Drug Delivery and Translational Research
https://doi.org/10.1007/s13346-018-0534-4

ORIGINAL ARTICLE

Cytosine arabinoside prodrug designed to bind plasma serum albumin for drug delivery

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Abstract

Rational design of anticancer prodrugs for efficient albumin binding can show distinct advantages in drug delivery in terms of high drug availability, long systemic circulation, potential targeting effect, and enhanced chemotherapy effect. In the present study, we reported a cytosine arabinoside (Ara-C) prodrug which could well formulate in solution and instantly transform into long-circulating nanocomplexes by hitchhiking blood-circulating albumin after i.v. administration. Specifically, Ara-C was conjugated with an albumin-binding maleimide derivative, the resulting Ara-C maleimide caproic acid conjugate (AM) was well formulated in aqueous solution, conferring high albumin-binding ability in vitro albumin-binding studies. Moreover, in vivo fluorescence images of sulfo-cyanine5 maleimide indirectly demonstrated that AM showed better accumulation in tumors, exhibiting superior tumor targeting ability and antitumor activity compared to Ara-C. Such a uniquely developed strategy, integrating high albumin-binding capability, has great potential to be applied in clinical cancer therapy.

Keywords Cytosine arabinosid · Bovine serum albumin · Albumin binding · Prodrug

Introduction

Cytosine arabinoside (Ara-C), a pyrimidine nucleoside analogue, is an attractive therapeutic drug for the treatment of acute and chronic myeloblastic leukemias and also therapeutically effective for solid tumors [1–3]. However, Ara-C presents a series of drawbacks; it is more likely to be deaminated by deoxycytidine deaminase to inactive metabolite uracil arabinoside (Ara-U) in the systemic circulation, resulting in short half-life time ($t_{1/2}$ is about 10–15 min) [4]. Ara-C is administered by infusion or by high doses of i.v. administration in clinic, resulting in severe adverse effects [5]. Therefore, poor pharmacokinetic properties of Ara-C strongly restrict its clinical applications. Rational design of Ara-C prodrugs will be an effective strategy to improve its drug availability.

The major soluble protein in blood circulation is serum albumin, and currently, it acts as an attractive versatile carrier for tumor target drug delivery [6–8], such as the albumin-bound paclitaxel nanoparticle (Abraxane), which was approved for metastatic breast cancer, non-small cell lung cancer, and pancreas adenocarcinoma [9, 10]. Albumin has a long plasma half-life with 19 days because of the large molecular size 66.5 kD and neonatal Fc receptor-mediated recycling by avoiding renal clearance. Besides, albumin can target and accumulate in tumor regions via the enhanced permeability and retention (EPR) effect [11–13]. Furthermore, a number of tumors overexpress SPARC protein, which has high affinity with albumin. So it can facilitate albumin to accumulate in the tumors [14, 15]. In addition, serum albumin is the most abundant protein in the plasma (40 mg/mL), and about 70% of it has an accessible free thiol on Cys-34, which is a very unique property among the plasma compartments [16, 17]. Therefore, thiol-reactive molecules can precisely hitchhike to the endogenous albumin when they enter the blood stream.

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Published online: 10 May 2018

Since covalent-binding (6-maleimidocaproyl) hydrazone derivative of doxorubicin (DOXO-EMCH) was applied in clinical practice, the thiol-binding prodrug platform technology which exploit endogenous albumin as a drug carrier has been designed for anticancer drug delivery [18–21]. Inspired by these, we present an Ara-C maleimide prodrug (AM), not only to take advantage of chemically defined synthesis but also harness endogenous abundant albumin as drug carrier. Followed by the special transport mechanism of albumin in vivo, the albumin-prodrug conjugate can target and accumulate in tumor tissues [13, 16, 17] to elicit excellent antitumor effect (Fig. 1). The prodrug AM could rapidly react with the thiol of plasma albumin in blood to form albumin-prodrug conjugate after intravenous administration. Then, it can avoid rapid metabolism, achieve high stability in blood circulation, and increase tumor accumulation via albumin-mediating EPR effect and significantly facilitate the therapeutic efficacy.

Materials and methods

Materials

Cytosine arabinoside (Ara-C, 99.3%) was purchased from Surui Chemical Corporation (Suzhou, China); maleic anhydride (AR, 99%), 6-aminocaproic acid (EMC),1-(3-fimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI), and *N*-hydroxysuccinimide (NHS) were obtained from Aladdin Industrial Corporation (Shanghai, China) (AR, 99%); EMC-Cy5 (sulfo-cyanine5 maleimide) was purchased from Nanjing Bioorth Biotech Co., Ltd. (Nanjing, China). Bovine serum albumin (BSA; 98%) was purchased from HarveyBioGene Technology Co. Ltd. (Beijing, China) containing approximately 40% free thiol groups as assessed with Ellman's test. 4T1 cells were obtained from Nanjing kaiji Biotech. Ltd. Co. (Jiangsu, China). All other reagents and solvents were analytical or HPLC grade and were used without further purification.

