

**Co-delivery of TLR agonist with hypomethylating agent via an ultrasmall pro-drug
micellar carrier for breast cancer chemoimmunotherapy**

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Immunotherapies harness the host immune system to elicit durable and safer antitumor effects. But due to the cancer immune escape mechanisms, the therapeutic outcomes of immunotherapies are under expectations. Researchers have struggled for decades to develop strategies to make this attractive idea work better and to figure out the underlying mechanisms. In recent years, numerous reports indicated that the altered epigenetic modifications may play a critical role in tumor immune escape. The agents that can reprogram cells to a more normal status like demethylating agents were also demonstrated to potentiate immunotherapies. In this study, we designed and synthesized an ultrasmall pro-drug nanocarrier for co-delivery of a DNA demethylating agent azacitidine and an immune modulator R848. The small size nanoparticles could efficiently penetrate and deliver the combination therapies to tumor core and reduce the distribution in other major organs. These nanoparticles PAza-R848 effectively inhibited the tumor growth. In addition, they significantly increased the numbers of tumor-infiltrated CD45⁺ cells, and the percentages of CD4⁺ and CD8⁺ T cells and dendritic cells, which may contribute to the improved antitumor efficacy.

Table of Contents

1.0 Introduction.....	1
2.0 Materials and Methods.....	4
2.1 materials	4
2.2 Cells and animals.....	4
2.3 Synthesis of azacitidine based polymer	5
2.3.1 Synthesis of VD monomer	5
2.3.2 Synthesis of POEG-co-PVD polymer	5
2.3.3 Synthesis and characterization of PAza polymer.....	6
2.4 Preparation and characterization of PAza-R848 micelles.....	6
2.5 Critical micelle concentration (CMC) of PAza micelles	7
2.6 <i>In vitro</i> bioactivity test.....	7
2.6.1 MTT test.....	7
2.6.2 Evaluation of maturation of dendritic cells (DCs)	8
2.7 Tissue biodistribution and penetration of PAza nanocarriers	9
2.8 <i>In vivo</i> therapeutic efficacy in 4T1 model.....	9
2.9 Quantification of tumor-infiltrating lymphocytes by flow cytometry	10
2.10 Safety evaluation.....	10
3.0 Results	12
3.1 Synthesis and characterization of PAza polymers	12
3.2 Physicochemical characterization of micelles	13
3.3 <i>In vitro</i> cytotoxicity assay	16

3.4 Evaluation of maturation of murine monocyte-derived dendritic cells	17
3.5 Biodistribution profiles of POEG-co-PVD and PAza nanoparticles	17
3.6 <i>In vivo</i> antitumor efficacy study of PAza-R848 nanoparticles	19
3.7 Quantification of tumor-infiltrating lymphocytes	21
4.0 Conclusion and discussion.....	23
Bibliography	25

List of Tables

Table 1	The size distribution, DLC and DLE of POEG-co-PVD, PAza and PAza-R848	14
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List of Figures

Figure 1 The synthesis route of PAza polymer	13
Figure 2 (A, B, C) The size distribution of POEG-co-PVD (A), PAza (B) and PAza-R848 (C). (D) The fluorescence intensity of PAza-NileRed at different concentration of PAza. (E, F) The TEM images of PAza (E) and PAza-R848 (F).....	15
Figure 3 (A) The cleavage of disulfide bond under chemical reduction. (B, C) Cytotoxicity assay of formulations in 4T1(B), and MDA-MB-231 (C). (D) The expression enhancement of CD80 and MHC-II after different treatments in murine monocyte- derived dendritic cells.....	16
Figure 4 Biodistribution and tumor penetration of PAza polymers. (A) The whole body NIR images of mice treated with different formulations. (B) The ex vitro NIR images of tumors and major organs of each mice treated with different formulations. (C) Quantitative	18
Figure 5 <i>In vivo</i> therapeutic effects in 4T1 breast cancer model. (A, B) Actual (A) and relative (B) tumor volume growth curve of mice treated with different formulations. (C) Representative images of excised tumor of mice treated with different fomulations. (D) Changes in bodyweight of mice treated with different formulations. (E) Tumor weights of mice in different treatment groups. (F, G) Histological analysis of H&E stained major organs (F) and tumors (G) sections in each treatment groups.....	20
Figure 6 Flow cytometry analysis (A, B, C, D), safety evaluation (E, F) and fluorescence images of CD45+/DAPI stained frozen tumor sections (G). The quantified percentage	

of tumor infiltrated immune cells including CD45+ cells (A), CD4+ T cells (B), CD8+ T cells.....	22
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1.0 Introduction

For a long time, the monotherapies prevailed in the cancer treatment. However, with single chemotherapy treatment, the cancer cell selection obeys the Darwin's law of evolution, leading to drug resistance[1]. The response rate of immunotherapies in clinic was also limited due to the heterogeneity and immune escape mechanism developed in cancer[2]. In recent decades, combination therapies were developed to improve the therapeutic efficacy of cancer treatments[3-5]. Compared to monotherapies, combination therapies may be more effective and less susceptible to drug resistance due to their impacts on multi targets[1, 6]. There have been increasing interests in developing various combination therapies that can achieve additive or synergistic effects in cancer treatment.

In this study, we attempted to develop a combination regimen of azacitidine and R848, and designed a nanocarrier to achieve the co-delivery of both drugs. Herein, azacitidine is a hypomethylating agent, a cancer chemotherapy drug, while R848 is a toll-like receptor 7/8 agonist, acting as a potent immune modulator.

DNA methylation is the major form of epigenetic modification in mammalian cells. A methyl group is added to the carbon-5 position of a cytosine in CpG island with an enzyme, DNA methyltransferase (DNMT) [7]. The DNA methylation observed in CpG island can silence the affected genes so as to regulate the expression level[8]. This modification helps to maintain the differentiation status and homeostasis in normal cells, but it becomes dysregulated in cancer cells[9]. The aberrant methylation of genes is a hallmark of cancer initiation and progression[10]. In cancer cells, DNA hypermethylation is frequent and widespread. It is reported that many tumor-suppressor genes and genes associated with mismatch repair, immune response were silenced by hypermethylation. For example, C. Stirzaker et al. reported that in some familial cases of unilateral retinoblastoma, the retinoblastoma gene, a tumor-suppressor gene, was featured as hypermethylated, promoting the tumor initiation and progression[11]. Also, the hypermethylation of the mismatch repair gene, hMLH1, has a high frequency in colorectal tumor[12]. The loss of antigen presenting molecules such as MHC class I molecules due to the hypermethylation is also reported in numerous kinds of cancer like lung cancer[13], breast cancer[14, 15] and colorectal

tumor[16]. It impairs the tumor associated antigen presentation and the induction of immune response. On the other hand, reversing the hypermethylation of these genes in cancer cells can impede the cancer progression through reactivating various pathways, and reprogram the cells back to a more normal status[14, 17]. Through reactivating tumor suppressor genes and immune response related genes, hypomethylating agents are able to elicit immunological and non-immunological effects to regulate tumor growth and metastasis[14, 18, 19]. Therefore, targeting the aberrant DNA methylation shall provide another attractive strategy for cancer therapy. Azacitidine is an analogue of cytidine, as well as an inhibitor for DNMT1. It can be incorporated into DNA as cytidine upon replication, and covalently bind to DNMT1. The trapped DNMT1 is then degraded in subsequent cell cycles, resulting in normalization of DNA methylation[20].

