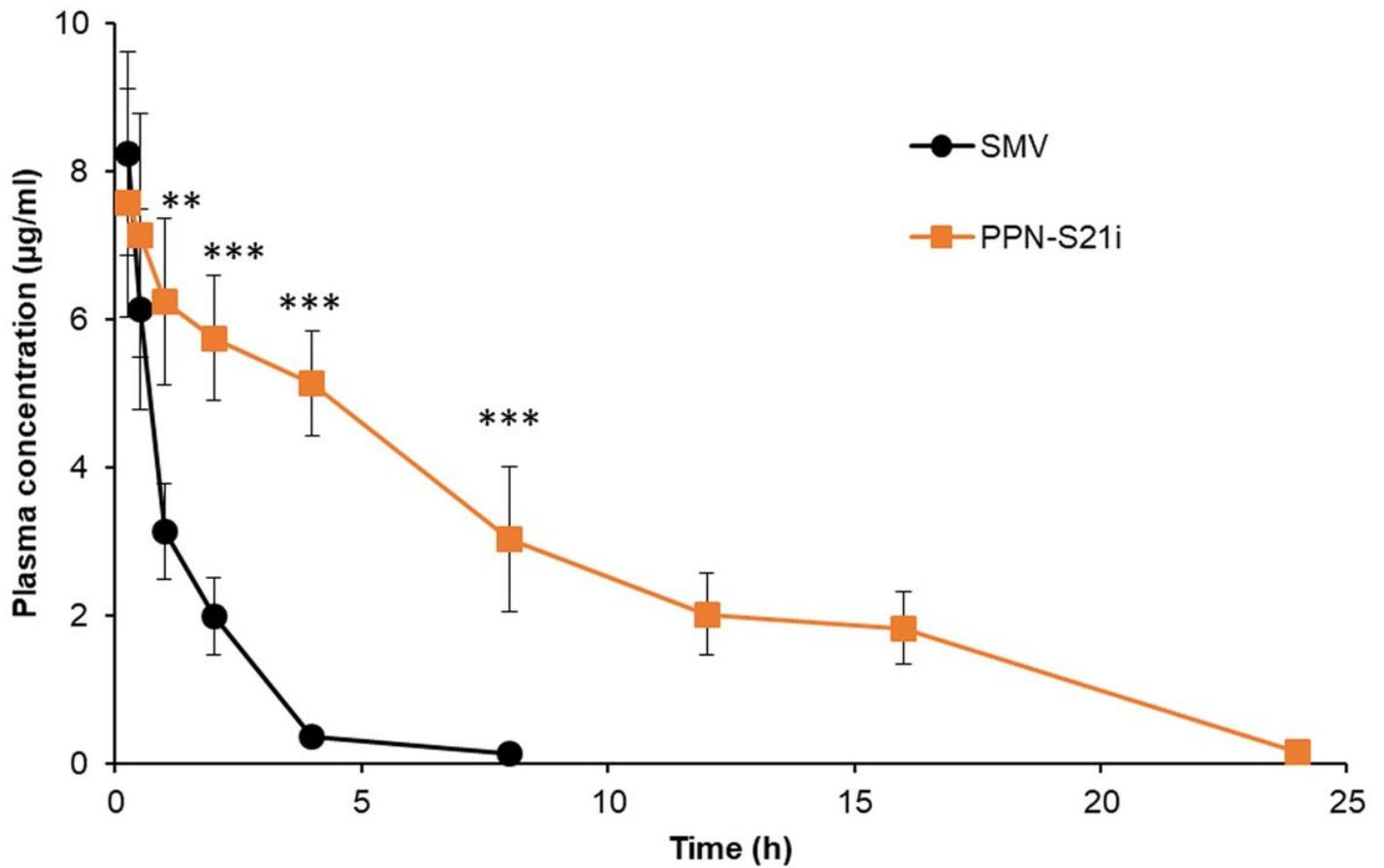


Figure 6

: In vivo plasma concentration-time profile of free SMV and PPN-S21i in SD rats (n=4). The plasma concentration of SMV was evaluated after intravenous administration of free SMV and PPN-S21i and samples were obtained until 24h. The blood samples were collected from femoral artery. PPN-S21i exhibited significantly (\*\*p<0.01 and \*\*\*p<0.0001) higher plasma drug concentration compared to free SMV.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementaryfile.docx](#)