

Article

Molecularly Tailored Artesunate Nanomedicine with Well-Balanced Nanoassembly and Anticancer Performance

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Abstract

Background: Artesunate (ART), a natural product derivative of artemisinin, exhibits striking antitumor activity. However, the clinical translation of ART is limited by rapid clearance, poor tumor selectivity, and severe off-target toxicity. To address these limitations, we developed an unsaturated aliphatic chain-driven nanoassembly strategy to optimize the therapeutic performance of ART. **Methods:** We designed and synthesized two ART derivatives by conjugating saturated aliphatic chains (ART-SAs) or unsaturated aliphatic chains (ART-LAs) to ART, which subsequently self-assembled into carrier-free nanoassemblies (NAs). These NAs were characterized for their self-assembly capacity and colloidal stability. Biological evaluations included studies on cellular uptake efficiency, in vivo pharmacokinetics, and antitumor efficacy in a tumor-bearing mouse model. **Results:** The saturated aliphatic chain is found to drive nanoassembly of ART-SA but significantly shields the antitumor activity of ART. Interestingly, the conjugate of an unsaturated aliphatic chain to ART (ART-LA) not only shows outstanding self-assembly capacities but also retains the native antitumor activity of ART. The P-AL NAs with improved pharmacokinetics and tumor-specific biodistribution exert potent antitumor activity and favorable safety. **Conclusions:** We successfully applied ART for highly effective antitumor therapy by employing an unsaturated aliphatic chain-driven strategy. This study is conducive to promoting the clinical application of ART.

Keywords: artesunate; linoleyl alcohol; nanoassemblies; oxidative stress; lipid peroxide



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1. Introduction

Cancer remains a formidable global health challenge, imposing a devastating global burden on society [1]. Current therapeutic paradigms encompass surgery, chemotherapy, and radiotherapy [2,3]. While surgical resection serves as the cornerstone for managing early-stage solid tumors, it is inefficient against metastatic or diffuse malignancies such as leukemia, leaving residual disease untreated [4]. Chemotherapy, as a systemic treatment,

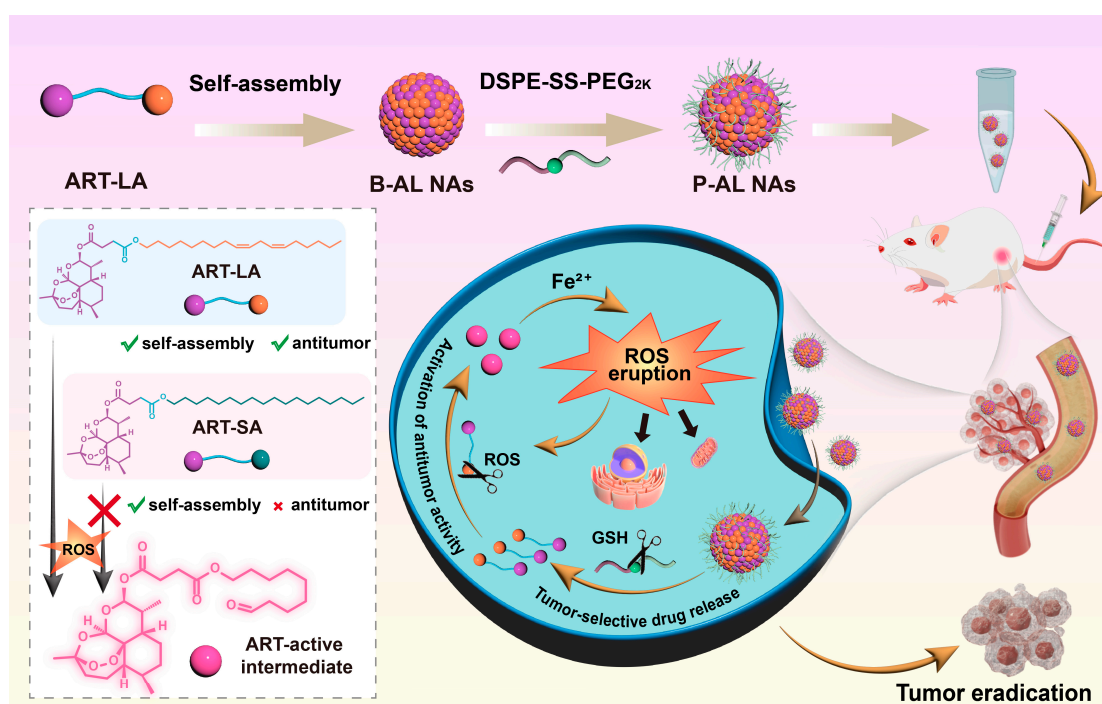
exerts broad-spectrum antitumor activity through pharmacologic agents [5], yet its lack of selectivity results in severe toxicity to normal tissues and the frequent emergence of drug resistance with prolonged use [6]. Radiotherapy achieves localized tumor control through high-energy radiation but inevitably damages adjacent healthy tissues [7,8]. Consequently, the development of novel tumor therapeutic strategies with both high efficiency and low toxicity remains an urgent priority.

Natural products have emerged as a pivotal resource for the development of novel antitumor therapies, attributed to their inherent advantages including extensive sources, excellent biocompatibility and diverse mechanisms of action. More importantly, against the backdrop of the growing emphasis on green pharmaceutical development, therapies based on natural products or their derivatives have become a research hotspot at present and even in the future due to their sustainability and renewability [9,10]. In recent years, ART, a derivative of artemisinin, has stood out among various natural products due to its unique antitumor mechanism. Specifically, the endoperoxide bridge within its molecular framework undergoes selective cleavage under the mediation of high Fe^{2+} levels in tumor cells. This reaction initiates an explosive burst of reactive oxygen species (ROS) [11–13]. When ROS generation overwhelms the scavenging capacity of endogenous antioxidant defenses in cells (e.g., superoxide dismutase), it disrupts intracellular redox homeostasis [14–16]. Subsequent oxidative stress-related damage encompasses lipid peroxidation, protein denaturation, and DNA impairment. Collectively, this cascade culminates in the activation of apoptotic signaling pathways, mediating the potent and selective killing of tumor cells. Despite this favorable antitumor mechanism and low risk of inducing drug resistance [17], the clinical translation of ART is impeded by critical limitations, including its short in vivo half-life and inadequate tumor cell selectivity [18,19]. Therefore, engineering advanced drug delivery systems to achieve efficient and precise tumor-targeted delivery of ART constitutes a pivotal scientific challenge for overcoming these limitations.

In recent years, a variety of advanced nanodrug delivery systems have been developed for tumor treatment [20–24], which also makes it possible to achieve efficient delivery of ART in vivo. Carrier-dependent nanodrug delivery systems, such as liposomes, can prolong drug circulation by encapsulating them within various carrier materials [25–27]. However, their broad application is constrained by several intrinsic drawbacks, including low drug loading capacity, limited encapsulation efficiency, potential carrier-related toxicity, and premature drug leakage [28]. In contrast, carrier-free small-molecule self-assembling nanodrug delivery systems leverage intermolecular noncovalent interactions (e.g., hydrophobic interactions, hydrogen bonding, and π – π stacking) to drive their spontaneous assembly into nanostructures, showing excellent biocompatibility and high drug-loading capacities [29–32]. Nevertheless, the intrinsic molecular architecture of ART limits its ability to form stable self-assembled nanomedicines for cancer therapy. A widely adopted strategy to address this issue is structural modification to enhance the self-assembly capacities of the compound. The pro-drug approach, which involves conjugating a poorly self-assembling parent drug (e.g., ART) to an assembly-promoting moiety (e.g., fatty acid chain) via a tumor-responsive linker (e.g., disulfide bond), is widely employed. However, this method often requires multistep synthetic routes, and the activation process may impair drug efficacy [33,34]. Consequently, developing simpler and more rational modification strategies that maintain both efficient self-assembly and the antitumor activity of ART remains a key challenge.

In this study, we observed that directly conjugating different aliphatic chains to ART via an ester bond exerts unexpected influences on its self-assembly behavior and antitumor activity. Specifically, ART was conjugated with either a saturated aliphatic chain (e.g., stearyl alcohol, SA) or an unsaturated aliphatic chain (e.g., linoleyl alcohol, LA). We found

that chemical modification indeed facilitated nanoassembly formation but simultaneously affected the antitumor activity of ART derivatives. Notably, the saturated derivative, artesunate–stearyl alcohol (ART-SA), could self-assemble into NAs, while its antitumor activity was markedly reduced compared with native ART. Conversely, the unsaturated derivative, artesunate–linoleyl alcohol (ART-LA), preserved both efficient self-assembly capability and the intrinsic antitumor potency of ART. To achieve the stable delivery of NAs and enhance tumor-selective drug release while minimizing off-target toxicity, we further modified DSPE-SS-PEG_{2K} on NAs, generating P-AL NAs and P-AS NAs. Furthermore, mechanistic studies revealed that the polyunsaturated double bonds in ART-LA released from P-AL NAs were susceptible to ROS-mediated cleavage in tumor cells, generating intermediates with pharmacological activity equivalent to ART. These intermediates subsequently underwent Fe²⁺-mediated cleavage of the endoperoxide bridge, producing abundant ROS, amplifying lipid peroxidation, and inducing a robust intracellular oxidative stress storm (Scheme 1 and Figure S1). In a 4T1 tumor-bearing mouse model, the P-AL NAs exhibited optimal antitumor efficacy with reduced systemic toxicity. This study presents an unsaturated aliphatic chain modification-directed molecular engineering strategy, which holds promise for providing a feasible approach to enable more natural small molecules to achieve highly effective and low-toxicity antitumor therapy.



