

Supplementary information

Dual-responsive, Methotrexate-loaded, Ascorbic acid-derived Micelles Exert Anti-tumor and Anti-metastatic Effects by Inhibiting NF- κ B Signaling in an Orthotopic Mouse Model of Human Choriocarcinoma

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Synthesis of PEG-ss-*cis*-aconityl 6-Pal-AA-2P palmitate ester

1. A mixture of L-ascorbic acid-2-*O*-phosphate (AA-2P) (10 mmol) and palmitic acid (12 mmol) in concentrated sulfuric acid (50 ml) was stirred for 30 h at room temperature, then diluted with ice water (250 ml). The precipitate was collected by filtration and washed with a small amount of cold water. Solid was extracted three times with dry diethyl ether. The extracts were combined and dried over anhydrous Na₂SO₄ *in vacuo*, and the residue was washed with n-hexane and dried under reduced pressure. In the end, 2.5 g of white powder L-ascorbic acid-2-*O*-phosphate palmitate ester (6-Pal-AA-2P, Figure S1) was obtained (57% yield).

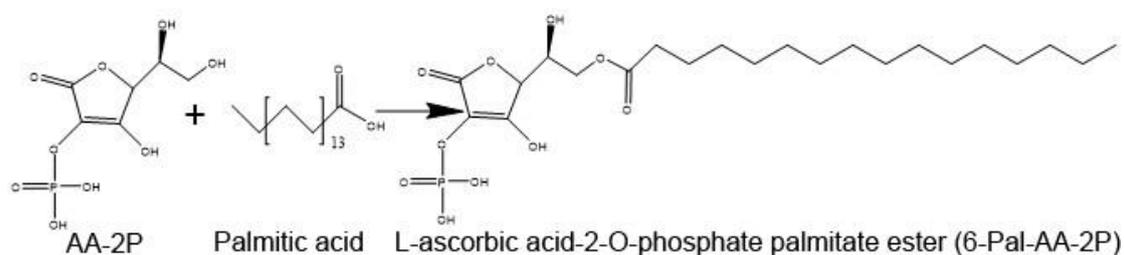


Figure S1. Chemical reaction of 6-Pal-AA-2P palmitate ester.

2. 6-Pal-AA-2P-NH₂ (1.76 g, 10 mmol) was added to a mixture of acetone (4.65 g) and POCl₃ (0.35 g) in the presence of molecular sieve (5 g). The reaction was

carried out for 4 h in an air bath shaker at 20 °C with mixing at 200 rpm. The resulting protected 6-Pal-AA-2P-NH₂ solid (Figure S2) was collected by filtration and used in further reactions.

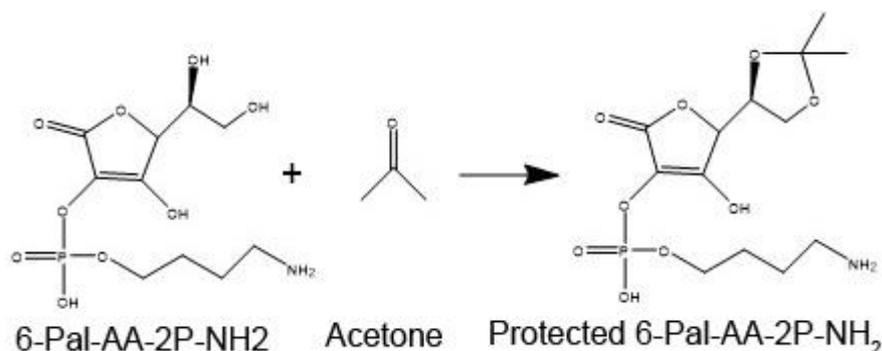


Figure S2. Chemical reaction of protected 6-Pal-AA-2P-NH₂.