Activation of host immune system represents another attractive strategy to inhibit tumor growth and metastasis in recent years[21]. Immunotherapies have been proved to be less toxic, more potent and durable comparing to conventional chemotherapies[22]. R848 is a toll-like receptor (TLR) 7/8 agonist. TLRs play an important role in pathogen-associated molecules recognition[23]. Through a myeloid-differentiation factor-dependent pathway, R848 stimulates TLR 7/8 expressed in infiltrated immune cells, leading to increased expression and release of several proinflammatory cytokines[24]. It is reported[25] that the activation of TLR7 preferentially leads to the release of interferon (IFN) α and C-X-C motif chemokine (CXCL) 10 and 11. The stimulation of TLR8 is able to boost the release of nuclear factor κ B (NF κ B)-regulated proinflammatory cytokines like tumor necrosis factor (TNF) α , interleukin (IL) 1 α , 1 β , 6, 8 and 12, as well as the macrophage inflammatory protein (MIP) 1 and 3. In mice, there is a lack of expression of functional TLR8. But in human, both TLR 7 and 8 facilitate the immune response like activation and maturation of dendritic cells[26], recruitment and activation of cytotoxic T cells[24], and polarization of M2 macrophage[27].

Considering that R848 is highly hydrophobic while azacitidine is water soluble, a nanodrug delivery system is designed for codelivery of the two drugs together. Nanotechnology exhibits many unique advantages in drug delivery. For example, it allows the efficient delivery of drugs of poor solubility and the co-delivery of multiple drugs in a controlled manner. With the small size, the loaded drugs can be effectively delivered to and accumulate in tumor tissues through enhanced permeability and retention effects (EPR)[28]. In addition, the stimuli-responsive nanocarriers enable the drugs to be released in certain tissues through responding to the inherent stimuli[29].

In this study, we designed and developed an azacitidine pro-drug-based micellar carrier PAza. Here, azacitidine is conjugated to the POEG-co-PVD backbone through a disulfide bond, which is sensitive to reductive GSH. PAza can self-assemble in aqueous solutions to form nanoparticles. With this nanocarrier, R848 can be efficiently loaded inside the hydrophobic core to form PAza-R848. The micellar structure becomes disintegrated following the cleavage of the disulfide bond by the relatively high concentrations of GSH in tumor microenvironment[30, 31], resulting in the release of both Aza and the loaded R848. By investigating the biodistribution profiles, it was shown that the nanoparticles can preferentially deliver the loaded drugs to the tumor site. The *in vivo* efficacy study was conducted in a highly aggressive and metastatic breast cancer model 4T1. PAza-R848 exhibited potent inhibition of tumor growth with little effects on the body weights and liver functions. With flow cytometry, it was determined that PAza increased the proportion of T cells, and PAza-R848 nanoparticles could much more effectively recruit CD45+ cells, T cells and dendritic cells to tumor tissue, which may account for the promising anti-tumor effects.

2.0 Materials and Methods

2.1 materials

Vinylbenzyl chloride, 4-Cyano-4-(phenylcarbonothioylthio)pentanoic acid (CTA), potassium carbonate, sodium sulfate, oligo(ethylene glycol) methacrylate (OEG950 monomer, average $M_n=950$), 4,4'-dithiodibutyric acid, 2,2-Azobis(isobutyronitrile) (AIBN), tetrahydrofuran, dimethyl sulfoxide, 5'-azacitidine, resiquimod, were all purchased from Sigma-Aldrich, (MO, U. S. A.). Purified AIBN was obtained by recrystallization in anhydrous ethanol. Diisopropylethylamine (DIPEA) was purchased from Acros Organics. 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and 1-hydroxybenzotriazole (HOBT) were purchased from GL Biochem (Shanghai, China). Fetal bovine serum (FBS) and penicillin-streptomycin solution were purchased from Invitrogen (NY, U. S. A.). All other reagents were analytical grade and purchased from various commercial sources.

2.2 Cells and animals

Human breast cancer cell line (MDA-MB-231) and mouse breast cancer cell line (4T1) were purchased from American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle's Medium containing 10% v/v fetal bovine serum (FBS) and 1% v/v penicillin/streptomycin. Dendritic cell line was obtained from C57BL/6 mice bone marrow, differentiated with rmGM-CSF and rmIL-4 and culture in RPMI 1640 medium containing 10% v/v FBS and 1% v/v penicillin/streptomycin. Trypsin-EDTA solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl

tetrazolium bromide (MTT) and the mentioned culture materials were all purchased from Sigma-Aldrich, (MO, U. S. A.).

The animal-related experiments were performed in full compliance with institutional guidelines and approved by the Animal Use and Care Administrative Advisory Committee at the University of Pittsburgh.

2.3 Synthesis of azacitidine based polymer

2.3.1 Synthesis of VD monomer

As reported previously[32], 4,4'-Dithiodibutyric acid (2.3g, 9.7mmol), potassium carbonate (1g, 7.2 mmol) and Vinylbenzyl chloride (305.2 mg, 2mmol) were dissolved in 10 mL dimethylformamide (DMF) and reacted overnight at 50°C under stirring. After the reactant mixture was cooled down to the room temperature, it was then resuspended in 40 mL methylene chloride. Then the mixture was centrifuged at 4500 rpm for 10 min. The supernatant was collected and the resuspension, centrifuge and collection processes were repeated again. All the collected supernatant was washed with water for three times. Then the organic phase was dried with anhydrous sodium sulfate. Subsequently the VD monomer was isolated by column chromatography purification. The eluent consisted of ethyl acetate and petroleum ether (v/v, 1/4~1/5).

2.3.2 Synthesis of POEG-co-PVD polymer

AIBN (3.6 mg, 0.022 mmol), OEG950 (480 mg, 0.51 mmol), VD monomer (450 mg, 1.27 mmol) and CTA (7.6 mg, 0.027 mmol) were dissolved in 1.5 mL dried tetrahydrofuran in a Schlenk

tube. Then the mixture was deoxygenated with three freeze-pump-thawing cycles and then stirred at 80°C under the N₂ protection overnight. Then, the reaction was ended by cooling down to room temperature. The POEG-co-PVD polymer was washed with ether and centrifuged at 4500 rpm for 10 min for 3 times. Then the purified POEG-co-PVD polymer was obtained. Conversion(OEG950 monomer) = 43%; Conversion(VD monomer) = 49%.