Scheme 1. The schematic diagram illustrates the composition of P-AL NAs and their antitumor mechanism.

2. Materials and Methods

2.1. Materials

Artesunate and Dithiothreitol (DTT) were purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Linolenol, DMAP (4-Dimethylaminopyridine), and EDCI (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) were purchased from Energy Chemical (Shanghai, China). NAC (N-acetyl-L-cysteine) was purchased from Macklin Biochemical Co., Ltd. (Shanghai, China). Fetal bovine serum, culture medium, stearyl alcohol, Cy3 (Cyanine 3), and Cy7 (Cyanine 7) were sourced from Meilun Biotechnology Co., Ltd. (Dalian, China). DSPE-SS-PEG_{2K} and Cy3/Cy7-labeled DSPE-SS-PEG_{2K}

were purchased from Xi'an Ruixi Biotechnology Co., Ltd. (Xi'an, China). Micro malondialdehyde (MDA) assay kits were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). NEST Biotechnology Co., Ltd. (Wuxi, China) provided the cell culture dishes and plates used in this study. All solvents and reagents employed in this work were of analytical or high-performance liquid chromatography (HPLC) grade.

2.2. Synthesis of Artesunate Derivatives

Artesunate (192.2 mg, 0.5 mmol) was accurately weighed out and subsequently dissolved in 10 mL of dichloromethane (DCM). After continuous stirring, DMAP (17.1 mg, 0.14 mmol), previously dissolved in DCM (500 μ L), was added dropwise to the reaction mixture. After thorough mixing, linolenic alcohol (111.9 mg, 0.42 mmol), also pre-dissolved in DCM (5 mL), was introduced into the system, followed by the addition of EDCI (95.9 mg, 0.5 mmol), which had likewise been dissolved in DCM (5 mL). Therefore, we obtained a reaction solution with a total volume of 20.5 mL. Finally, the mixed system reacted for 48 h at 25 °C under nitrogen protection. Upon completion, the generating product ART-LA was purified using preparative HPLC (LC-3050N, CXTH, Beijing, China). Structural confirmation of the target compound was achieved through mass spectrometry (MS) (G2-XS Qtof, Waters, Shanghai, China) and proton nuclear magnetic resonance (^{1} H NMR) (400MHZ AVIII, Bruker, Karlsruhe, Germany) analyses. In addition, we also confirmed the structure of ART-LA using Fourier transform infrared spectroscopy (FTIR) (Thermo Fisher Scientific Nicolet iS20, Waltham, MA, USA). The artesunate–stearyl alcohol derivative (ART-SA) was synthesized and purified following the same procedures as described above.

2.3. Preparation of Artesunate Derivative NAs

Stock solutions of ART-LA and ART-SA were prepared in ethanol, respectively (3.3 mg/mL). Subsequently, 300 μ L of each stock solution was added dropwise into deionized water (2 mL) at a constant rate while stirring at 800 rpm. To intensify the colloidal stability and tumor responsiveness of the NAs, DSPE-SS-PEG_{2K} (20% *w/w*, dissolved in ethanol) was added promptly during the stirring process. After stirring for 3 min, the organic solvent was removed from the system to obtain P-AL NAs and P-AS NAs, respectively. Measurement of the Zeta potential and particle size for these NAs was performed via the Zetasizer (NS-90Z Plus, OMEC, Zhuhai, China) (particle size was calculated using the Stokes–Einstein equation; Zeta potential was measured by Doppler electrophoresis method). Their morphologies were observed by transmission electron microscopy (TEM) (HT7800, HITACHI, Tokyo, Japan). Additionally, the preparation method of Cy3- or Cy7-labeled NAs for cell uptake and in vivo pharmacokinetic studies was identical to the above. It only required adding a certain concentration of Cy3 stock solution to the system or using specially labeled Cy7-DSPE-SS-PEG_{2K}.

2.4. Colloidal Stability Investigation of NAs

The colloidal stability of P-AL NAs and P-AS NAs was evaluated based on the changes in particle size under different conditions. Specifically, we mixed 1 mL of each formulation (0.5 mg/mL) with 9 mL of phosphate-buffered saline (PBS, pH 7.4) or PBS supplemented with 10% fetal bovine serum (FBS). The mixtures were incubated in a 37 °C constant-temperature shaker at 100 rpm. At preselected time points (0, 1, 2, 4, 8, and 12 h), samples were collected, and the particle diameter variations in the NAs were detected via the Zetasizer (NS-90Z Plus, OMEC, Zhuhai, China).

2.5. Assembly Mechanisms of NAs

Molecular docking simulations were conducted using the YinFu cloud computing platform (<http://cloud.yinfotek.com/>) (accessed on 25 April 2025) to analyze the intermolecu-

lar interactions of ART-LA and ART-SA during the self-assembly process. Furthermore, the sodium dodecyl sulfate (SDS, which disrupts hydrophobic interactions), urea (which disrupts hydrogen bonding interactions), and sodium chloride (NaCl, which disrupts electrostatic interactions) were used to further verify the assembly mechanism of the NAs as the force-destroyed objects. The specific operation was as follows: Prepare 10 mM solutions of SDS, NaCl, and urea in water, respectively. And then, 0.5 mL of non-PEG-modified ART-LA NAs and ART-SA NAs were respectively added to 4.5 mL of the prepared SDS, NaCl, and urea solutions, and then incubated in a constant temperature shaker at 37 °C (with a rotational speed of 100 rpm) for 8 h. The particle size changes in the NAs before and after incubation were measured.

2.6. Reduction-Sensitive Drug Release of NAs

Prepare PBS (pH 7.4) containing different concentrations of DTT (0, 5 mM). Separately took 1 mL of P-AL NAs and P-AS NAs each at a concentration of 0.5 mg/mL, and co-incubated them with 9 mL of the aforementioned medium. Finally, the particle size of each NA was detected at different incubation time points (0, 1, 2, 4, 8, and 12 h). The dialysis method was adopted for the quantitative analysis of the release of ART-LA and ART-SA from the NAs. Specifically, first boil the dialysis bag (with a molecular weight cutoff of 8000–30,000) for activation. Subsequently, 1 mL each of P-AL NAs and P-AS NAs was taken and placed in the activated dialysis bags, then the dialysis bags were immersed in the pre-prepared release medium containing different concentrations of DTT (0, 5 mM) (PBS containing 30% ethanol, pH 7.4) for co-incubation at 37 °C. At different time points of co-incubation (0, 2, 4, 8, 12 h), 200 µL of the release medium was taken, tested, and the release amount of ART-LA or ART-SA was determined by HPLC (LC-3050N, CXTH, Beijing, China).

2.7. Cell Culture

4T1 cells and 3T3 cells were purchased from the cell bank of the Chinese Academy of Science (Shanghai, China). 4T1 cells were cultured in RPMI-1640 Medium supplemented with 10% FBS and 1% antimicrobial solution. In contrast, 3T3 cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) Medium. All cell cultures were subsequently incubated at 37 °C under an atmosphere of 5% CO₂.

2.8. Cellular Uptake Efficiency of NAs

We selected 4T1 cells to investigate the cellular uptake efficiency of NAs. The cells were maintained in RPMI-1640 medium supplemented with 10% FBS and 1% antibiotic solution at 37 °C under 5% CO₂. Subsequently, fluorescently labeled Cy3-DSPE-SS-PEG_{2K} was applied as the fluorescent probe to fabricate fluorescently labeled Cy3/P-AL NAs and Cy3/P-AS NAs. The uptake behavior of 4T1 cells was qualitatively analyzed by confocal microscopy (CLSM) (NIS 4.13, Nikon, Tokyo, Japan): Firstly, the pre-cultured cells (seeded at 2×10^5 cells per well in 24-well plates and cultured for 12 h) were co-incubated respectively with the media containing Cy3 Sol, Cy3/P-AL NAs and Cy3/P-AS NAs for 0.5 h and 4 h, where the equivalent concentration of Cy3 was 8 µg/mL. After incubation, quickly discard the drug-containing medium and wash it three times with cold PBS (pH 7.4) to terminate the uptake. After that, add Hoechst dye and stain for 10 min, then wash it again. Finally, the cell uptake results were observed using CLSM (C2, Nikon, Tokyo, Japan), and related fluorescence images were captured (Cy3 was excited at 550 nm with an emission wavelength of 570 nm, while Hoechst was excited at 350 nm with an emission wavelength of 460 nm). Similarly, the same strategy could be adopted to quantitatively analyze the fluorescence intensity within the cells after they take up the NAs using flow cytometry (BD FACSCelesta, San Jose, CA, USA).