Synthesis and purification of the prodrug AM

The albumin-binding Ara-C prodrug (AM) was synthesized by a two-step reaction. Firstly, 6-maleimidocaproic acid (EMC) was synthesized as described previously [17]. Maleic anhydride (3.92 g, 40 mmol) and 6-aminocaproic acid (5.25 g, 40 mmol) were refluxed in 70 mL acetic acid at 130 °C for 10 h. Acetic anhydride (3.78 ml, 40 mmol) was added dropwise and further reflux for 1 h. Upon completion, the reaction mixture was cooled to room temperature, and the solvent was removed with anhydrous toluene in high vacuum three times at 30 °C to obtain yellow solidified syrup. Then, the product was purified by silica column chromatography

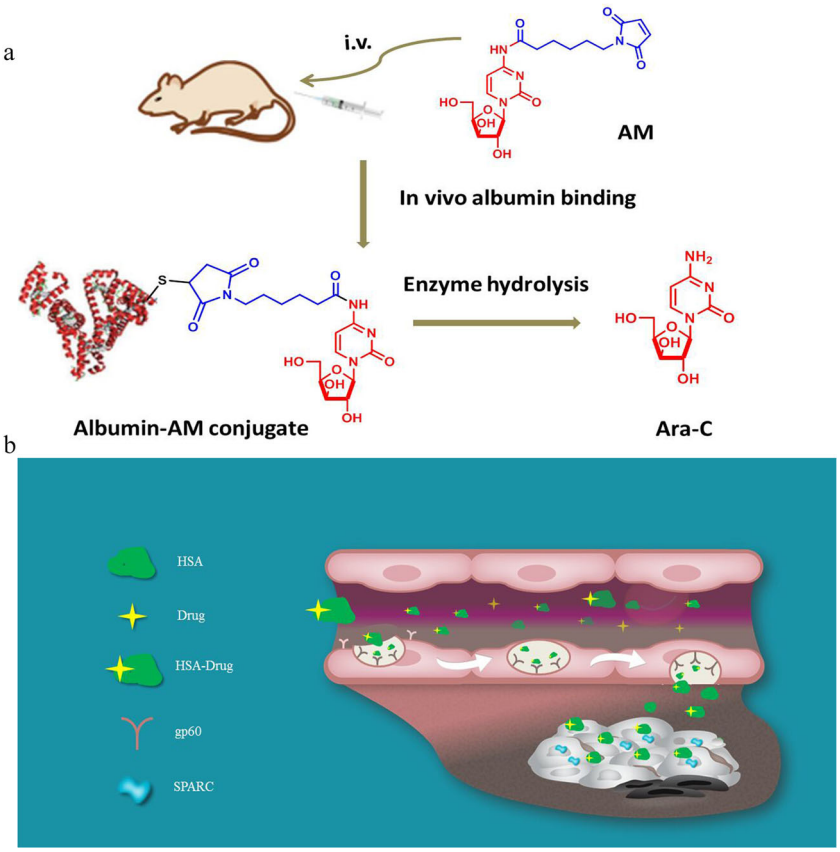

Fig. 1 Schematic diagram of albumin-binding Ara-C maleimide prodrug (AM) delivery strategy

(dichloromethane/methanol = 200: 1, *v*/*v*) affording white powder in 60% yield (5.07 g).

EMC: percent purity, 98%; ^{1}H-NMR (600 MHz, $CDCl_3$) 6.69(s, 2H), 3.51 (t, 2H,), 2.33 (t, 2H,), 1.65 (m, 4H), 1.33 (m, 2H); ESI-MS m/z 209.6 $[M-H]^-$.

Next, EMC (1.06 g, 5 mmol), NHS (0.575 g, 5 mmol), and EDCI (1.92 g, 10 mmol) were dissolved in 20 ml acetonitrile, stirred at 70 °C for about 8 h, then 8 ml Ara-C dimethyl formamide solution was added dropwise to the reactive mixture. The reaction was maintained under stirring for 6 h under nitrogen. TLC was utilized to monitor the reaction process and at the end of reaction, solvent was evaporated and the crude product was purified by silica gel column (dichloromethane/methanol = 100: 8, *v*/*v*) to obtain a purity of 98.3% product in 40% yield (0.873 g).

AM: percent purity, 98%; ^{1}H-NMR (600 MHz, DMSO): δ 10.78 (s, 1H), 8.03 (d, 1H), 7.2 (d, 1H), 6.9 (s, 2H), 6.04 (s, 1H), 5.48 (s, 2H), 5.06 (m, 1H), 4.06 (m, 1H), 3.92 (s, 1H), 3.82 (m, 1H), 3.60 (t, 2H), 3.39 (t, 2H), 2.49 (t, 2H), 1.55 (m, 4H), 1.46 (m, 2H); ESI-MS m/z $[M-H]^-$ 435.0; $[M+H]^+$ 437.2; $[M+Na]^+$ 459.1.

In vitro stability of Ara-C prodrug AM

The chemical stability of the prodrug AM was investigated in different pH phosphate buffers (pH 4.0, 7.4, and 10.0) [22]. Briefly, AM was incubated in 3 mL phosphate buffers at 37 °C for 24 h with a final concentration of 100 μg/mL. Then, 100 μL sample was withdraw at predesigned time intervals. The amount of non-degraded AM was determined by high-performance liquid chromatography (HPLC) and the concentration of AM was obtained by using standard curve.