2.3.3 Synthesis and characterization of PAza polymer

POEG-co-PVD polymer (50 mg, 0.073 mmol -COOH), azacitidine (60 mg, 0.25 mmol), HOBT (80 mg, 0.59 mmol), EDC (100 mg, 0.52 mmol) and DIPEA (100 μL) were dissolved in 10 mL DMSO. After stirring at 30°C for 72 h, the mixture was first dialyzed against DMSO overnight and then water for 2 days. After the lyophilization, the PAza polymer was obtained. The chemical structure and the grafting rate of PAza was determined by ¹H NMR.

2.4 Preparation and characterization of PAza-R848 micelles

The PAza and PAza-R848 micelles were both prepared by film hydration method. Briefly, PAza and R848 were mixed in methylene chloride/ethanol with a 50:3 ratio. By removing the organic solvents completely, a thin film was formed. The micelles were obtained by resuspending the film in saline

As reported previously[32], the drug loading capacity (DLC) and drug loading efficiency (DLE) were measured by HPLC and calculated according to the following equations

$$\text{DLC (\%)} = [\text{weight of drug loaded}/(\text{weight of polymer} + \text{drug used})] \times 100$$

$$\text{DLE (\%)} = (\text{weight of loaded drug} / \text{weight of input drug}) \times 100$$

The sizes and morphologies of PAza and PAza-R848 micelles were examined by DLS (dynamic light scattering) and TEM (transmission electron microscopy).

2.5 Critical micelle concentration (CMC) of PAza micelles

The CMC value of the PAza micelles was measured with Nile red as the fluorescence probe. Briefly, PAza micelles (0.0625 mg/ml) were firstly diluted into different concentrations and then 250 μL of each were added in different vials containing 1.5×10^{-7} mmol dried Nile red. After overnight incubation, fluorescence intensity of solutions was measured by fluorescence spectrometer. Finally, the CMC value was determined by the cross-point of the extrapolated line of the intensity in low and high concentration region.

2.6 *In vitro* bioactivity test

2.6.1 MTT test

The *in vitro* cytotoxicity of free Aza, free R848, Free combination of Aza and R848, POEG-co-PVD, PAza and PAza-R848 against 4T1 and MDA-MB-231 cell lines were measured and compared by MTT assay. 4T1 and MDA-MB-231 were seeded in 96-well plate at 800 and 1000 cells/well. After 12h, cell culture medium was removed and different concentration of free

Aza, free R848, Free combination of Aza and R848, POEG-co-PVD, PAza and PAza-R848 were added to the cells. After 96h incubation, 50 μ L MTT (2 mg/mL) was added to each well and incubated for 3 h. Then, the medium in each well was removed and the MTT crystal was dissolved by 100 μ L DMSO.

Absorbance was measured by a micro-plate reader at wavelength at 550 nm. Untreated cells were used as a positive control. Cell viability was calculate as $((OD_{treated} - OD_{blank}) / (OD_{control} - OD_{blank})) * 100\%$

2.6.2 Evaluation of maturation of dendritic cells (DCs)

The maturation of murine monocyte-derived dendritic cells (MDDCs) induced by the loaded R848 was evaluated by the measuring the expression level of CD80 and MHC-II via flow cytometry. Briefly, the murine monocytes were isolated from C57BL/6 mice bone marrow and treated with 10 ng/ml IL-4 and 20 ng/ml GM-CSF for ten days to give MDDCs. The MDDCs were then seeded in 6-well plate and treated with free Aza, free combination of Aza and R848, PAza or PAza-R848 at R848 and azacitidine concentration of 500 ng/ml. Saline and LPS acted as negative and positive control. After a 72h incubation, cells were collected and fixed with 4% paraformaldehyde for 30 min. And then the cells were stained with antibodies against CD45, GR-1, CD80 and MHC-II for 30 min. Next the cells were washed with PBS for twice and finally analyzed by a flow cytometer.

2.7 Tissue biodistribution and penetration of PAza nanocarriers

First prepare POEG-co-PVD-DiR and PAza-DiR at polymer concentration of 5 mg/ml and DiR concentration of 0.1 mg/ml by film hydration method. And free DiR was dissolved in 5% ethanol at concentration of 0.1 mg/mL. Then 200 μ L of free DiR, POEG-co-PVD-DiR and PAza-DiR were intravenously injected into Balb/C mice bearing ~ 300 mm³ tumor. After 24 h, mice were sacrificed and hair was removed. The near-infrared imaging of whole body was carried out with an IVIS imaging system. Subsequently, the main organs and tumors were removed and imaged ex vivo. To investigate the tumor penetration and accumulation more directly, the tumors of POEG-co-PVD-DiR group and PAza-DiR group were prepared as frozen sections and stained with DAPI. The immunofluorescence intensity were quantified with Image J.

2.8 *In vivo* therapeutic efficacy in 4T1 model

4T1 cell line, a murine breast cancer cell line isolated from Balb/C mice strain, can well mimic the tumor growth and metastasis of stage IV human cancer cell line. Here, an orthotopic 4T1 breast cancer was established by inoculate 5×10^5 4T1 cells into the mammary fat pad of female Balb/C mice. When the tumor reached 40 mm³ in size, the mice were randomly divided into 4 groups (n=4) and received treatments of saline, free combination of Aza and R848, PAza and PAza-R848 respectively. All the treatments were administered by intravenous injection every three days and a total for 6 times. The dose of Aza and R848 were both 3 mg/kg. Every three days, the tumor volumes were measured and body weights were recorded. The tumor volume was calculated as $V = (\text{length of tumor}) \times (\text{width of tumor})^2 / 2$. At 19 days after the first treatment, all

mice were sacrificed and the tumors, main organs were excised for the following histopathological, immunohistochemical and quantification of tumor-infiltrating lymphocytes examination.

For histopathological examination, the tumor and main organs sections were fixed with 10% paraformaldehyde and then embedded in paraffin. The paraffin blocks were sectioned into 7- μ m-thick slices and then stained with hematoxylin and eosin (H&E) and imaged with a fluorescence microscope.

For immunohistochemical examination, the tumor sections were frozen sectioned at 7- μ m-thick slices and stained with a primary antibody against CD45, and then a secondary antibody of FITC goat anti-rat IgG. The stained sections were investigated under a fluorescence microscopy.