2.9. *In Vitro* Assessment of Cytotoxicity

The cytotoxicity of the different formulations against 4T1 and 3T3 cells was determined by the MTT assay. The specific operation process was as follows: 4T1 cells were inoculated into 96-well plates at a density of 2×10^3 cells per well, and then placed in a constant temperature incubator at 37 °C with 5% CO₂ for 12 h to ensure adhesion. The old culture medium was discarded. Subsequently, the culture continued for 48 h using medium containing a series of concentrations of ART Sol, LA Sol, SA Sol, ART/LA Sol, ART/SA Sol, ART-LA Sol, ART-SA Sol, P-AL NAs, and P-AS NAs (equal concentration of ART from 0 to 4000 nM). Subsequently, 20 µL of MTT (5 mg/mL) was dispensed to each well of the 96-well plate, followed by incubation at 37 °C for 4 h. The culture medium containing MTT was discarded, and 200 µL DMSO was added to each well to completely dissolve the formed formazan. Finally, the ultraviolet absorbance at 490 nm of different administration groups was determined using a multifunctional microplate reader (Varioskan Flash 3001, Thermo Scientific, Waltham, MA, USA). Meanwhile, using mouse 3T3 cells as the normal cell model, the same method was used to verify the cytotoxicity of each administration group against normal cells. Furthermore, to verify that the cytotoxic effect of P-AL NAs (equal amounts of ART from 0 to 4000 nM) on 4T1 cells was indeed ROS dependent, the ROS scavenger NAC (5 mM) was co-administered with the treatment. The cell viability was then assessed following the same experimental procedure.

2.10. Detection of LPOs

Fluorescent detection of lipid hydroperoxide (LPO) generation in cells was performed using the C11 BODIPY 581/591 probe. The specific operation process was as follows: 4T1 cells were seeded in 24-well plates with pre-set cell slides at a density of 5×10^4 cells per well. Subsequently, cell culture medium containing the formulations (ART Sol, LA Sol, SA Sol, ART-LA Sol, ART-SA Sol, P-AL NAs, and P-AS NAs) at a consistent ART concentration of 4 µM was added, with further incubation for 8 h. After that, the cells were rinsed three times with cold PBS (pH 7.4) to remove the drug-containing medium and incubated in the presence of 10 µM C11 BODIPY 581/591 for 1 h. Finally, the fluorescence signals of each group were observed under CLSM (C2, Nikon, Tokyo, Japan).

2.11. Detection of MDA

The detection of MDA within cells could be conducted through MDA detection kits. The specific operation was as follows: 4T1 cells were seeded in 24-well plates with pre-set cell slides at a density of 5×10^4 cells per well. Subsequently, spent medium was replaced with fresh cell culture medium supplemented with the formulations (ART Sol, LA Sol, SA Sol, ART-LA Sol, ART-SA Sol, P-AL NAs, and P-AS NAs) at a consistent ART concentration of 4 µM, with further incubation for 8 h post-respective addition. Cells were rinsed three times with cold PBS (pH 7.4) to remove the drug-containing culture media. Then, following the kit instructions, cells were lysed with the extraction buffer using sonication. After adding the corresponding reagents in order and mixing, the samples were heated in a 100 °C water bath for 60 min, then cooled on ice and centrifuged. Next, 200 µL of supernatant was transferred to a 96-well plate. Finally, the absorbance of MDA was measured at wavelengths of 532 nm and 660 nm via a multifunctional microplate reader (Varioskan Flash 3001, Thermo Scientific, Waltham, MA, USA), and the content of MDA was calculated according to the formula provided in the kit manual.

2.12. Detection of Intermediates from ART-LA

The intermediates produced by ART-LA after lipid peroxidation in cells could be monitored by mass spectrometry, and the mechanism by which ART-LA exerts pharmacological

activity *in vivo* had been analyzed. The specific operation was as follows: Same cell culture procedures were as described above. Here, ART-LA Sol and ART-SA Sol (equal to 4 μM of ART) were added to the culture medium, respectively, to incubate the cells for 8, 12, 24, and 48 h. After incubation, the cells were scraped into the culture medium with a scraper and then transferred to EP tubes. The cells were disrupted by ultrasound, proteins were precipitated with acetonitrile, and the intermediate was extracted by centrifugation. The molecular weight of the intermediate was determined by high-performance liquid chromatography-mass spectrometry (HPLC-MS) (LCMS-8060, Shimadzu, Kyoto, Japan).

2.13. Detection of ROS

The DCFH-DA fluorescent probe was employed to detect intracellular ROS. Use the same operation mentioned above to enable the cells to adhere and grow on the surface (5×10^4 per well). Then, cells were incubated for 8 h with cell culture medium containing ART Sol, LA Sol, SA Sol, ART-LA Sol, ART-SA Sol, P-AL NAs, and P-AS NAs at a consistent 4 μM ART concentration. After incubation, the old drug-containing medium was discarded, cells were rinsed three times with cold PBS (pH 7.4), then incubated with 10 μM DCFH-DA probe for 30 min, and then washed three times with cold PBS (pH 7.4) again. Finally, the intracellular ROS level was quantitatively determined by flow cytometry (BD FACSCelesta, San Jose, CA, USA).

2.14. Animal Studies

Sprague–Dawley (SD) rats and BALB/C mice were acquired from the Animal Center of Shenyang Pharmaceutical University (Shenyang, China) without genetic modification. All animal experimental protocols had been approved by the Animal Ethics Committee of Shenyang Pharmaceutical University prior to the study (Approval Code: SYPU-2ACUC-S2024-0919-201; Approval date: 19 September 2024). This study employed adult male SD rats (8–12 weeks of age, weighing approximately 200 g) and adult female BALB/c mice (6–8 weeks of age, weighing around 20 g). A total of 15 SD rats and 40 BALB/c mice were maintained under standard laboratory housing conditions with *ad libitum* access to standard chow and drinking water. Randomization of treatment regimens and cage placements was performed, and blinded evaluations were conducted to mitigate handling-related and environmental biases.

2.15. Pharmacokinetics

SD rats were randomly divided into 3 groups ($n = 5$): (i) Cy7 Sol, (ii) Cy7/P-AL NAs, and (iii) Cy7/P-AS NAs (equal to 2 mg/kg of Cy7). This division occurred after administering the formulations by the tail vein, respectively. Blood was collected through the ocular vein at the preselected time points (0.033, 0.083, 0.25, 0.5, 1, 2, 4, 8, and 12 h). Whole blood specimens were harvested in heparin sodium-containing EP tubes. The specimens were promptly centrifuged at 8000 rpm for 3 min to recover plasma. Lastly, plasma drugs were extracted via the protein precipitation technique. The drug concentration was detected under the condition of $\text{Ex/Em} = 744/776 \text{ nm}$, and a drug concentration-time curve was plotted.

2.16. In Vivo Biodistribution of NAs

BALB/C mice xenograft model (4T1) was established and randomly divided into 3 groups ($n = 3$). After that, Cy7 Sol, Cy7/P-AL NAs and Cy7/P-AS NAs (equal to 2 mg/kg of Cy7) were administered intravenously into the rats. At preselected time points (1, 2, 4, 6, 8, 12, and 24 h), the fluorescence signals in the mice were dynamically monitored by means of a small animal *in vivo* imaging system. And at the time, when the NAs accumulated

maximally in the tumor, the mice were euthanized, and the drug distribution in their heart, liver, spleen, lung, and kidney was photographed.

2.17. *In Vivo* Antitumor Activity

Once the tumor volume in mice reached approximately 100 mm³, the mice were randomly assigned into 6 groups ($n = 5$), comprising the blank control group (saline), ART Sol, ART-SA Sol, ART-LA Sol, P-AL NAs, and P-AS NAs. During the treatment period, we administered the drugs via the tail vein every 48 h for a total of five administrations, with the dosage of ART at an equal concentration of 10 mg/kg. The tumor volume and body weight of mice were systematically evaluated and recorded daily at predetermined time points. No animals, experimental units, or data points were omitted from the final analysis, given that all animals exhibited unimpaired health status, stable body weight, and experimental data free of anomalies. The formula for calculating the tumor volume: $V = (a \times b^2)/2$ (“V” represents the tumor volume, “a” represents the long diameter of the tumor, and “b” represents the short diameter of the tumor). The formulation for calculating the tumor burden rate is as follows: $S = W_T/W_M \times 100\%$ (“S” represents the tumor burden rate, “W_T” represents the tumor weight, and “W_M” represents the weight of the tumor-bearing mice).