Albumin-binding studies of AM with BSA

Prodrug AM (300 μM) was added to a solution of BSA (700 μM in PBS, pH 7.4) and incubated at 37 °C for albumin-binding studies, Then, 50 μL samples were directly analyzed using capillary electrophoresis (CE) after an incubation time of 1, 2, 5, 10, and 20 min. Samples were injected into the capillary by hydrodynamic flow at a height differential of 10 cm for 5 s. The prodrug AM and albumin were detected at 247 nm. Similarly, for blocking study, the BSA solution was preincubated with an excess EMC at 37 °C for 30 min before adding AM.

Capillary electrophoresis: CE for the investigation of albumin binding was performed on a CE apparatus (CL1030, Beijing Cailu Science Apparatus, China) equipped with a UV detector. An uncoated capillary (50 μm ID, effective separation length 40 cm) was used. Detection was performed online by UV absorption at 247 nm. The following conditions were used: running buffer 30 mM sodium phosphate, pH 7.4; separation voltage 20 kV; rinse prior to each analysis: separation buffer 2 min; rinse after each analysis 0.1 M NaOH 2 min, water 1 min.

Computational docking studies

In this study, the Msketch software was used to draw AM molecular structures which were defined as the ligands. The crystal structure of BSA (PDB ID: 3V03) was obtained from Protein Data Bank (http://www.rcsb.org) [23], and the protein molecular was defined as the receptor. The "active site" was defined to include any amino acid closer than 10 Å from Cys-34. Sequence alignment was performed in Discovery Studio 3.0 to obtain the best-fit alignment. As a next step, the ligands were separately docked in HSA protein by using AutoDock 4.0 software package. The Auto Docking parameters were as follows: the energy evaluation was set to 25,000,000 per run, and the number of docking runs was 500. All other parameters were set as default values. The conformations with more negative values of the binding energy were superimposed on to reference ligands after running the AutoDock 4.0. Then, the results were sorted from low to high according to the amount of energy. The program Discovery Studio 4.0 was applied to calculate the root-mean-square deviation (RMSD) of a selected element by least-square fitting the structure to the reference structure. At last, the docking results and the schematic plan of the receptor-ligand interactions were analyzed by Discovery studio 4.0 software.

Preparation of the albumin-prodrug (BSA-AM) conjugate

The preparation method of the BSA-AM conjugate was established consistently with the above albumin-binding studies, just incubating compound AM (300 μM) with BSA (700 μM) in PBS (pH 7.4) for 2 h until no free prodrug could be detected by CE. Then, the solutions were collected and freeze-dried directly to obtain BSA-AM conjugate as white powder. The samples were kept at − 80 °C and thawed prior to use.

Fluorescence and circular dichroism spectra of the albumin-AM conjugate

For fluorescence spectroscopic study, BSA and BSA-AM conjugate were prepared in phosphate buffer (pH 7.4) with the concentration of 1 μM [24]. Then, 200 μL of the above solutions were taken in a micropipette to 96-well plate and the measurement was performed on Model 500 Microplate Reader (BioRed, USA). The spectrum was recorded in the range from 300 to 500 nm using excitation wavelength of 280 nm, with the silt widths set at 5 nm.

For circular dichroism (CD) measurements, all circular dichroism spectra were recorded from 190 to 250 nm at 1-nm

intervals on a BioLogic MOS450 CD spectrometer (French) at room temperature (25 °C). BSA and BSA-AM conjugate were prepared in phosphate buffer (pH 7.4) and diluted to 1 μM [25–27].

Tumor accumulation of EMC-Cy5

One hundred microliters PBS of 4T1 cells (1×10^6 cells per mouse) was transplanted subcutaneously into the right flank region of 6-week-old female BALB/c mice. When the tumor volume reached 400 mm^3, free Cy5 and EMC-Cy5 were administrated via the tail vein at a dose equivalent to 2 mg/kg of Cy5, for which the Ara-C moiety of AM had been substituted with the Cy5 NIR dye for ex vivo imaging. The mice were killed at 4, 24, 48, and 72 h post-injection, and the main tissues of mice were collected for ex vivo fluorescence imaging evaluation using an IVIS lumina series III pre-clinical in vivo imaging system (PerkinElmer, Massachusetts, America).

In vivo antitumor effect

One hundred microliters PBS of 4T1 cells (1×10^6 cells per mouse) were transplanted subcutaneously into the right flank of 6-week-old female BALB/C mice. When the tumor reached 150–200 mm^3, the 4T1 tumor-bearing mice were randomly divided into three groups ($n = 5$). Then, the mice were treated with saline (control), Ara-C, and AM solutions at a dose equivalent to 20 mg/kg of Ara-C five times every other day via tail vein. (Ara-C and AM was dissolved in 0.9% NaCl solution). Body weight and tumor volume ($V = w^2 \times l/2$, where w and l are the width and length of tumor as measured by caliper) were recorded every day. At the end of experiment, the tumors were isolated and weighed. The major organs (heart, liver, spleen, lung, and kidney) were harvested for histopathological analyzing via hematoxylin and eosin (H&E) staining.