2.9 Quantification of tumor-infiltrating lymphocytes by flow cytometry

The tumor excised in the *in vivo* efficacy study was roughly cut, incubated with 0.25 mg/ml TL liberase for 30 min, grounded and filtered to give single cell suspension. Then the cells were activated and stained with different antibodies for flow cytometry analysis with FlowJo software.

2.10 Safety evaluation

To determine the safety profiles of different treatments, the bodyweight was measured throughout the *in vivo* efficacy study and was plotted as a line chart. At the completion of the *in vivo* efficacy study, the serum of different treatment groups was collected for measuring alanine

transaminase (ALT), aspartate transaminase (AST). ALT and AST are biological markers of liver functions.

3.0 Results

3.1 Synthesis and characterization of PAza polymers

The PAza polymer was synthesized as shown in Figure 1 A. First the vinylbenzyl chloride and the 4,4'-dithiodibutyric acid reacted to yield the vinyl benzyl monomer (VD monomer) with a disulfide linkage. Then the VD monomer and OEG950 were co-polymerized to yield POEG-co-PVD polymer via a reversible addition-fragmentation chain-transfer polymerization (RAFT). And the PAza polymer was synthesized by conjugating azacitidine to the POEG-co-PVD polymer via EDC-HOBt coupling reaction.

¹H NMR was conducted to determine the structures of POEG-co-PVD and PAza, and the grafting rate of PAza. As shown in Fig 1B, the characteristic peak (-O-CH₃) of OEG950 (both free OEG950 and OEG950 residues) appeared at 3.38 ppm in the reactant mixture. The integral was set as 3.00. The characteristic peaks (-C=CH₂) of free OEG950 were found at 6.13 and 5.58 ppm. Normalized to the methyl group, the integral of each peak was 0.57. The characteristic peak of total VD monomer (-CH₂-O-) appeared at 5.10 ppm and the integral was set as 2.00. The peaks of free VD monomer located at 5.78 and 5.75 ppm and integrated as 0.51 on average. As calculated, 43% of the input OEG950 and 49% of the input VD monomer was yield to POEG-co-PVD.

After the removal of free reactants, the ¹H NMR of POEG-co-PVD was shown in Fig 1C. The characteristic peak of VD residues was found at 4.98 ppm. According to the yield rate, amount of input CTA, the average degree of polymerization of OEG950 and VD monomer was 8 and 23.

Fig 1D. characterized the structure of PAza. The featured peak of azacitidine (peak e) located at 5.76 ppm. As calculated, the loading content of azacitidine is 6.06%.

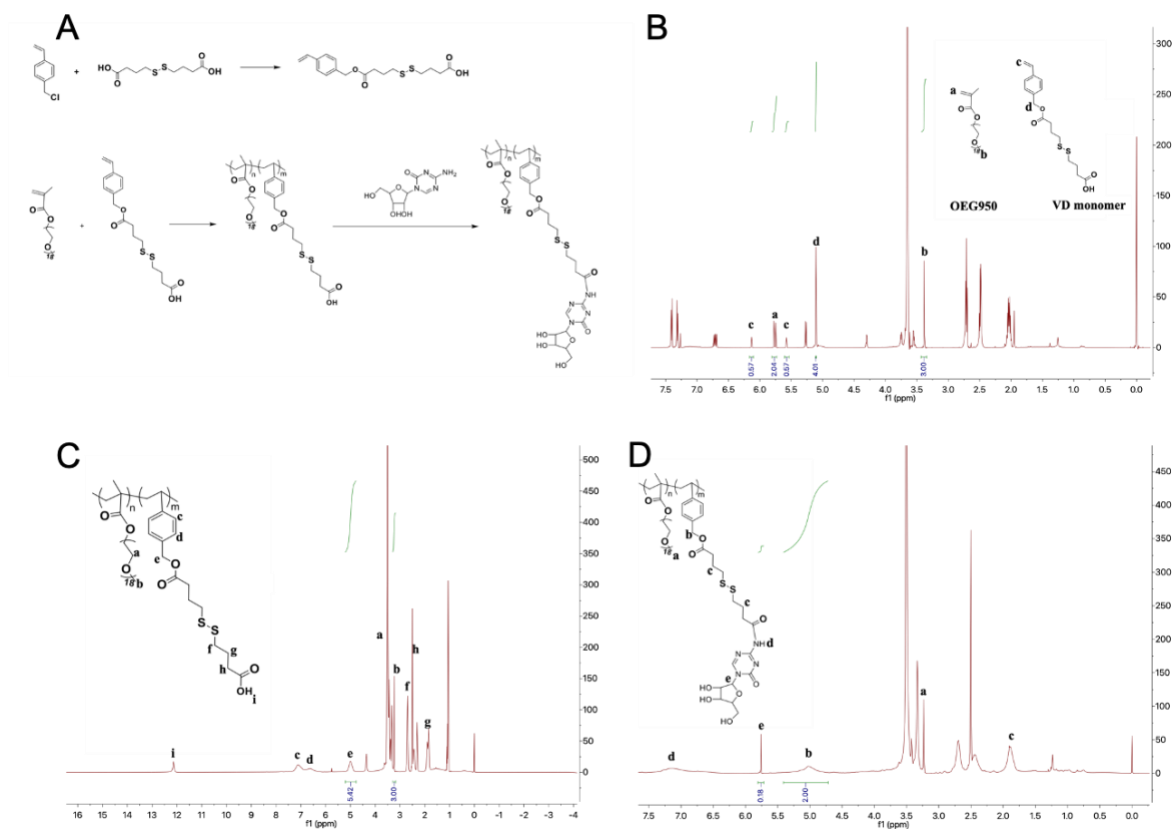


Figure 1 The synthesis route of PAza polymer

3.2 Physicochemical characterization of micelles

POEG-co-PVD, PAza and PAza-R848 micelles were prepared through film hydration method and dissolved in saline. The size of POEG-co-PVD is 125.3 nm (Fig 2A). After the conjugation of azacitidine, the size decreased to 18.83 nm (Fig 2B), indicating that the azacitidine structure may contribute to the formation of small size nanoparticles. The drug loaded nanoparticles PAza-R848 maintained the small size as 16.79 nm (Fig 2C). The TEM images (Fig 2E, F) showed the spherical structures of PAza and PAza-R848, which showed similar sizes as those measured by DLS.

The CMC value of PAza nanoparticles was measured through fluorescence measurement using Nile red as the fluorescence probe. As shown in Fig 2D, PAza polymer has a CMC value of 0.0097 mg/ml, indicating a good colloidal stability after the dilution in the blood circulation following systemic administration.

The DLC & DLE values of PAza-R848 nanoparticles were measured through high-performance liquid phase chromatography (HPLC). As shown in Table 1, the DLC of PAza-R848 nanoparticles is 5.4% and the DLE is 95.34%, demonstrating the effective loading capacity and efficiency of PAza nanocarriers.