2.18. *Statistical Analysis of Animal Experimental Data*

In this study, animals were randomly assigned to the control and treatment groups to ensure equitable allocation, prevent selection bias (e.g., unintentional assignment of healthier animals to a particular group), balance confounding variables (such as body weight, age, and litter effects), and satisfy statistical assumptions (e.g., independence of observations). For basic comparative analyses (e.g., *t*-test or one-way ANOVA), a sample size of five animals per group provides approximately 80% statistical power to detect large effect sizes (e.g., Cohen’s $d > 1.0$) when variability is low. Healthy animals meeting predefined criteria for age, body weight, and baseline health status were enrolled, whereas those exhibiting severe adverse reactions (e.g., >20% body weight loss) or associated with technical failures were excluded—all criteria were established a priori. Outliers identified by predefined statistical methods (>2 standard deviations) were excluded, and data resulting from technical errors were discarded. The entire experimental and data analysis process adhered strictly to these pre-specified inclusion/exclusion protocols. During allocation, only the randomization coordinator was aware of group assignments and did not participate in subsequent procedures; all other personnel, including treatment administrators, outcome assessors, and statisticians, remained blinded throughout the study. Coded identifiers were used, and data were analyzed in a blinded manner until final results were generated.

2.19. *Statistical Analysis*

All data in this study were presented as the mean $\pm$ standard deviation (SD). Statistical analyses were conducted using GraphPad Prism 8.0 software, and intergroup significant differences were evaluated using Student’s *t*-test or one-way analysis of variance (ANOVA). Exact *p* values were given in the corresponding figures, with values < 0.05 deemed statistically significant.

3. Results and Discussion

3.1. *Synthesis of ART Derivatives*

Owing to the intrinsic structural constraints of ART, it is challenging to form stable nanoassemblies via small-molecule self-assembly technology. To address this, we designed an artesunate–linoleyl alcohol derivative (ART-LA) and an artesunate–stearyl alcohol

derivative (ART-SA) bridged by ester bonds based on our findings, respectively (Figures S2 and S3). The synthesized products were purified using preparative HPLC. As shown in Figures S4 and S5, the HPLC purity percentages of ART-LA and ART-SA were 98.6% and 93.7%, respectively. The molecular structures of the two target compounds were confirmed through ^1H NMR and MS analyses, validating the successful synthesis of ART-LA and ART-SA (Figures S6 and S7). Furthermore, we also used FTIR to confirm the successful synthesis of ART-LA and ART-SA. As shown in Figure S8, the retention of the characteristic functional group peaks of ART in the spectra of ART-LA and ART-SA, the attenuation of the -OH peaks in LA and SA, and the newly emerged characteristic ester group peaks collectively confirm the successful synthesis of ART-LA and ART-SA via the esterification reaction between ART and LA/SA.

3.2. Nanoassembly Performance of ART Derivatives

In a preliminary investigation, we found that aliphatic alcohols exhibited excellent assembly-promoting effects due to their hydrophobicity. Accordingly, we systematically investigated the self-assembly properties of the synthesized ART-LA and ART-SA. Notably, to further improve the colloidal stability of the prepared NAs and their capability for tumor-selective drug release, DSPE-SS-PEG_{2K} was employed for surface modification, affording P-AL NAs and P-AS NAs, respectively (Figure 1A). The particle size of P-AL NAs was 78.78 ± 1.35 nm, while that of ART-SA NAs was 80.60 ± 0.66 nm. Moreover, the Zeta potentials of both P-AL NAs and P-AS NAs were approximately -25 mV (Table S1), which was conducive to preventing abnormal aggregation of the NAs. TEM observations revealed that both P-AL NAs and P-AS NAs exhibited uniform spherical morphologies (Figure 1B,C). The appearances of the prepared NAs are shown in Figure S9.

To further elucidate the impacts of DSPE-SS-PEG_{2K} modification on the colloidal stability of NAs, PBS (pH 7.4) and PBS supplemented with 10% FBS (pH 7.4) were used to mimic the *in vivo* environment for assessing the stability of P-AL NAs and P-AS NAs. Following PEGylation, both P-AL NAs and P-AS NAs exhibited favorable colloidal stability in the respective media (Figure 1D,E). No significant variation in particle size was observed over the incubation time, which was beneficial for their stable delivery *in vivo*.

Additionally, the molecular docking simulation technique was employed to investigate the self-assembly mechanism of P-AL NAs and P-AS NAs. As shown in Figure 1F, to clearly depict intermolecular interactions, the two ART-LA molecules were shown in blue and green, with their oxygen atoms highlighted in red to indicate potential polar sites. Hydrophobic interactions were denoted by gray dashed lines. The results revealed that hydrophobic interactions were the primary driving force for P-AL NAs formation, and the P-AS NAs also exhibited similar simulation outcomes (Figure S10). Moreover, intermolecular forces were further validated through force-disruption assays, where SDS, NaCl, and urea served as disruptors of hydrophobic interactions, electrostatic interactions, and hydrogen bonds, respectively. The results indicated that incubation with SDS led to a significant increase in the particle size of the NAs, whereas no remarkable fluctuation was observed following incubation with NaCl or urea (Figure 1G). This indicates that hydrophobic interactions played a dominant role in the self-assembly process of the drug, which was consistent with the molecular docking simulation findings.

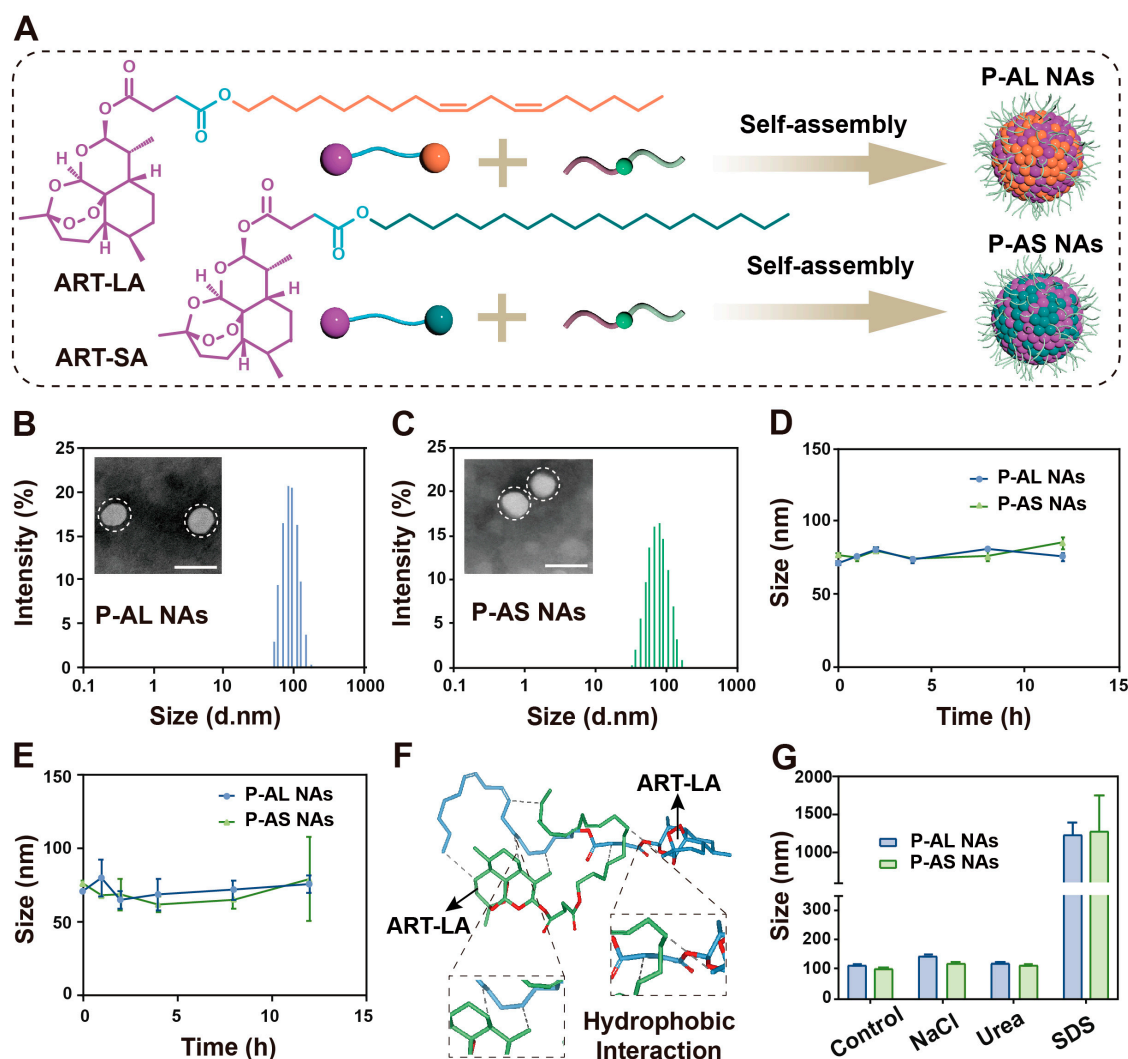


Figure 1. Preparation and characterization of self-assembled P-AL NAs and P-AS NAs. (A) Schematic illustration of the preparation process for P-AL NAs and P-AS NAs; (B,C) particle size distributions and TEM morphologies of P-AL NAs and P-AS NAs, respectively (scale bar = 100 nm); (D) colloidal stability profiles of NAs in PBS ($n = 3$); (E) colloidal stability of NAs in PBS supplemented with 10% FBS ($n = 3$); (F) molecular docking simulation model of P-AL NAs; and (G) particle size variations in P-AL NAs and P-AS NAs after incubation with NaCl (10 mM), Urea (10 mM), and SDS (10 mM), respectively ($n = 3$).