- The protected 6-Pal-AA-2P-NH₂ (10 g) was dissolved in 4 L of deionized water. *Cis*-aconitic anhydride (13.46 g) in 100 mL of 1,4-dioxane was slowly added, and the mixture was stirred overnight at 4 °C. The solution was mixed with 500 mL of chloroform and 5 mL of 5 % aqueous sodium bicarbonate. The chloroform phase was decanted, and the residual solution was extracted with ethyl acetate. The extract was concentrated on a rotary evaporator and dried at room temperature under vacuum to get protected *cis*-aconitic 6-Pal-AA-2P-NH₂ (Figure S3).

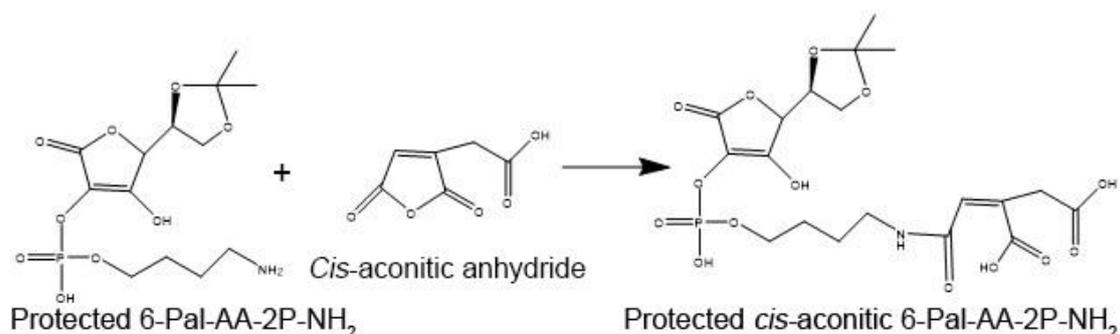


Figure S3. Chemical reaction of protected *cis*-aconitic 6-Pal-AA-2P-NH₂.

- HOOC-PEG2000 (2.0 g, 1 mmol) and mono-Boc-cystamine (0.3 g, 1.20 mmol)

were dissolved in anhydrous dichloromethane (30 mL), and DCC (0.25 g, 1.21 mmol) was added to the solution. The reaction was allowed to proceed at room temperature for 24 h and then the insoluble dicyclohexylurea was removed by filtration. The product was precipitated with diethyl ether, filtered, and vacuum-dried at room temperature to obtain Boc-ss-PEG (Figure S4) with a yield of 2.1 g.

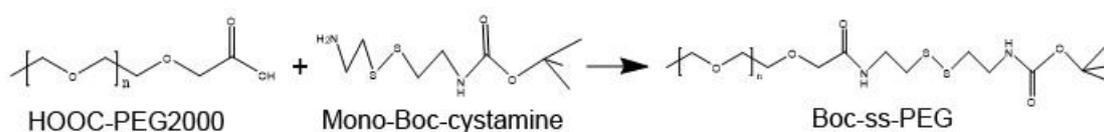


Figure S4. Chemical reaction of Boc-ss-PEG.

5. Cystamine-modified PEG (NH_2 -ss-PEG) was prepared by dissolving Boc-ss-PEG (2 g, 1 mmol) in anhydrous dichloromethane (20 mL) and TFA (10 mL). The reaction was allowed to proceed at room temperature for 3 h, the reaction solvent was evaporated, and the resulting product was precipitated in an excess amount of cold diethyl ether. The precipitate was filtered, washed several times with diethyl ether, and vacuum-dried to obtain PEG-ss- NH_2 (Figure S5) with a yield of 1.77 g.

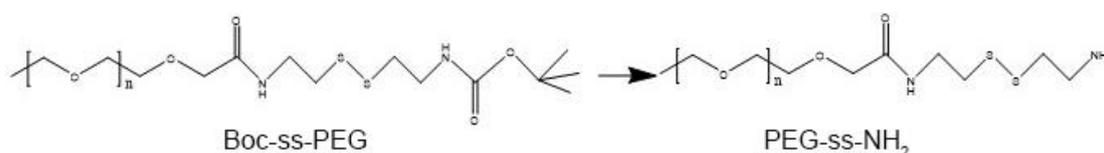


Figure S5. Chemical reaction of PEG-ss- NH_2 .