Experimental animals

The female BALB/C mice (18–22 g) were supplied by the Animal Centre of Shenyang Pharmaceutical University (Shenyang, China). All animal experiments were performed in compliance with the Animal Management Rules of the Ministry of Health of the People's Republic of China (Document no. 55, 2001) and the guidelines for the Care and Use of Animals approved by Shenyang Pharmaceutical University Committee of Ethics.

Statistical analysis

All the experiments performed were repeated at least three times, and the quantitative data were expressed as mean ± SD. Comparison between groups was performed with Student's t test and $p < 0.05$ was considered statistically significant.

Results and discussion

Synthesis and characterization of AM

The prodrug AM was successfully synthesized by conjugating maleimide to Ara-C via amide bond according to the synthetic routes in Fig. 2. The ^{1}H-NMR spectra in Fig. 3 confirmed the successful synthesis of the conjugate. Compared to the ^{1}H-NMR spectra of AM with EMC and Ara-C, the peaks of EMC and Ara-C could be found in the AM spectrum. And the peak of $-NH_2$ at δ 7.06 was vanished, and a new peak of AM (–CONH–) appeared at δ 10.78, demonstrating the successful synthesis of prodrug AM.

Stability and albumin-binding studies

The chemical stability of the prodrug AM in different pH phosphate buffers (pH 4.0, 7.4, and 10.0) was monitored over 24 h using analytical RP-HPLC. The prodrug AM was relatively stable at pH 4.0 and pH 7.4, showing slow hydrolysis (see Fig. 4g). However, it was rapidly hydrolyzed at pH 10.0 with about 90% degradation after 2 h incubation.

To investigate the binding ability of AM to albumin, AM (300 μM) were incubated in PBS (pH 7.4) at 37 °C with BSA (700 μM). The samples were directly analyzed by CE after an