To further verify that P-AL NAs and P-AS NAs modified with DSPE-SS-PEG_{2K} can respond to the relevant GSH concentration in tumor cells and achieve drug release, we first co-incubated each NAs with PBS (pH 7.4) containing different concentrations of DTT (0, 5 mM) and dynamically monitored the particle size changes in them. As shown in Figure S11, within 12 h of co-incubation, the particle sizes of P-AL NAs and P-AS NAs co-incubated with DTT-containing PBS (pH 7.4) both increased significantly. This indicates that P-AL NAs and P-AS NAs do have reduction sensitivity, and the high GSH in tumor cells is expected to destroy the structure of NAs and trigger drug release. More importantly, we used the dialysis method to quantitatively detect the free ART-LA and ART-SA released from the NAs. As shown in Figure S12, under the condition of 5 mM DTT, the cumulative release amount of free ART-LA from P-AL NAs exceeded 90% at 12 h of incubation, and P-AS NAs showed a similar release trend at the same time. These results collectively confirm that P-AL NAs and P-AS NAs modified with DSPE-SS-PEG_{2K} can effectively achieve drug release in the tumor reductive microenvironment.

3.3. Cellular Uptake

The cellular uptake efficiency of NAs is a critical factor determining their antitumor efficacy. To explore the cellular uptake efficiency, we employed Cy3 to label the P-AL NAs and the P-AS NAs, and subsequently assessed the uptake efficiency of those Cy3-labeled formulations (Cy3/P-AL NAs and Cy3/P-AS NAs) by 4T1 cells using CLSM for qualitative analysis and flow cytometry for quantitative assessment. As shown in Figure 2A, the Cy3 fluorescence signals in cells treated with different groups exhibited a time-dependent pattern, with the intracellular fluorescence signal at 4 h being stronger than that at 0.5 h in all groups, indicating cells were continuously engaging in uptake. Notably, compared with free Cy3 Sol, cells treated with Cy3/P-AL NAs or Cy3/P-AS NAs showed stronger intracellular Cy3 fluorescence signals at both 0.5 h and 4 h, which was attributed to the inferior transmembrane transport efficiency of small molecules compared to NAs. Additionally, the quantitative flow cytometry data were consistent with the qualitative observations, indicating that the formation of NAs enhances cellular uptake efficiency and thereby ensures the potent exertion of the drugs' antitumor activity (Figure 2B).

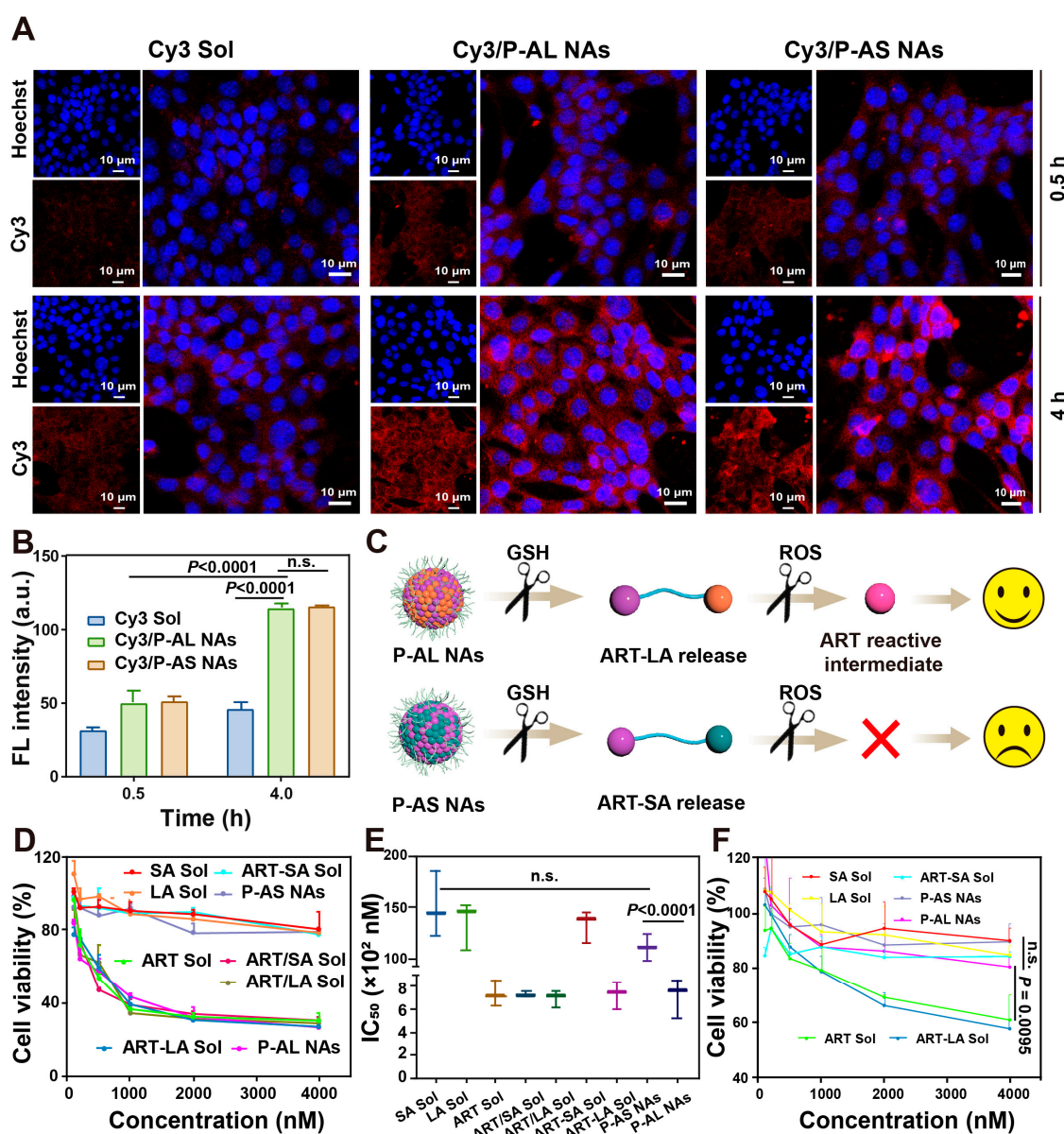


Figure 2. Cell uptake and in vitro antitumor activities evaluation. (A) Intracellular Cy3 fluorescence images of 4T1 cells treated with Cy3 Sol, Cy3/P-AL NAs, or Cy3/P-AS NAs at different uptake time

points (scale bar = 10 μm); (B) quantitative analysis of intracellular Cy3 fluorescence intensity ($n = 3$). Data were presented as mean $\pm$ SD. Two-way ANOVA with Tukey's multiple comparisons test was used for the analysis of data and adjusted p value. n.s. represents no significance. (C) Schematic illustration of intracellular response release and subsequent active activation of P-AL Ns and P-AS NAs, respectively; (D) in vitro anti-4T1 cell activities of ART and its derivatives preparations at different concentrations ($n = 3$). (E) IC_{50} values of the anti-4T1 activities of ART and its derivative preparations ($n = 3$). (F) In vitro anti-3T3 cell activities of ART and its derivatives preparations at different concentrations ($n = 3$). Data are presented as mean $\pm$ SD. One-way ANOVA with Tukey's multiple comparisons test was used for the analysis of data and adjusted p value. n.s. represents no significance.