6. *N-cis*-aconityl-protected 6-Pal-AA-2P was conjugated to NH_2 -ss-PEG in an EDC/NHS coupling reaction. NH_2 -ss-PEG (100 mg) was dissolved in distilled water (10 mL), followed by dilution with 10 mL of methanol. A predetermined

amount of *N-cis*-aconityl-protected 6-Pal-AA-2P was dissolved in 1 mL of dimethylformamide, then equal amounts of EDC and NHS (two equivalents of carboxyl group) were added to the solution. After stirring for 15 min, the polymer solution obtained above was added. The resulting solution was stirred for 1 day at room temperature. The solution was dialyzed against excess distilled water. The product was freeze-dried to obtain PEG-ss-*cis*-aconityl-protected 6-Pal-AA-2P (Figure S6).

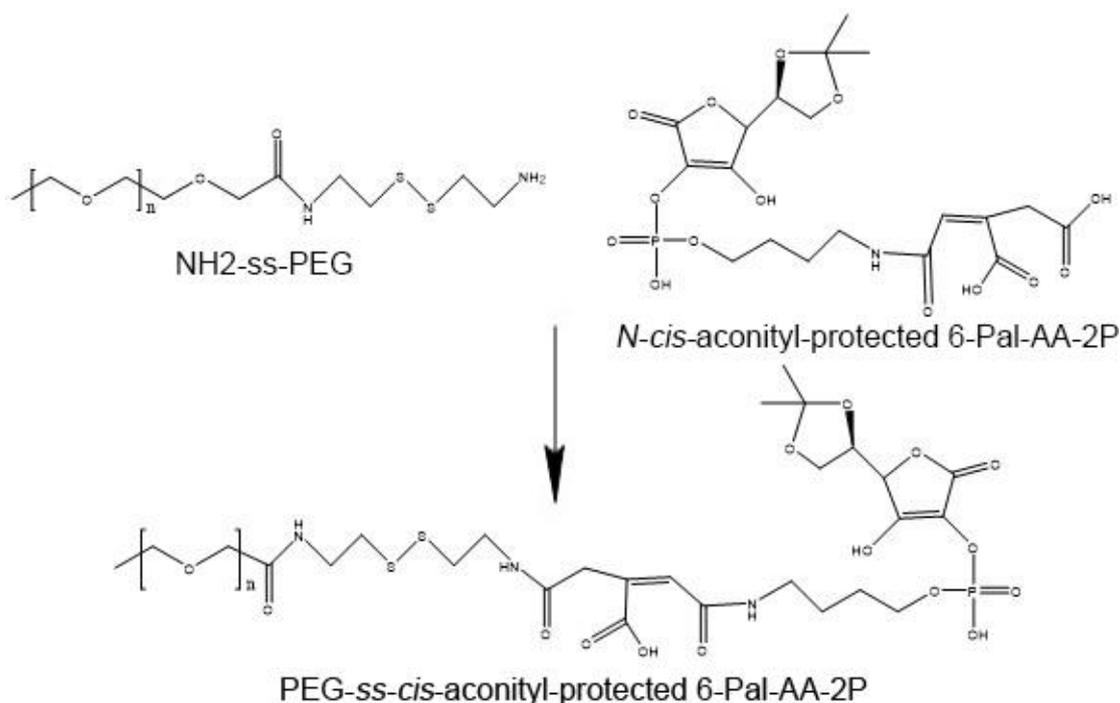


Figure S6. Chemical reaction of PEG-ss-*cis*-aconityl-protected 6-Pal-AA-2P.