Table 1 The size distribution, DLC and DLE of POEG-co-PVD, PAza and PAza-R848

Micelles	Size (nm)	PDI	DLC(%)	DLE(%)
POEG-co-PVD	125.3±0.625	0.238±0.013		
PAza	18.83±0.076	0.219±0.003		
PAza-R848	16.79±0.287	0.114±0.011	5.4	95.34

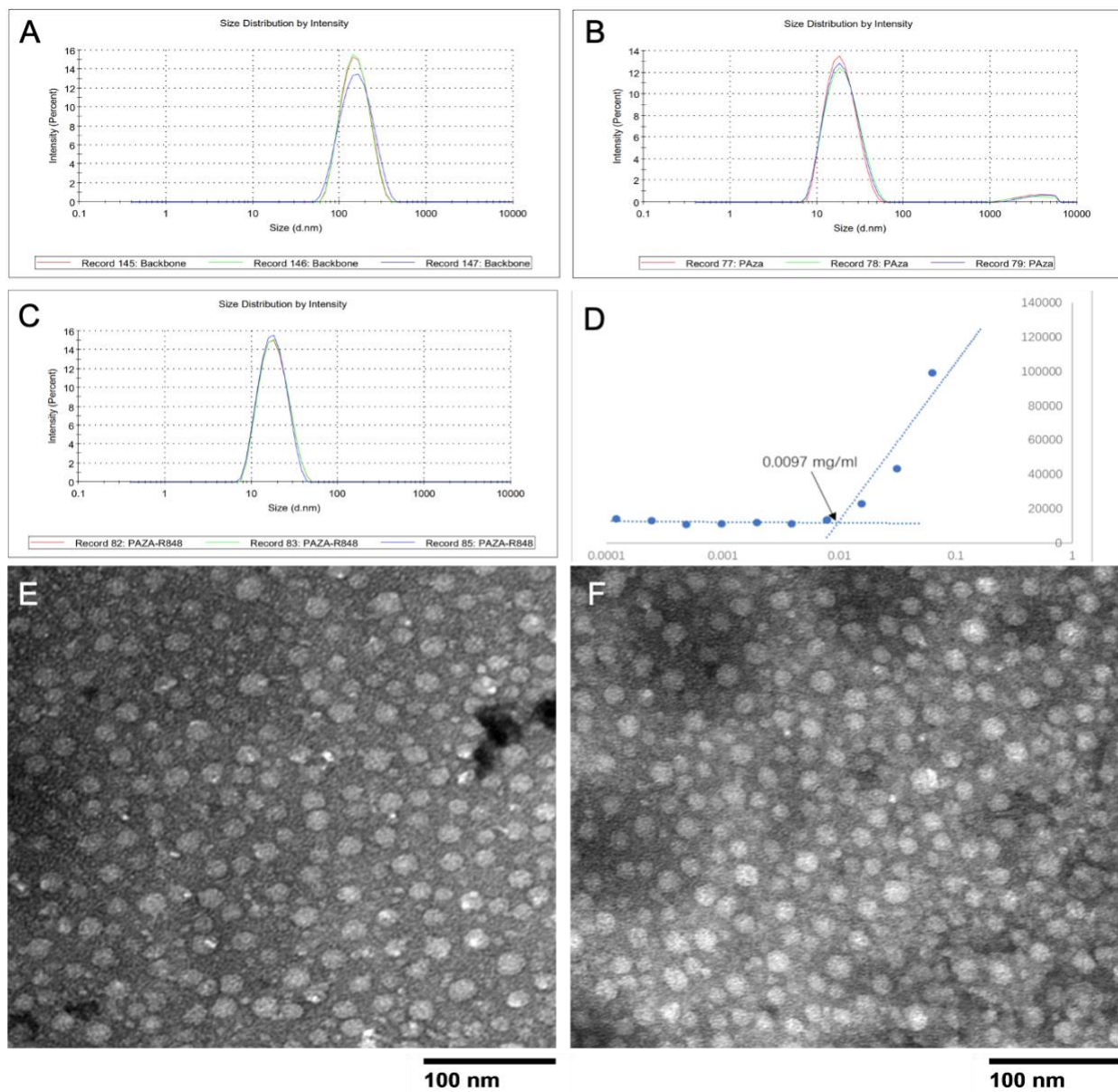


Figure 2 (A, B, C) The size distribution of POEG-co-PVD (A), PAza (B) and PAza-R848 (C). (D) The fluorescence intensity of PAza-NileRed at different concentration of PAza. (E, F) The TEM images of PAza (E) and PAza-R848 (F)

3.3 *In vitro* cytotoxicity assay

As previously reported[31], the disulfide bond cleaved through chemical reduction would immolate and release the parent drug as shown in Fig 3A. To further confirm that the parent drug can be well released and maintain its bioactivity, here we conducted the *in vitro* cytotoxicity test with MTT assay. The *in vitro* cytotoxicity of free azacitidine, free R848, combination of free azacitidine and R848, POEG-co-PVD, PAza and PAza-R848 was examined in 4T1 and MDA-MB-231 cell lines. As shown in Fig 3B and C, in both cell lines, free R848 and POEG-co-PVD showed minimal cytotoxicity and the other treatments exhibited a dose-dependent cell killing effect. In 4T1 cell line, Aza, combination of free Aza and R848, PAza and PAza-R848 exhibited similar cytotoxicity. In MDA-MB-231 cell line, azacitidine and combination of free azacitidine and R848 exhibited similar cytotoxicity, and were higher than PAza and PAza-R848.