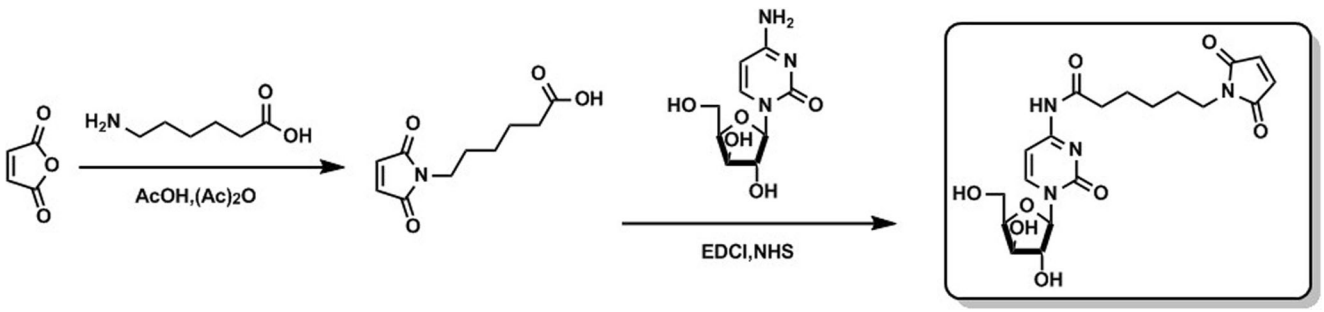

Fig. 2 Synthetic routes and chemical structures of AM

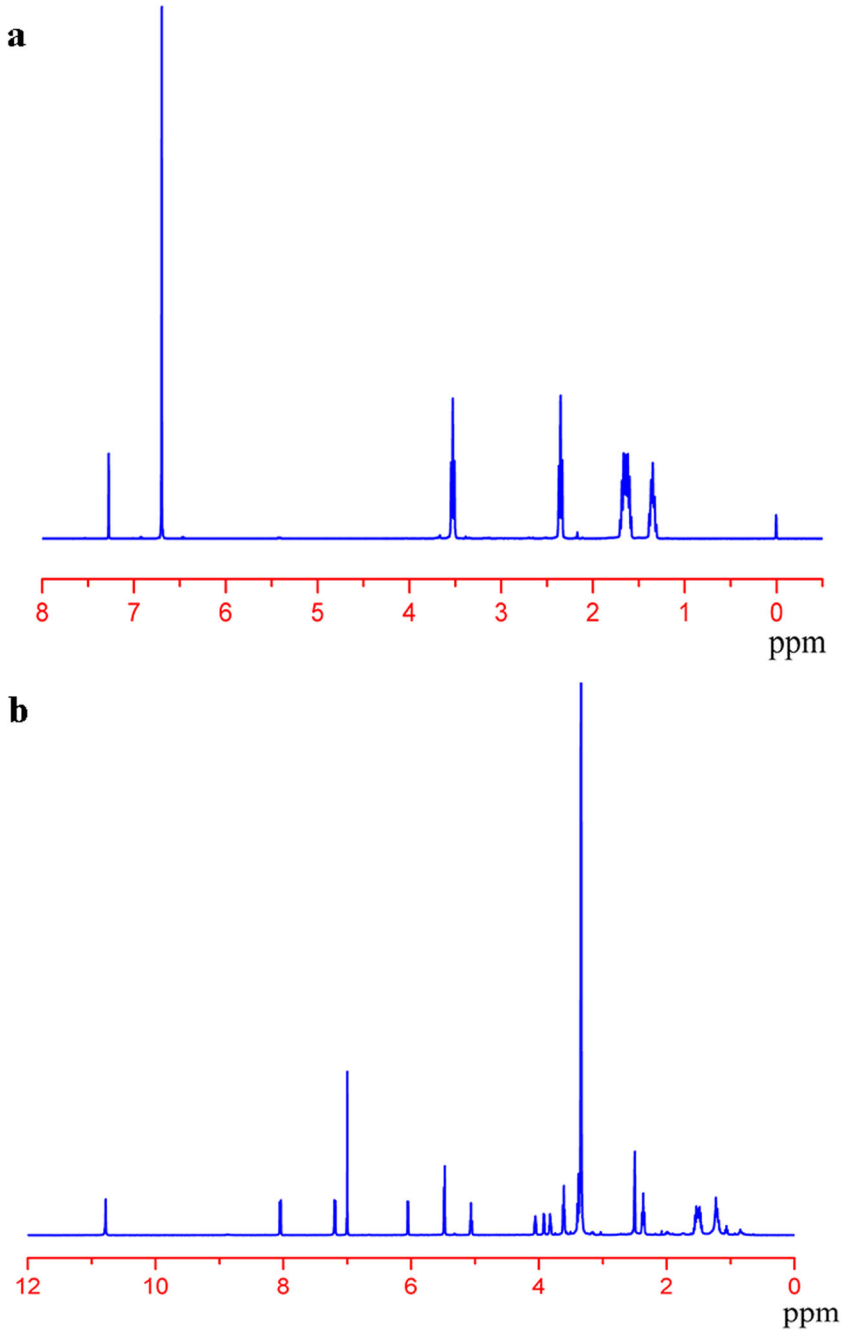

Fig. 3 ^{1}H-NMR spectra of **a** EMC and **b** AM

incubation time of 1, 2, 5, 10, and 20 min. The residual amount of AM was depicted in Fig. 4f, and the electropherograms recorded at 2 and 20 min after incubation with BSA are also shown in Fig. 4a, b. Approximately 60% of AM were bound to BSA within the first minute and after 20 min, less than 10% of the free prodrug remained (Fig. 4f). However, when the thiols of BSA were blocked with an excess of EMC, negligible binding to BSA could be detected after incubating AM with BSA for 20 min (Fig. 4c), demonstrating that the Michael addition between the sulfhydryl group of albumin and the maleimide group of AM led to the covalent-binding process.

Computational docking studies

Molecular docking has widely gained applications in structural molecular biology and computer-assisted drug design [22, 23, 28]. In this study, the molecular simulation experiment was performed by using the Discovery Studio 3.0 in order to furnish theoretical analysis about energy variation of the BSA-