7. PEG-ss-*cis*-aconityl-protected 6-Pal-AA-2P was deprotected using SbCl_3 (0.2 equiv.) in methanol with water in 1.0 equivalents at room temperature for 15 min, yielding PEG-ss-*cis*-aconityl 6-Pal-AA-2P in 90% yield. Palmitate acid was dissolved in 1 mL of dimethylformamide and equal amounts of EDC and NHS (two equivalents of carboxyl group) were added to the solution. After stirring for 15 min, the polymer solution obtained above was added. The resulting

solution was stirred for 1 day at room temperature. The solution was dialyzed against excess distilled water. The product was freeze-dried to obtain PEG-ss-*cis*-aconityl 6-Pal-AA-2P palmitate ester.

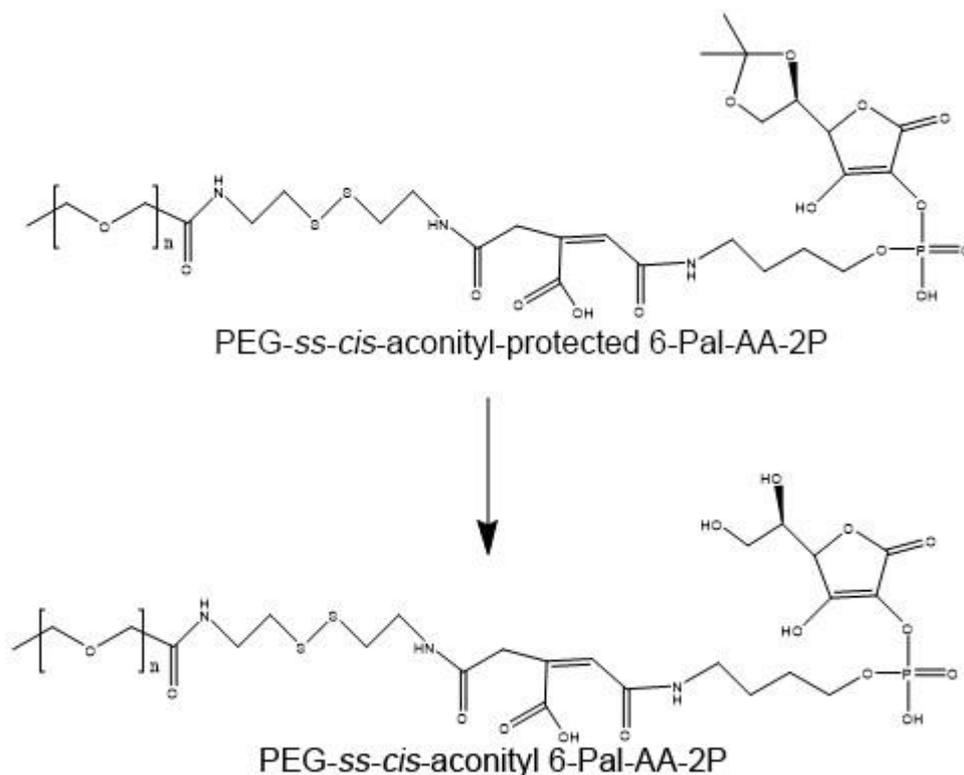


Figure S7. Chemical reaction of PEG-ss-*cis*-aconityl 6-Pal-AA-2P.

Quantification of MTX using HPLC

The HPLC system consisted of an LC20A VP solvent pump, an SPD-20A UV spectrophotometric detector (Shimadzu, Kyoto, Japan), and a 250×4.6 mm (i.d.) XTerra MS C18 column (5 μ m particle size; Waters, MA, USA). The mobile phase was a mixture of 40 mM potassium phosphate dibasic (pH 4.5) and acetonitrile (88:12, v/v), and the flow rate was 1.0 mL/min. Absorbance of the eluate was measured at 313 nm. Under these conditions, the retention time of MTX was 5.47 min and the standard curve for absorbance as a function of MTX concentration was $y = 25.21x + 2.18$ ($r = 0.999$).