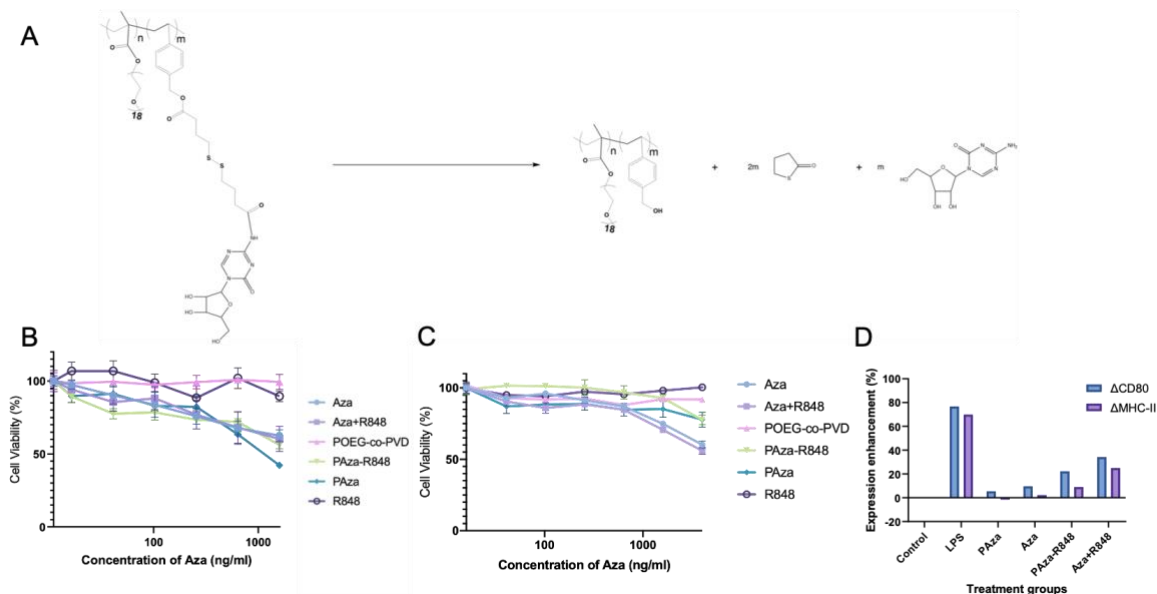


Figure 3 (A) The cleavage of disulfide bond under chemical reduction. (B, C) Cytotoxicity assay of formulations in 4T1(B), and MDA-MB-231 (C). (D) The expression enhancement of CD80 and MHC-II after different treatments in murine monocyte-derived dendritic cells

3.4 Evaluation of maturation of murine monocyte-derived dendritic cells

DCs are the most effective antigen presenting cells (APCs) which contribute significantly to adaptive immune responses. And R848 is reported to be able to elicit the maturation of DCs through activating the pattern recognition receptor TLR 7/8. The mature DCs are able to migrate to lymph nodes and induce the downstream immune response such as the activation of T cells and recruitment to tumor site. Here the maturation of MDDCs induced by the drug-loaded nanoparticles was measured by flow cytometry. As shown in Fig 3D, the PAza-R848 and combination of free azacitidine and R848 can both effectively enhance the expression level of the surface molecular markers CD80 and MHC II. These two markers are highly expressed on the mature DCs and are known to be involved in the activation of helper T cells and antigen presentation.

3.5 Biodistribution profiles of POEG-co-PVD and PAza nanoparticles

Briefly, we used DiR as the fluorescence probe and prepared free DiR solution, POEG-co-PVD-DiR and PAza-DiR micelles to determine their tissue distribution and tumor accumulation profiles in 4T1 tumor-bearing mice. Twenty-four h following the injection through the tail vein, whole-body NIR imaging was conducted. The mice were then sacrificed and major organs and tumors were excised for ex vivo imaging. Fig 4A shows the whole body NIR imaging of mice treated with different formulations. There was minimal accumulation at tumor site following injection of free DiR. I.V. injection of POEG-co-DiR led to concentrated fluorescence signals at tumors. However, injection of PAza-DiR led to highest levels of fluorescence signals at the tumor sites. Fig 4B and C show the representative fluorescence images and quantitative analysis of fluorescence intensities of removed organs and tumors. In free DiR group, fluorescence was mostly observed in spleen, liver and lung with minimal signals in the tumor tissues. POEG-co-PVD was

largely concentrated in liver and spleen and the levels of fluorescence were significantly higher than those in tumor tissues. In contrast, PAza-DiR was preferentially targeted to tumor sites with concomitant decreases in accumulation in liver and spleen.

The excised tumors in POEG-co-PVD-DiR and PAza-DiR groups were further frozen sectioned for the tumor penetration study. As shown in Fig 4D & E, The slices of the tumor tissues were stained with DAPI and the fluorescence intensities of both groups were normalized to DAPI and quantified with ImageJ software. In the tumor core, more DiR signals were observed in PAza-DiR group, indicating that the PAza nanocarrier can better penetrate into tumor tissue.

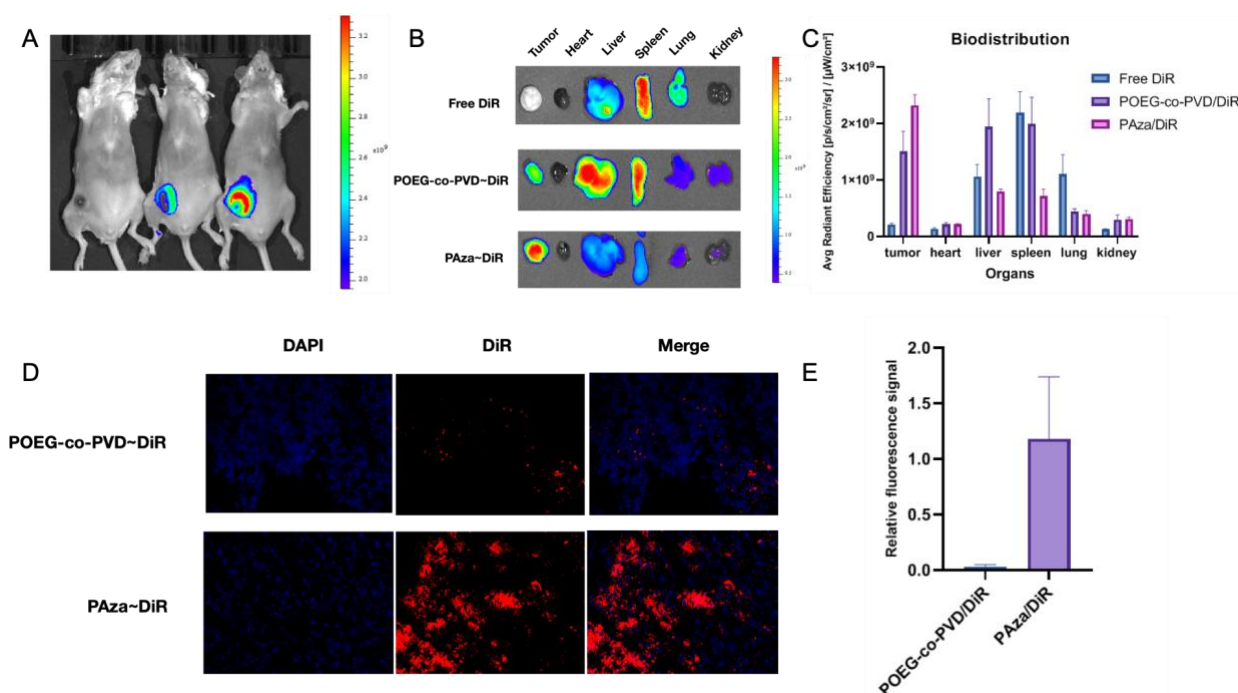


Figure 4 Biodistribution and tumor penetration of PAza polymers. (A) The whole body NIR images of mice treated with different formulations. (B) The ex vivo NIR images of tumors and major organs of each mice treated with different formulations. (C) Quantitative

3.6 *In vivo* antitumor efficacy study of PAza-R848 nanoparticles

As for the *in vivo* antitumor efficacy study, we examined the efficacy of PAza-R848 in an 4T1 xenograft model. 4T1 is a murine triple negative breast cancer model, in which the growth and the spontaneous metastatic spread can well mimic stage IV human mammary cancer.