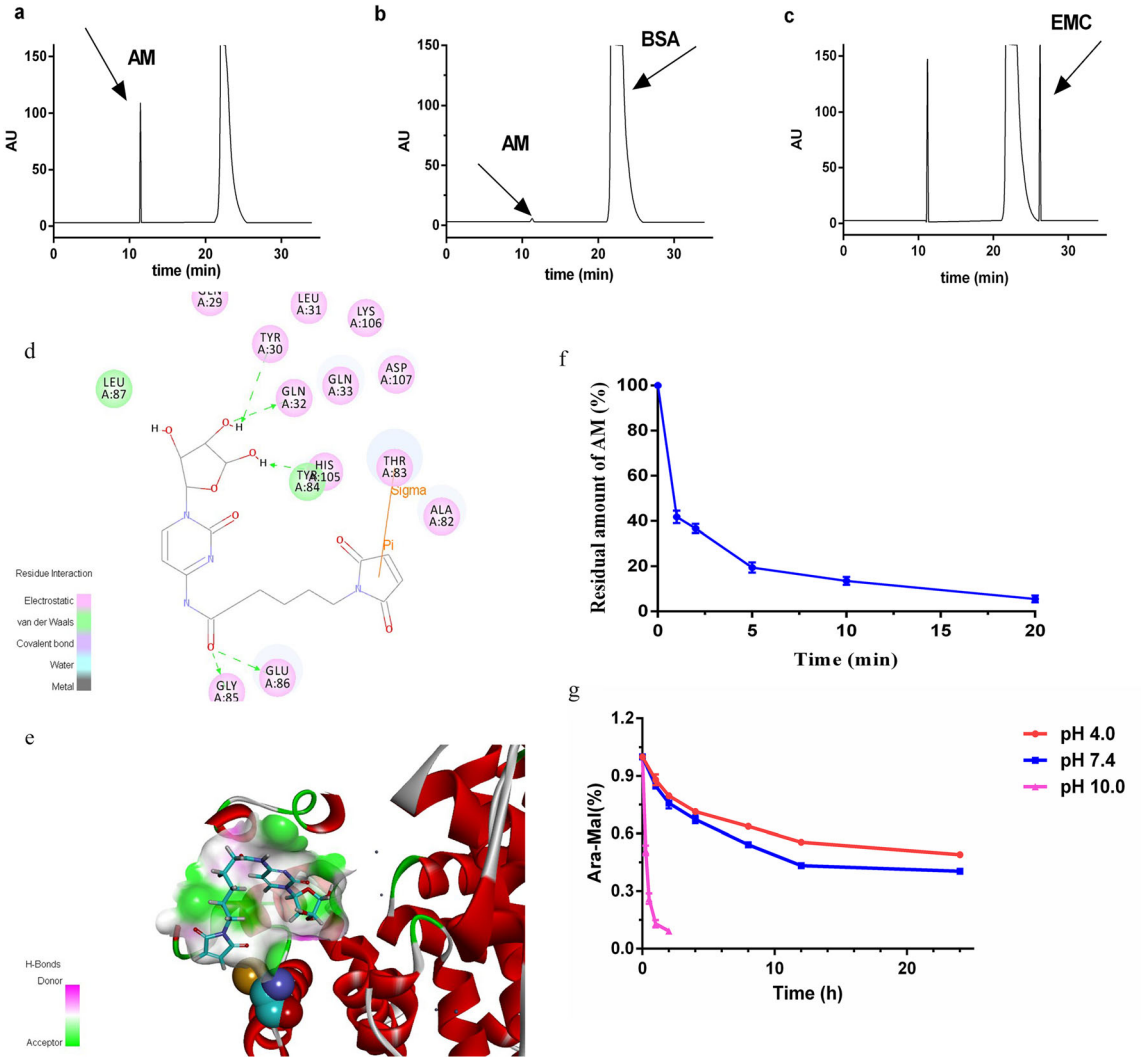

Fig. 4 Albumin-binding studies of AM. CE chromatograms of AM incubated with bovine serum albumin for **a** 2 min, **b** 20 min, and **c** after a 20-min incubation with bovine serum albumin which is preincubated with an excess of 6-maleimidocaproic acid. **d**, **e** Docking of AM inside the Cys-34 subdomain of BSA. The model structure of BSA (with PDB entry 3V03 as the template) is displayed as a solid ribbon colored by secondary structure. The weak H-bond is displayed as blue dotted lines, the ion-dipole H-bond is displayed as green dotted lines, and the π-π interactions is displayed as yellow dotted lines. **f** Decrease of free AM after incubation with BSA at 37 °C and pH 7.4. **g** Stability profiles of AM in PBS with different pH values. Data are presented as the mean ± SD ($n = 3$)

drug complex and to evaluate the binding affinity on a molecular level. As the best ranked docking poses, the binding energy which contained intermolecular energy and torsional free energy was minimized and provided the substrate in an optimal orientation for non-covalent-binding. From a thermodynamic point of view, a negative free energy ($\Delta G < 0$) indicates a favorable interaction system. The calculated binding energy values were − 5.7 kJ/mol for AM, demonstrating that prodrug AM had strong affinity to BSA. Moreover, because of the long-chain lipophilic linkage and the maleimide group, the hydrogen bond and Van-der Waal forces significantly contributed to stabilization of the conformations of the protein-prodrug complex (Fig. 4d, e). As a result, the structural analysis indicated that the 34-cysteine subdomain of BSA had a unique binding ability to AM which was favorable for the formation of the covalent bond between the prodrug and BSA. The trend in the binding affinities was well consistent with the results of the albumin-binding studies presented above.

Preparation and characterization of the albumin-AM conjugate

AM could bind albumin quickly in PBS at 37 °C according to the above binding studies, so we prepared the albumin-AM conjugate for subsequent experiments. The intrinsic fluorescence spectrum of BSA-AM was analyzed to determine if AM conjugation can alter the conformation of BSA around the

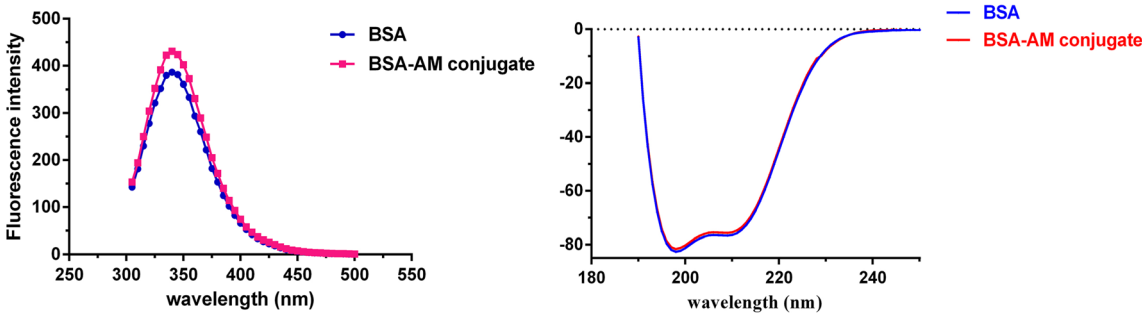

Fig. 5 Fluorescence emission spectra (left) and CD spectra (right) of BSA and BSA-AM conjugate

tryptophan residue (Trp 214). Figure 5 showed that upon excitation at 280 nm, the native BSA and the conjugate BSA-AM had the same maximum emission intensity at 340 nm and the nearly identical emission profiles, indicating no quench of the intrinsic fluorescence of Trp-214 residue. This demonstrated that conjugation with the prodrug AM did not affect the conformation of the hydrophobic binding pocket in the second a-helix domain of BSA.