In vitro ROS induced by APP

Asada et al. have demonstrated that APP has a marked carcinostatic advantage over AA. This benefit may be due to the addition of the of 2-*O*-phosphatidyl moiety, which adjusts the molecular LHB (lipophilicity-hydrophilicity balance) to be more hydrophilic.^[1] More importantly, in our preliminary experiments (**Figure.S8**), APP induced higher levels of oxygen radicals than ascorbic acid, which may due to the phosphate groups in APP carrying greater negative charge than hydroxyl groups.

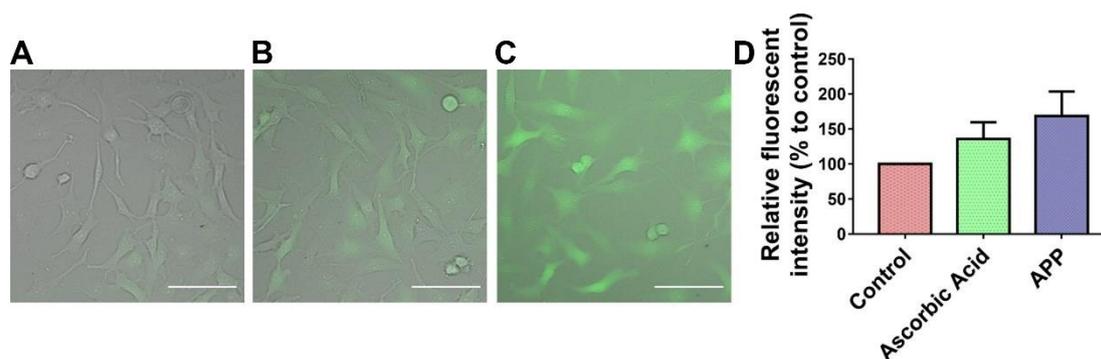


Figure S8. Confocal microscopy of intracellular ROS (green) in JEG3 cells treated for 12 h with (A) PBS (control), (B) ascorbic acid and (C) APP. (D) Quantified intracellular ROS levels.

Preliminary evaluation of hydrolytic stabilities of PEG-ss-aAPP/MTX and APP/MTX micelles in different concentrations of dithiothreitol

To explore the possible reason for the different MTX release behaviors between the PEG-ss-aAPP/MTX and APP/MTX micelles, we investigated their hydrolytic stabilities by DLS measurements in PBS containing different concentrations of dithiothreitol.

