Preparation and Biological Evaluation of Tumor-Specific Ara-C Liposomal Preparations Containing RGDV Motif

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ABSTRACT: Arginine–glycine–aspartate (RGD) has been shown to be essential for the recognition of integrins overexpressed in tumor cells, especially during tumor invasion, angiogenesis, and metasis. In this study, a novel tetrapeptide, RGD–valine (RGDV), was designed and attached to the N position of 1-β-D-arabinofuranosylcytosine (Ara-C) at the valine end, as a homing device for the delivery of Ara-C to tumor cells. Furthermore, fatty acids of various chain lengths ($C_nH_{2n+1}COOH$, n = 7, 9, 11, 13, and 15) were attached to the arginine end of RGDV to form a series of $C_nH_{2n+1}CO$–RGDV–Ara-C compounds. The structures of $C_nH_{2n+1}CO$–RGDV–Ara-C compounds were confirmed using mass spectrometry and nuclear magnetic resonance. The liposomal preparations of the synthesized $C_nH_{2n+1}CO$–RGDV–Ara-C compounds were obtained using the film dispersion method in the presence of phospholipids. The particle size, zeta potential, and dispersity index of the liposomes formed were found to be approximately 215 nm (diameter), approximately −30 mV, and <0.3, respectively. The antitumor activity of the liposomal preparations containing the respective $C_nH_{2n+1}CO$–RGDV–Ara-C compounds was evaluated in mice inoculated with sarcoma S_{180}. Liposomal Ara-C preparation, liposomal $C_{11}H_{23}CO$–V–Ara-C preparation, Ara-C, and $C_{11}H_{23}CO$–V–Ara-C sodium carboxymethyl cellulose (CMC-Na) suspensions were used as controls. $C_nH_{2n+1}CO$–RGDV–Ara-C containing liposomal preparations were shown with an enhanced antitumor activity, likely because of the targeting effect of RGDV. © 2012 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 101:4559–4568, 2012

Keywords: drug delivery systems; drug targeting; liposomes; prodrugs; 1-β-D-arabinofuranosylcytosine (Ara-C); RGD; antitumor

INTRODUCTION

Although drugs may cross cell membranes by various mechanisms such as passive diffusion, carrier-mediated transport, and phagocytosis, most drugs rely on passive diffusion. Consequently, drug's solubility characteristics are essential for drug absorption.[1] Drugs of high hydrophilicity are usually associated with a poor membrane-penetrating ability, whereas drugs of high lipophilicity generally have a poor affinity for biological fluids resulting in poor distribution. Drugs with a balanced solubility are, therefore, preferred.

Cytarabine (1-β-D-Arabinofuranosylcytosine, Ara-C, Fig. 1), a pyrimidine analogue, is an antimetabolic antitumor agent. It has been used for the treatment of leukemia for over 40 years. It is also used for acute nonlymphocytic leukemia. However, Ara-C suffers from a low bioavailability due to its poor lipophilicity. Following absorption, Ara-C undergoes deamination resulting in 1-β-D-arabinofuranosyluracil, which is inactive. It is also known that solid tumors are not sensitive to Ara-C.[2]

Various strategies have been explored to improve the effectiveness of anticancer agents including Ara-C. There have been many reports wherein chemical modifications of Ara-C have been attempted and various moieties have been attached to the NH_2 and/or 3′, 5′-OH groups[3–9] among which are Enocitabine and N-palmitoyl-Ara-C. It has been reported that the

Additional Supporting Information may be found in the online version of this article. Supporting Information

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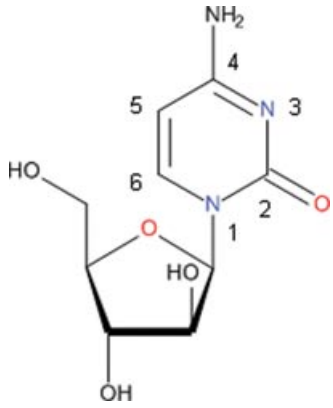

Figure 1. The structure of Cytarabine (1-β-D-Arabinofuranosylcytosine or Ara-C). Systematic (IUPAC) name: {4-amino-1-[(2R,3S,4R,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl] pyrimidin-2-one}.