4T1 cells were subcutaneously injected into the mammary fat pad of Balb/c mice. After 5 days, the tumor-bearing mice were intravenously injected with saline, combination of free azacitidine and R848, PAza and PAza-R848 once every three days for xxx times.

Fig 5 A ~ E show the tumor volume growth curve, body weight change, tumor weights and the representative images of excised tumor. PAza pro-drug micelles displayed comparable anti-tumor activity as the combination of free azacitidine and R848. PAza-R848 was able to effectively delay the tumor growth and had much better anti-tumor activity comparing to any other treatment group.

We further stained the tumor and other tissue sections with hematoxylin and eosin (H&E). As for those main organs (Fig 5F), we observed metastasis in liver and lung in control group. The metastasis to liver was also observed in free combination group. The morphologies of the main organs maintained normal in all treatment groups. As for the tumor sections (Fig 5G), different extents of necrosis were observed in three treatment groups. The altered morphology of cancer cells with shrunk nuclei and cell damage was also observed. Among the 4 groups, PAza-R848 group exhibited the highest extent of intra-tumor tissue damage.

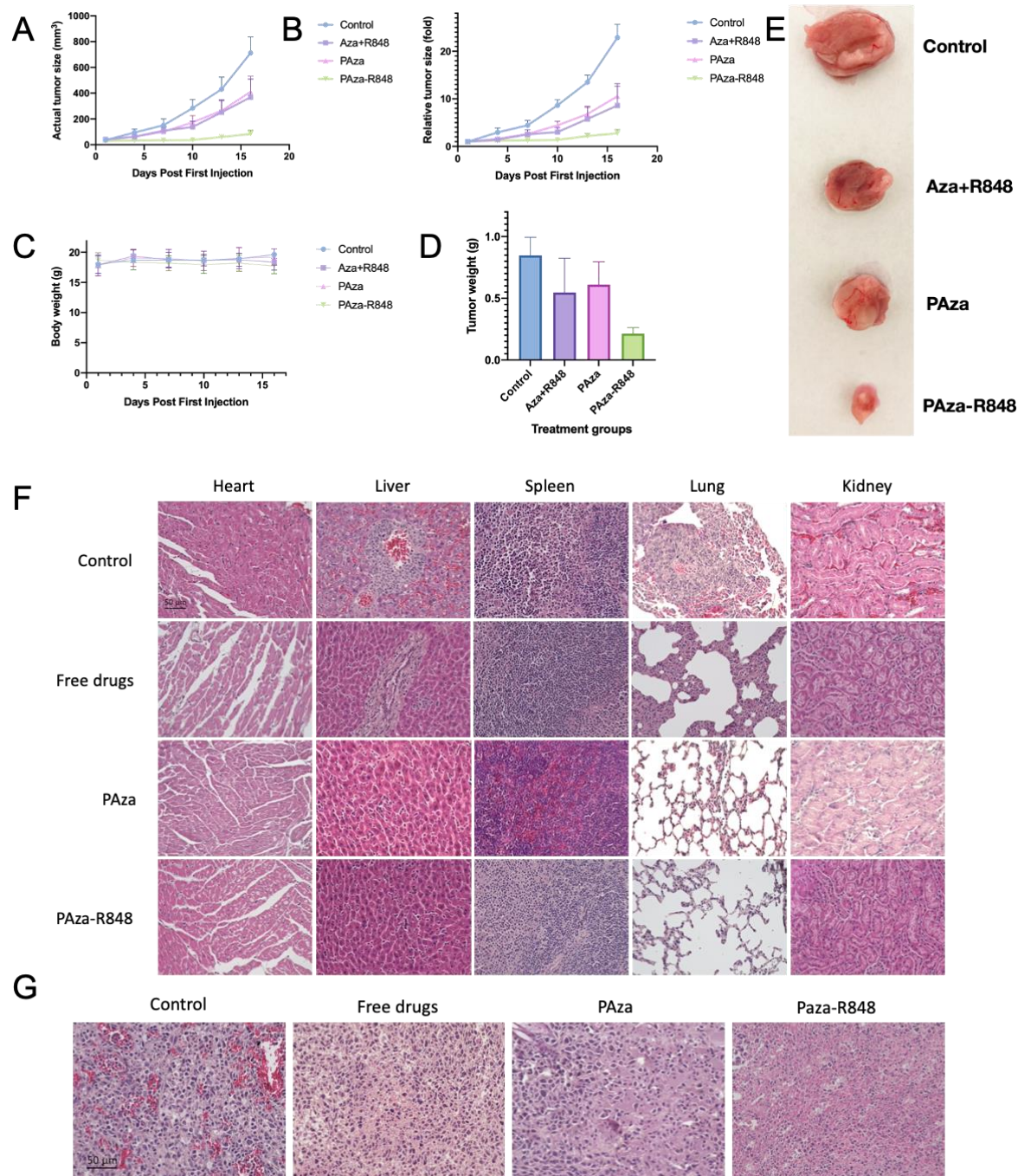


Figure 5 *In vivo* therapeutic effects in 4T1 breast cancer model. (A, B) Actual (A) and relative (B) tumor volume growth curve of mice treated with different formulations. (C) Representative images of excised tumor of mice treated with different fomulations. (D) Changes in bodyweight of mice treated with different formulations. (E) Tumor weights of mice in different treatment groups. (F, G) Histological analysis of H&E stained major organs (F) and tumors (G) sections in each treatment groups.

3.7 Quantification of tumor-infiltrating lymphocytes

The profile of tumor-infiltrating lymphocytes of different treatment groups were investigated by flow cytometry analysis. As shown in Fig 6A, the CD45⁺ cell population was significantly enhanced in PAza-R848 treatment group while those of the other three groups were similar. Within the CD45⁺ cell population, the CD4⁺, CD8⁺ and DCs populations were identified and quantified (Fig 6 B~D). Comparing to the control group, there were significant increases in the proportions of CD4⁺ and CD8⁺ T cells in the groups treated with PAza-R848 and the free combination. PAza treatment also led to slight increases in the numbers of CD4⁺ and CD8⁺ T cells. Treatment with free combination or PAza-R848 also led to significant increases in the proportions of DCs while there were no significant changes in PAza treatment group.