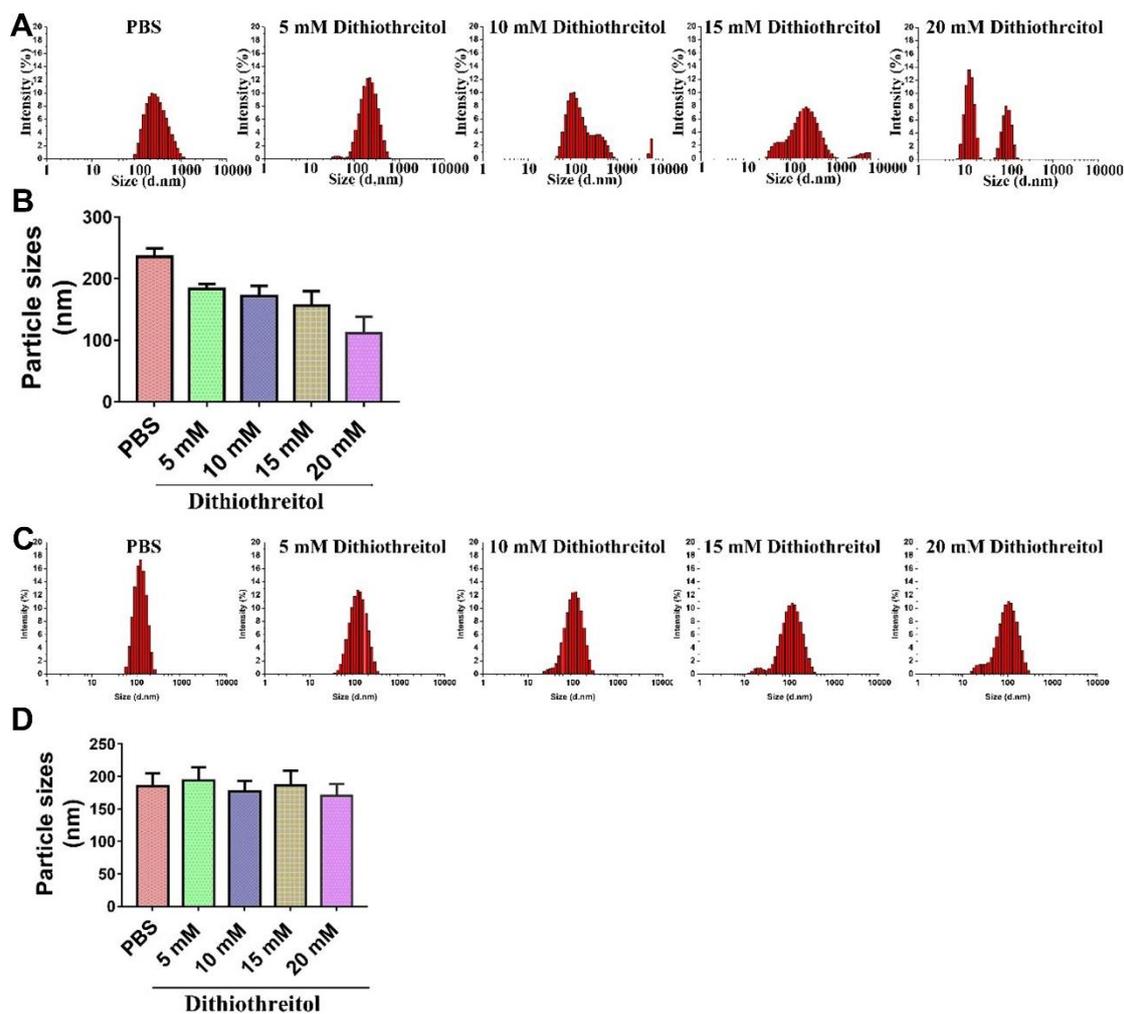


Figure S9. The H_2O_2 concentration-dependent responsiveness of PEG-ss-aAPP/MTX and APP/MTX micelles. Changes in size distribution profile of (A) PEG-ss-aAPP/MTX and (C) APP/MTX, and changes in mean diameter of (B) PEG-ss-aAPP/MTX and (D) APP/MTX.

As shown in **Figure S9**, the hydrolytic stability of PEG-ss-aAPP/MTX micelles was poorer than that of APP/MTX micelles. After 48h incubation with 20 mM dithiothreitol, the PEG-ss-aAPP/MTX micelle disintegrated into smaller particles. In contrast, the size distribution of APP/MTX micelles remained narrow, indicating its high hydrolytic stability under the same conditions. We hypothesize that the

ROS-mediated hydrolysis of disulfide bonds disrupted the PEG-ss-aAPP/MTX micelle assembly, leading to higher release of its MTX payload.

Table S1. Purchase, dilution and storage condition of primary antibodies and secondary antibodies

Antibody	Dilution factor
p-I κ B α	1:1000
I κ B α	1:1000
P65	1:1000
MMP-9	1:500
MMP-2	1:1000
HRP-linked Antibody	1:1000

All antibodies were purchased from CST and stored at 4 °C.

Table S2. Primer sequences for PCR amplification

Target gene	Forward or reverse	Sequence
MMP-2	F	5'-TGATCTTGACCAGAATACCATCGA-3'
	R	5'-GGCTTGCGAGGGAAGAAGTT-3'
MMP-9	F	5'-CCTGGAGACCTGAGAACCAATC-3'
	R	5'-CCACCCGAGTGTAACCATAGC-3'
GAPDH	F	5'-TTGTGACAAAGTGGACATTGTTG-3'
	R	5'-TCTCGCTCCTGGAAGATGGTGAT-3'