3.4. In Vitro Antitumor Activities

To evaluate the in vitro antitumor efficacy of the NAs, the MTT assay was employed to assess their proliferation inhibitory effects on 4T1 cells. As shown in Figure 2D,E, P-AL NAs exhibited cytotoxicity comparable to ART Sol. This observation is hypothesized to stem from the efficient release of ART-LA from P-AL NAs under the action of GSH within tumor microenvironments. Subsequent lipid peroxidation of the polyunsaturated fatty alcohol moiety in ART-LA occurs in the high ROS environment of tumor cells, triggering chain scission and restoration of the original ART drug activity; this also accounts for why ART-LA Sol exerts cytotoxicity comparable to ART Sol (Figure 2C). In contrast, cells treated with P-AS NAs and ART-SA Sol showed no significant decrease in viability, indicating minimal cytotoxicity. This may be attributed to the absence of ROS-sensitive sites in ART-SA, which makes it difficult to initiate a series of processes (e.g., lipid peroxidation and cleavage) to generate active ART intermediates, as observed in ART-LA, thereby manifested as reduced anti-tumor activity (Figures 2C and S1). To more directly demonstrate that the anti-tumor effect of P-AL NAs indeed stems from the ROS produced by the activated intermediates, we added NAC simultaneously during the co-incubation of P-AL NAs with 4T1 cells. As shown in Figure S13, after the addition of NAC (5 mM), the antitumor activity of P-AL NAs was significantly suppressed. The viability of 4T1 cells remained above 85% across all concentrations tested, indicating that the antitumor effect of P-AL NAs indeed depends on the robust production of ROS.

In addition, we evaluated the cytotoxicity of different formulations on normal cells (3T3 cells) (Figure 2F). The viabilities of 3T3 cells treated with SA Sol, LA Sol, ART-SA Sol, P-AS NAs, and P-AL NAs remained above 80% across various equivalent ART concentrations, with no statistically significant differences among the groups, indicating negligible intrinsic toxicity of these formulations. Notably, at an equivalent ART concentration of 4000 nM, treatment with ART Sol and ART-LA Sol led to a significant reduction in 3T3 cell viability (approximately 60%), which may reflect the inherent cytotoxicity of ART and suggest that ART-LA can be activated under normal physiological ROS levels. The difference in cytotoxicity between P-AL NAs and ART-LA Sol can be attributed to the DSPE-SS-PEG_{2K} modification of P-AL NAs. This modification confers GSH-responsive release of free ART-LA. In 3T3 cells with low GSH levels, the structure of P-AL NAs remains stable, impeding drug release and effectively shielding the cytotoxicity of free ART-LA, thereby demonstrating negligible cytotoxicity. These results strongly suggest that the DSPE-SS-PEG_{2K}-modified P-AL NAs can effectively distinguish between normal and tumor cells through their unique GSH-responsive mechanism, achieving favorable tumor-selective drug release. These lay the foundation for realizing the efficient and low-toxicity antitumor application of ART.

3.5. Insights into the Antitumor Mechanism of P-AL NAs

Based on the above findings, the P-AL NAs we prepared demonstrated significant cytotoxicity against 4T1 cells. To this end, we further explored a panel of biomarkers

involved in tumor-cell killing. First, we examined the levels of LPOs and MDA during lipid peroxidation. As shown in Figure 3A, cells treated with ART Sol, ART-LA Sol, and P-AL NAs exhibited significantly enhanced fluorescence signals, indicating their ability to induce LPOs accumulation in cells. In contrast, cells treated with LA Sol, SA Sol, ART-SA Sol, and P-AS NAs exhibited inferior fluorescence intensity, suggesting negligible LPO-inducing activity. Consistently, MDA content measurements corroborated the LPO production outcomes (Figure 3B). Collectively, among the two derivatives we synthesized, ART-LA effectively triggered intracellular lipid peroxidation to exert antitumor effects, whereas ART-SA failed to induce this response.

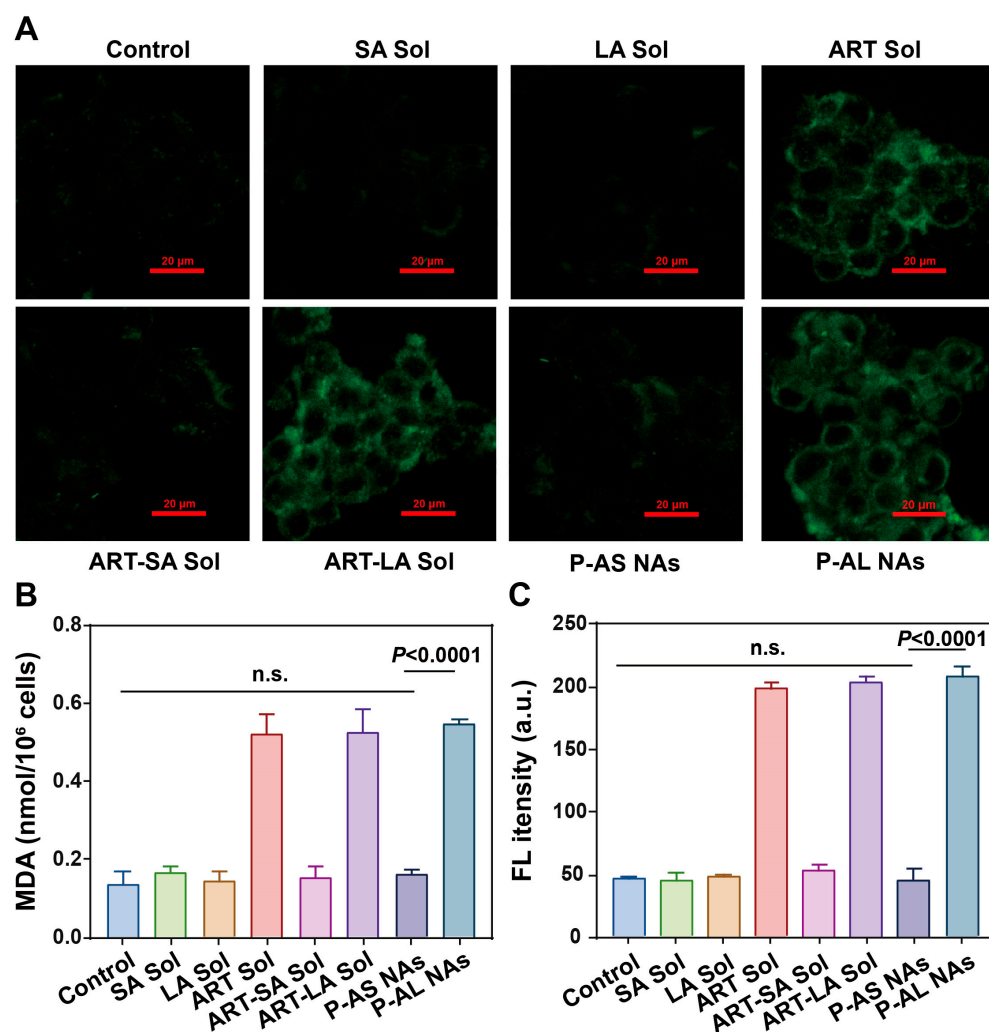


Figure 3. Detection of oxidative stress markers and ROS levels in 4T1 cells. (A) Intracellular levels of LPOs in 4T1 cells as visualized by CLSM (scale bar = 20 μ m); (B) intracellular MDA amounts in 4T1 cells following various treatments ($n = 3$); and (C) intracellular ROS amounts in 4T1 cells after treatments ($n = 3$). Data are presented as the mean $\pm$ SD. One-way ANOVA with Tukey's multiple comparisons test was used for the analysis of data and adjusted p value. n.s. represents no significance.

To further verify the ROS-generating capacity of the NAs, the DCFH-DA probe was employed for intracellular ROS detection. As shown in Figure 3C, in comparison with the control group, cells treated with ART Sol, ART-LA Sol, and P-AL NAs exhibited marked ROS generation. Conversely, cells treated with LA Sol, SA Sol, ART-SA Sol, and P-AS NAs generated almost no ROS, consistent with the cytotoxicity results. Additionally, we detected the intermediates formed after lipid peroxidation-induced chain scission of P-AL

NAs and P-AS NAs, respectively. Briefly, to verify the actual activation of ART-LA in cells, we employed HPLC-MS to characterize the intermediates generated from the derivatives following lipid peroxidation, aiming to validate the pharmacological mechanism of ART-LA in vivo. Results showed that ART-LA exhibited high response signals for aldehyde intermediates at 8, 12, and 24 h with lower aldehyde intermediates and trace ester bond hydrolysis product ART detected at 48 h (Figure S14). In contrast, ART-SA showed no aldehyde intermediates throughout the detection period but also yielded trace ART via ester bond hydrolysis at 48 h. These findings further validated our hypothesis that the difference in antitumor activity between ART-LA and ART-SA indeed stems from the modification with a structurally distinct aliphatic chain.