Circular dichroism (CD) has been extensively utilized to determine the conformational change of BSA structure. There are two negative bands at 208 and 222 nm which correspond to π-π^* and n-π^* transition and are performance features of α-helical structures of BSA. So, to explore if there was some influence on the secondary structure of BSA when covalently conjugating with the prodrug, circular dichroism measurement was performed for native BSA and BSA-AM conjugate. As shown in Fig. 5, circular dichroism spectra of the BSA-prodrug conjugates indicated no obvious changes in the 190–250 nm regions especially in the negative bands at about 208 and 220 nm, indicating that AM had little effect on the secondary structure of BSA.

Tumor accumulation of EMC-Cy5

We then used a probe which had the same maleimide structure but Ara-C moiety substituted with an NIR dye Cy5 to explore the in vivo biodistribution of the prodrug following i.v. administration in tumor-bearing 4T1 mice. When the tumor volume reached about 400 mm^3, the mice were administrated intravenously with free Cy5 and EMC-Cy5 solutions. At predetermined time points, mice were sacrificed and the major organs and tumors were harvested for ex vivo imaging. As shown in Fig. 6, compared with free Cy5, the EMC-Cy5 solution exhibited dramatically enhanced fluorescence signal in tumors from 4 to 72 h. At 72 h post-administration, the fluorescence level in the tumor was even maintained in the EMC-Cy5 group, while very small amount of fluorescence signal could be seen in the tumor in the free Cy5 group. These results indirectly indicated that in the long systemic circulation, the albumin-prodrug adducts could successfully facilitate the intratumor accumulation of AM via albumin-mediating EPR effects and active target in vivo.

In vivo antitumor study

Encouraged by the excellent in vitro albumin-binding capacity and efficient tumor accumulation, prodrug AM was further investigated for tumor therapy in a 4T1 tumor-bearing xenograft model. As shown in Fig. 7a, compared with PBS group, Ara-C showed a moderate tumor-inhibiting ability. As expected, the mice treated with AM achieved the most significant antitumor activity, with barely growth in tumor volume. In

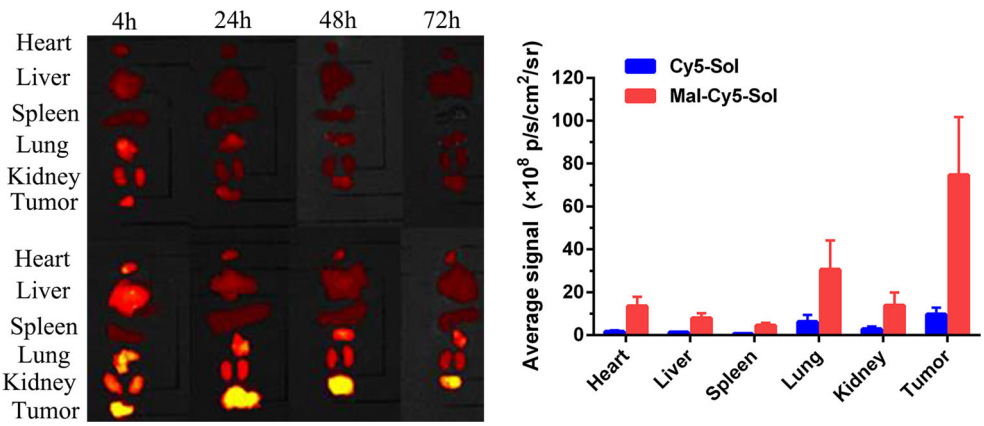

Fig. 6 Ex vivo biodistribution of free Cy5 and Mal-Cy5 IVIS spectrum small-animal in vivo imaging system. Fluorescent imaging (left) and quantitative results (right) of relative organ and tumor accumulation at 4, 24, 48, and 72 h