The excised tumors were further frozen sectioned for staining of CD45⁺ as shown in Fig 6E. Consistent with the data obtained from flow cytometry analysis, the immunofluorescence images revealed the significant increases of CD45⁺ cells in PAza-R848 treatment group.

The results indicate that the PAza itself may contribute to the activation and recruitment of CD4⁺ and CD8⁺ T cells. The R848 loaded nanoparticles was able to effectively recruit CD45⁺ cells to tumor site. The combination of azacitidine and R848 effectively altered the proportions of different lymphocyte populations. The increase of CD45⁺ T cells following PAza-R848 treatment may be due to the enhanced codelivery of both Aza and R848 to the tumor tissues. The powerful immune modulation of PAza-R848 is likely to play a critical role in the improved antitumor efficacy.

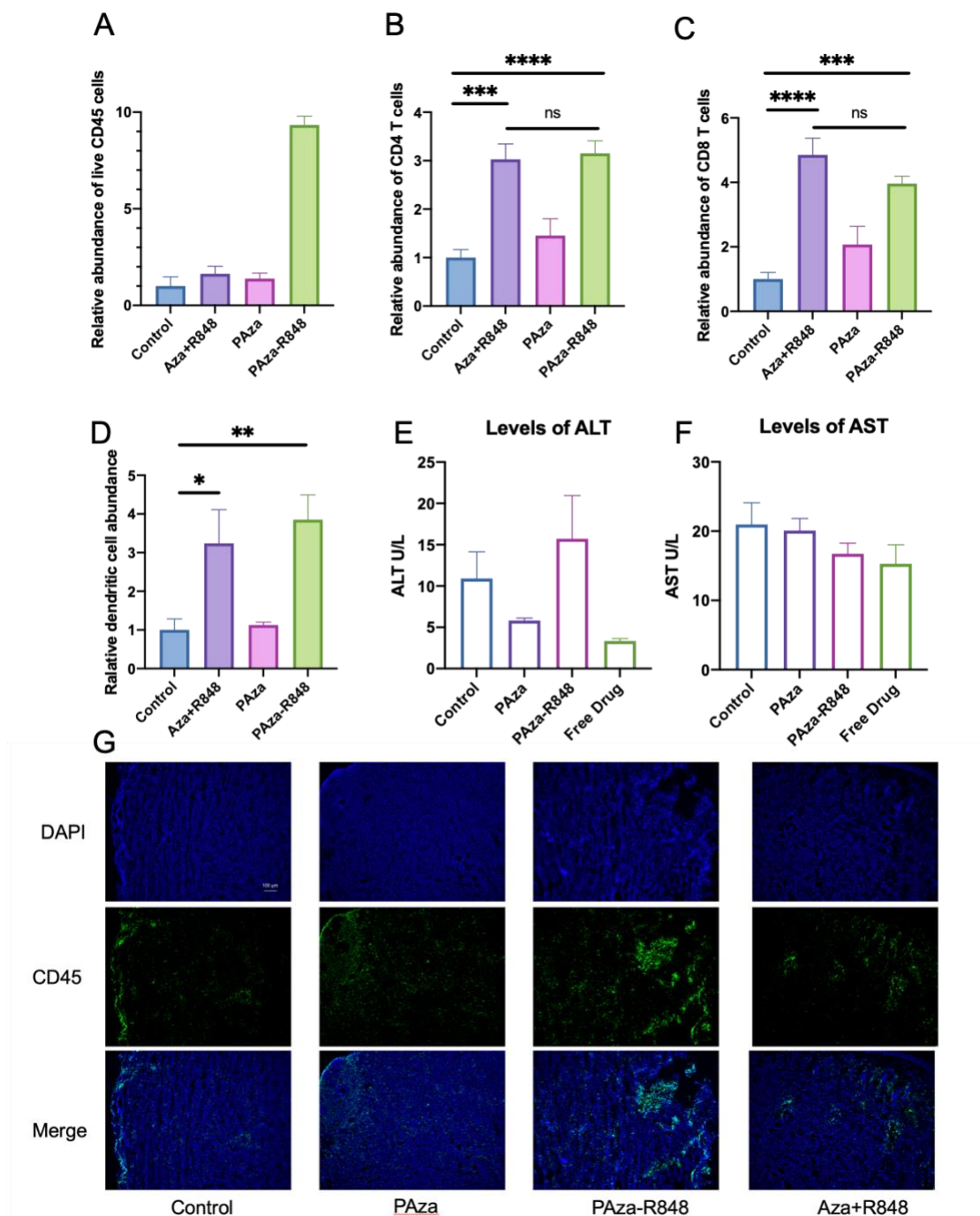


Figure 6 Flow cytometry analysis (A, B, C, D), safety evaluation (E, F) and fluorescence images of CD45+/DAPI stained frozen tumor sections (G). The quantified percentage of tumor infiltrated immune cells including CD45+ cells (A), CD4+ T cells (B), CD8+ T cells

4.0 Conclusion and discussion

In this study, we designed and synthesized an azacitidine pro-drug-based nanocarrier and used it to co-deliver Aza and R848. The structure of the PAza nanocarrier was confirmed with ^1H NMR. The polymerization degree of OEG and VD monomer were 8 and 23, respectively, and the azacitidine loading content was 6.06%. PAza-R848 has a small size as 16.79 nm and can well maintain its small size after loading R848 inside the hydrophobic core. In the meantime, the azacitidine conjugated to the backbone and the R848 loaded inside the micelles can both maintain their bioactivity. The small size help the nanoparticles to penetrate and accumulate into tumor core and induce a durable and effective antitumor effects. The improved antitumor effect may be due to the changes in tumor immune microenvironment. PAza nanocarriers can increase the percentages of CD4⁺ and CD8⁺ T cells in tumor infiltrated lymphocyte population. The PAza-R848 formulation could significantly enhance the total number of tumor infiltrated CD45⁺ cells, and further increase the percentages of CD4⁺ and CD8⁺ T cells, and dendritic cells.

We are aware that more studies are needed to further define the mechanism of the improved antitumor activity of PAza-R848. For example, Azacitidine is known to be quickly degraded by hydrolysis and enzymes. Thus the conjugation to POEG-co-PVD polymer allows selective and sustained release at tumor tissues, providing a durable therapeutic effect. Our current treatment regimen involved injection once every 3 days. A stability test and a release profile analysis need to be done in the future to evaluate how the prodrug design protects azacitidine from degradation and controls the release under highly reductive environment.

In the therapeutic efficacy study, we observed that PAza nanocarrier can increase the proportion of CD4⁺ and CD8⁺ T cells. To further study the underlying mechanisms, we will

evaluate the changes in expression levels of MHC-I *in vitro* via qPCR and Western blot. These studies may further explain how azacitidine facilitate the antigen presentation process.

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