3.6. Pharmacokinetics and Biodistribution

The in vivo delivery of NAs is intricate, with prolonged circulation and tumor-targeted accumulation being critical for maximizing therapeutic efficacy. Consequently, we evaluated the pharmacokinetics and tissue distribution of the P-AL NAs and P-AS NAs, respectively. First, Cy7 labeling was used to trace their metabolic clearance. As shown in Figures 4A and S15, Cy7 Sol exhibited rapid blood clearance after tail vein injection, whereas Cy7/P-AL NAs and Cy7/P-AS NAs showed significantly prolonged circulation, with area under the curve (AUC_{0-12h}) increased by 3.51-fold and 3.25-fold versus Cy7 Sol (Table S2). This is attributed to their high stability and surface modification of DSPE-SS-PEG_{2K}, which together help the NAs resist rapid clearance from the bloodstream while potentially enhancing their accumulation at tumor sites through improved pharmacokinetic behavior.

Using Cy7 fluorescence, we performed real-time small-animal imaging to assess tumor targeting in 4T1-bearing mice. Cy7/P-AL NAs and Cy7/P-AS NAs reached peak tumor accumulation at 6 h post-administration, followed by slow clearance, whereas Cy7 Sol peaked at 1 h with faster elimination (Figure 4B). In addition, we also investigated the fluorescence signal of the heart, liver, spleen, lung, kidney, and tumor in the tested mice at the time point of maximum tumor accumulation of the Cy7. As expected, due to the better pharmacokinetic properties of NAs (Figure 4A), their accumulation level in the tumor was notably superior to that of the free Cy7 solution, with the total fluorescence signal of Cy7 from the NAs being approximately 4-fold that of the Cy7 solution group (Figure 4C,D). These results showed that NAs possessed enhanced tumor accumulation, attributed to their stability, optimized pharmacokinetics, and the enhanced permeability and retention (EPR) effect. Notably, significant NAs accumulation was also observed in the liver (Figures 4C,D and S16). This can be attributed mainly to the high hepatic blood flow, which enhances nanoparticle exposure, as well as to the efficient uptake by resident macrophages that recognize nanoparticles as foreign substances. Since NAs are also distributed in tissues other than tumors, especially in the liver, this may raise potential concerns about safety. Although in normal tissues, the P-AL NAs and P-AS NAs modified with DSPE-SS-PEG_{2K} may not exert pharmacological effects, verifying the safety of each formulation remains a very important task in the subsequent in vivo studies.

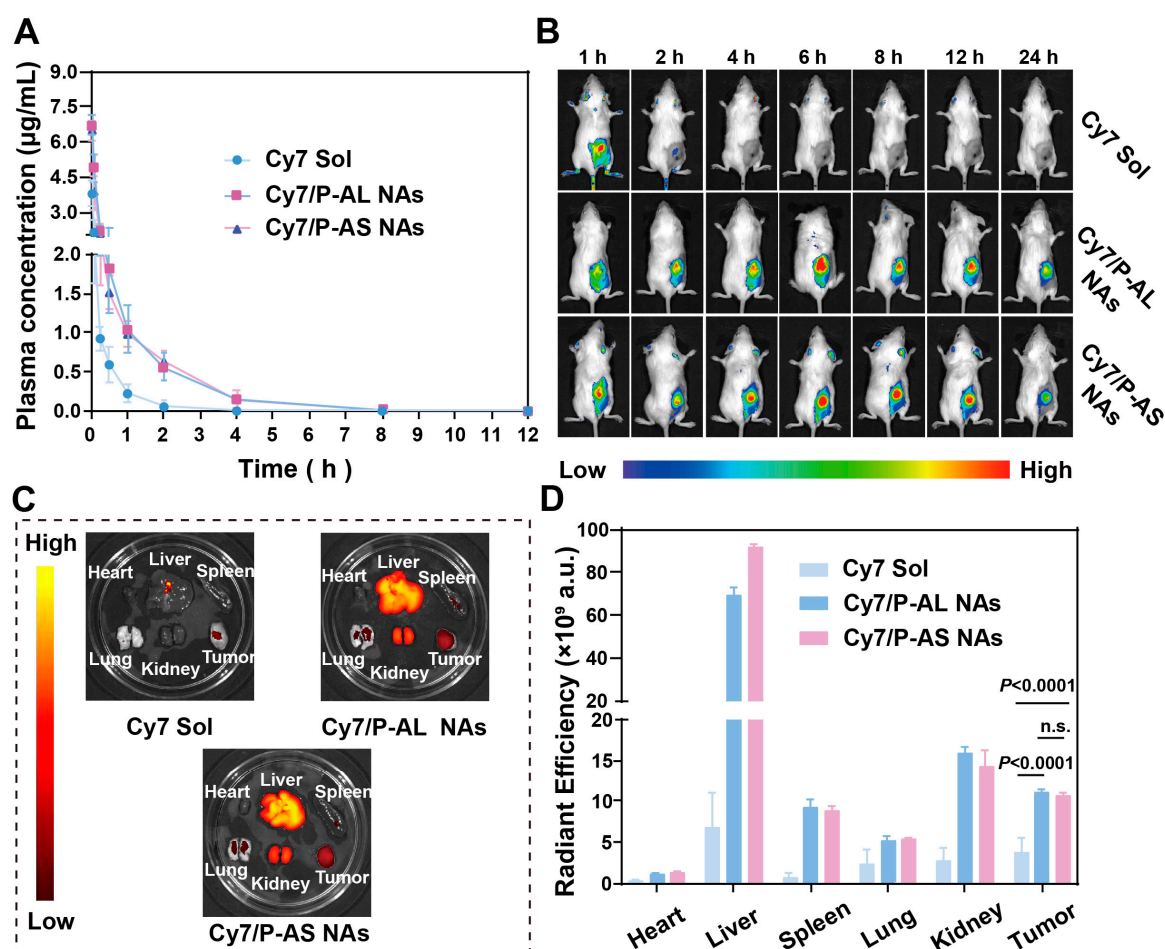


Figure 4. Pharmacokinetic properties and in vivo biodistribution. (A) Pharmacokinetic profiles of Cy7 Sol, Cy7/P-AL NAs and Cy7/P-AS NAs (administered at an equivalent Cy7 dose of 2 mg/kg) in vivo ($n = 5$); (B) in vivo fluorescence imaging of 4T1 tumor-bearing mice treated with Cy7 Sol, Cy7/P-AL NAs and Cy7/P-AS NAs (Cy7 equivalent dose of 2 mg/kg) in vivo during 24 h ($n = 3$); (C) ex vivo fluorescent imaging of major organs and tumors following of different formulations at 6 h after administration; and (D) quantitative analysis of fluorescence signal within principal organs and tumors at 6 h after administration ($n = 3$). Data are presented as mean $\pm$ SD. Two-way ANOVA with Tukey's multiple comparisons test was used for the analysis of data and adjusted p value. n.s. represents no significance.

3.7. In Vivo Antitumor Activities

We evaluated the in vivo antitumor efficacy and safety of P-AL NAs using a BALB/c 4T1-bearing mouse model for 10 days (Figure 5A). Tumors in saline-treated mice grew rapidly. ART-SA Sol and P-AS NAs showed poor antitumor activity, primarily due to the poor pharmacokinetic behavior of ART-SA and the inability of ART-SA to undergo lipid peroxidation in vivo, with steric hindrance from its long chain restricting activity. By contrast, ART-LA Sol induced lipid peroxidation to restore activity, similar to ART Sol, but both showed limited efficacy due to rapid clearance and insufficient tumor accumulation in vivo. Notably, P-AL NAs demonstrated significant tumor growth inhibition, exhibiting the smallest tumor volume and lowest incidence rate (Figures 5B,D and S17). Histological analysis showed maximal tumor damage (Figure 5G), attributed to the superior self-assembly of P-AL NAs, stability, and cellular uptake—properties that prolong circulation, enable EPR-mediated tumor accumulation, and optimize therapeutic delivery.