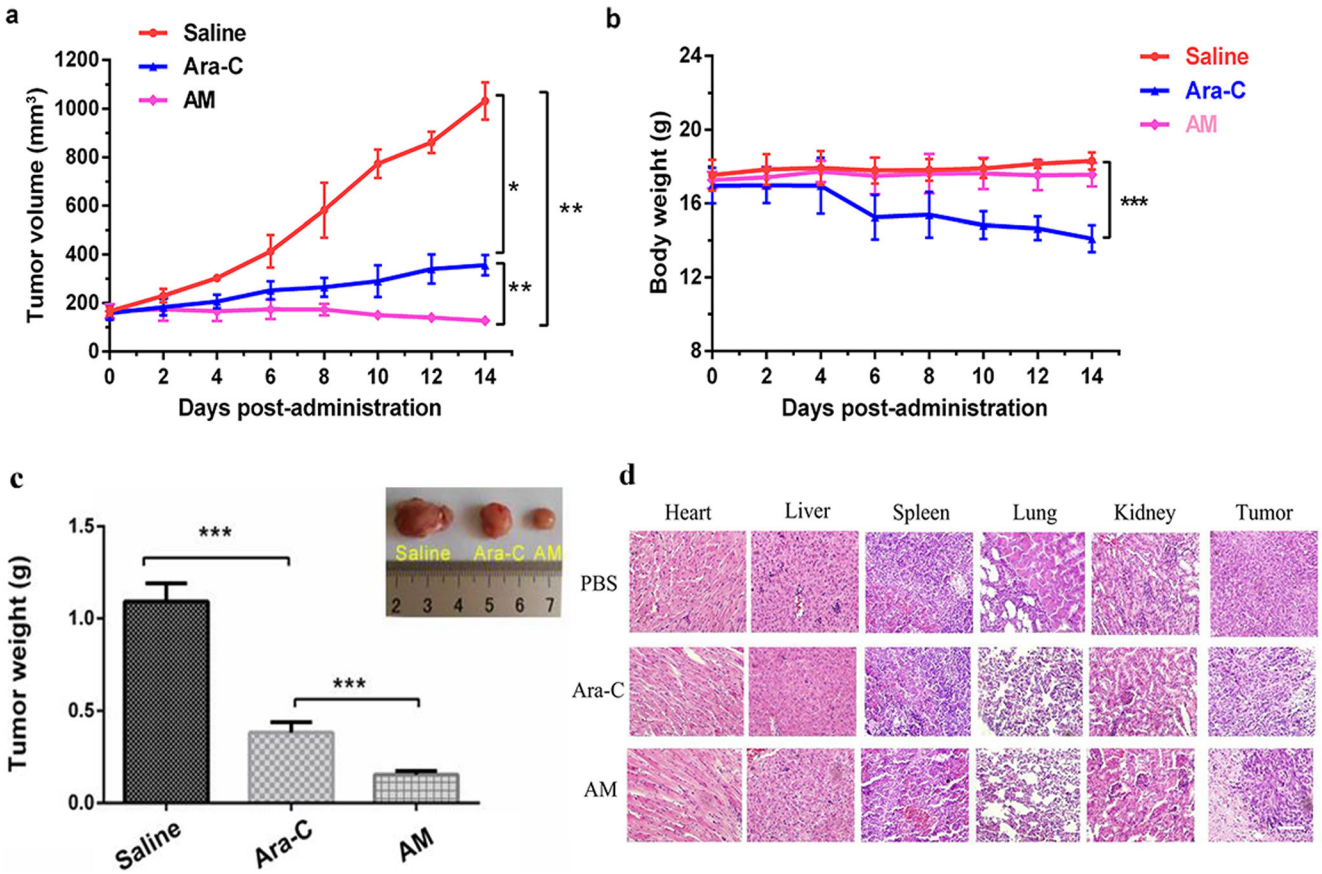

Fig. 7 The results of in vivo antitumor studies. **a** Tumor growth profiles treated with different formulations. **b** Body weight changes in BABL/C mice bearing 4T1 cells every other day. **c** Tumor weight and representative images of excised tumors on day 14. **d** H&E staining of the major organs and tumors after treatments. Scale bar represents 500 μm. Data are presented as the mean ± SD ($n = 3$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

addition, mice treated with Ara-C suffered from serious weight loss (***$p < 0.001$), but no obvious change in body weight was observed in AM-treated mice (Fig. 7b). The results suggested that the albumin-binding prodrug AM had preferable antitumor activity.

At the end of the study on 14th day, the excised tumors were weighed and calculated the inhibition rates (Fig. 7c). The mean tumor weight of saline, Ara-C, and AM groups was of 1.09 ± 0.10, 0.38 ± 0.060, and 0.15 ± 0.021 g, respectively, corresponding to the inhibition rate of $65.04 \pm 5.13\%$ (Ara-C group) and $85.98 \pm 1.88\%$ (AM group). Next, as shown in Fig. 7, H&E-stained pathological section results showed that obvious tumor metastasis was observed in the liver and lung of mice treated with PBS, but no metastatic lesion was found in Ara-C and AM groups. The potent antitumor efficacy of the prodrug AM could be attributed to the following distinct advantages: (i) quickly bind with plasma albumin after intravenous administration, (ii) achieve long blood circulation by forming BSA-AM conjugate, (iii) show high stability in blood circulation, and (iv) increase tumor accumulation via albumin-mediating EPR effect and active target in vivo. It is known that the anticancer efficacy of chemotherapy is greatly limited by short blood circulation and poor tumor selectivity. Thus, anticancer prodrugs with prolonged systemic circulation, tumor-specific distribution could significantly strengthen the chemotherapy efficacy. In this work, we propose such a uniquely developed strategy, integrating albumin-binding capability to achieve prolonged blood circulation and high intratumor accumulation, will have great potential to be applied in clinical cancer therapy.

Conclusions

To enhance the stability and antitumor efficacy of the anticancer drug Ara-C, the albumin-binding prodrug AM was prepared to employ endogenous albumin as drug carrier for delivery. The results suggested that the novel conjugate AM could rapidly and selectively bind to the cysteine-34 position of endogenous albumin. Furthermore, AM exhibited superior antitumor efficacy to that of Ara-C with less side effects. Such an albumin-based prodrug strategy is a promising method in the rational design of antitumor drugs for efficient chemotherapy.

Funding information This work was financially supported by the National Basic Research Program of China (973 Program, No. 2015CB932100) and the innovation team project of education department of Liaoning province (LT 2014022).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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