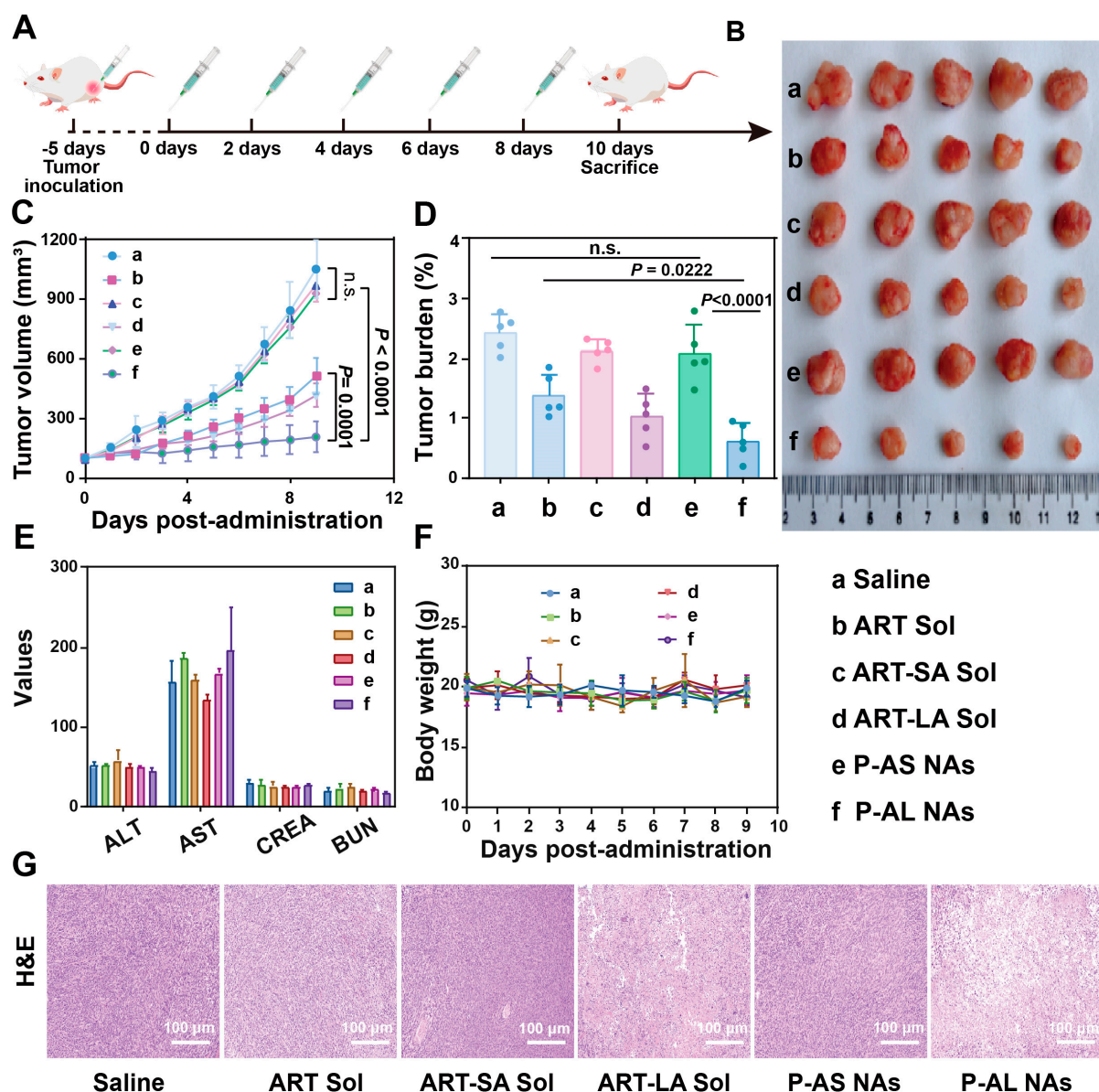


Figure 5. In vivo evaluation of antitumor effect. (A) Schematic diagram of drug administration schedule in tumor-bearing mice; (B) images of tumors after the last treatment ($n = 5$); (C) quantified tumor growth profiles across distinct treatments ($n = 5$); (D) tumor burden changes following therapeutic interventions ($n = 5$); (E) assessment of hepatic and renal function indices after the last treatment session ($n = 3$); (F) body weight fluctuations during the treatment period ($n = 5$); and (G) H&E staining images of tumors after various treatments (scale bar = 100 μm). Data are presented as mean $\pm$ SD. One-way ANOVA with Tukey's multiple comparisons test was used for the analysis of data and adjusted p value. n.s. represents no significance.

Building on the excellent in vivo antitumor efficacy of P-AL NAs, we further conducted an in-depth evaluation of its in vivo safety profile. As shown in Figure 5F, no notable variation in body weight was observed among the treatment groups throughout the experimental period. Post-treatment, major organs (heart, liver, spleen, lung, and kidney) were collected for H&E staining and pathological analysis. H&E sections showed no obvious morphological lesions in any organ (Figure S19). Additionally, hepatic and renal function assays confirmed that all relevant indicators remained within the normal ranges (Figures 5E and S18). Collectively, P-AL NAs exert therapeutic effects without causing damage to major organs or impairing hepatic/renal functions, demonstrating high antitumor activity with a favorable safety profile.

4. Conclusions and Discussion

To address the issues of poor bioavailability and tumor selectivity of ART caused by its unfavorable physicochemical properties, this study designed and synthesized a novel derivative, ART-linolenol alcohol (ART-LA), by conjugating LA to ART via an ester bond. The polyunsaturated double bonds in ART-LA undergo ROS-triggered chain scission within the tumor microenvironment, resulting in the formation of aldehyde intermediates with equivalent ART activity. Furthermore, ART-LA was formulated into NAs (P-AL NAs) through a one-step nano-precipitation technique, with its surface modified using 20% (*w/w*) DSPE-SS-PEG_{2K} to improve stability, tumor selectivity, and prolong systemic circulation. This work comprehensively evaluated the self-assembly capacity, colloidal stability, *in vitro* antitumor activity, pharmacokinetic behavior, *in vivo* biodistribution, and antitumor efficacy of the NAs. As expected, P-AL NAs exhibited favorable pharmacokinetic profiles and tumor selectivity, demonstrating great potential in effective and low-toxicity antitumor therapy. This strategy not only facilitates the clinical translation of ART but also provides a paradigm for developing diverse tumor therapies using ART-like natural products.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/pharmaceutics18020240/s1>. Figure S1: Schematic diagram of the oxidative cleavage mechanism of ART-LA. (A) The process of lipid peroxidation in ART-LA. (B) Degradation of lipid peroxides. Figure S2: The synthetic route of ART-LA: (a) DMAP, EDCI, 25 °C. Figure S3: The synthetic route of ART-SA: (a) DMAP, EDCI, 25 °C. Figure S4: (A) The high-performance liquid chromatogram of ART-LA. (B) The HPLC purity percentages of ART-LA. Figure S5: (A) The high-performance liquid chromatogram of ART-SA. (B) The HPLC purity percentages of ART-SA. Figure S6: (A) MS of ART-LA. $[M + Na]^+ = 655.42$. (B) ^1H NMR of ART-LA. Figure S7: (A) MS of ART-SA. $[M - H]^+ = 635.45$. (B) ^1H NMR of ART-SA. Figure S8: FTIR of ART, LA, SA, ART-LA, and ART-SA. Figure S9: Appearance of the preparation: (A) P-AL NAs, and (B) P-AS NAs. Figure S10: Molecular docking simulation of P-AS NAs. Figure S11: (A) The particle size of P-AL NAs varies under different DTT concentrations. (B) The particle size of P-AS NAs varies under different DTT concentrations. Figure S12: (A) The release curves of ART-LA in P-AL NAs under different DTT concentrations. (B) The release curves of ART-SA in P-AS NAs under different DTT concentrations. Figure S13: The effect on the antitumor activity of P-AL NAs (equal amounts of ART from 0 to 4000 nM) under the conditions with or without NAC (5 mM) ($n = 3$). Figure S14: Intermediates of ART-LA and ART-SA produced after lipid peroxidation at different times. Figure S15: Detailed mathematical statistical chart of pharmacokinetic parameters. (A) AUC_{0-12h} ; (B) MRT_{0-12h} ; (C) $T_{1/2}$. Figure S16: The quantitative tumor-to-organ fluorescence ratio of Cy7 Sol, Cy7/P-AL NAs, and Cy7/P-AS NAs at 6 h after administration ($n = 3$). Figure S17: Detailed mathematical plots of tumor volumes treated by different treatment groups at day 9. Figure S18: Serum biochemical analysis for ALT (A), AST (B), BUN (C), and CREA (D) indicators ($n = 3$). Figure S19: The H&E staining images of heart, liver, spleen, lung, and kidney after different treatments (scale bar = 100 μm). Table S1: Characterization of P-AL NAs and P-AS NAs. Table S2: Pharmacokinetic parameters of SD rats after various treatments ($n = 5$).

Author Contributions: C.L., X.Z. and H.W. conceived this work. H.W. and X.Z. prepared and characterized the materials. H.W., X.Z. and X.S. carried out the *in vitro*, *ex vivo*, and *in vivo* experiments. H.Z., W.Z., S.Z. and C.L. contributed technical support. H.W., X.Z., X.S. and H.Z. analyzed the data and discussed the results. H.W., H.Z. and C.L. wrote and edited the manuscript. C.L. supervised the project. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal studies were carried out in compliance with the Guidelines for Care and Use of Laboratory Animals of Shenyang Pharmaceutical University. The experimental protocols received prior approval from the Animal Ethics Committee of Shenyang Pharmaceutical University (Approval No: SYPU-2ACUC-S2024-0919-201, 19 September 2024).

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in this article. Further inquiries can be directed to the corresponding authors.

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