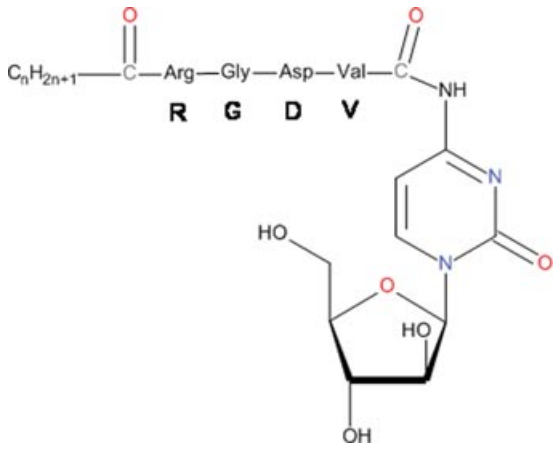

Figure 2. General structure of $C_nH_{2n+1}CO$–RGDV–Ara-C compounds synthesized.

introduction of fatty acids into Ara-C resulted in generally improved antitumor activities.[10]

It is known that the growth of many types of cancer is closely related to the integrity of integrins,[11,12] a group of transmembrane proteins that mediates the attachment between a cell and the tissues surrounding it including other cells or the extracellular matrix. Integrins are heterodimers containing two distinct chains named α- and β-subunits,[13,14] and play an important role in tumor invasion and metastasis, a complex multistage process involving tumor–host interaction, which includes adhesion, angiogenesis, and proteolysis. Increased expression of integrins facilitates the adhesion of tumor cells to the endothelial linings of blood vessels to colonize host organs.[15] It is known that tripeptide, arginine–glycine–asparate (Arg–Gly–Asp, RGD), is essential for the recognition of integrins. Synthetic inhibitors that contain an RGD motif demonstrated an inhibitory effect on the adhesion and angiogenesis of tumor cells.[15–20] RGD, however, is also known to promote the detachment of invasive tumor cells from the primary tumor site leading to the colonization of other organs.[21,22] Such dual and opposite effects on the progression of tumor limit the therapeutic potential of RGD in cancer treatment. Nevertheless, elevated expression of integrins in many types of tumor is well documented.[23,24] RGD's affinity for integrins has led researchers to investigate the possibility of using RGD to achieve site-specific delivery of liposomes by conjugating RGD to phospholipids and polyethylene glycols in the liposomes.[20,25] However, studies so far are nonconclusive.

Studies previously reported by this group revealed that fatty acid–amino acid–Ara-C compounds were more effective than Ara-C in solid tumors.[26] This study is a natural progression of the previous work. A novel tetrapeptide, RGD–valine (RGDV), was designed as a tumor-specific homing device and attached to the N^4 position of Ara-C via valine, which was added to the RGD motif. It has been demonstrated that nonpolar amino acids flanking the RGD moiety increased the binding affinity of RGD to integrins[27,28] and valine is a hydrophobic amino acid. In addition, fatty acids of various chain lengths ($C_nH_{2n+1}COOH$) were attached to the arginine end of RGDV to form a series of $C_nH_{2n+1}CO$–RGDV–Ara-C compounds (Fig. 2). As such, the amine group on Ara-C is protected from deamination, whereas RGDV is used for tumor recognition. Liposomal systems of anticancer drugs have been marketed and shown with reduced toxicity.[29,30] The fatty acids in the design were used to improve the overall lipophilicity of the compounds synthesized so that they can be easily incorporated into liposomes.

MATERIALS AND METHODS

Materials

All chemicals were of chemical grade unless otherwise specified. Ara-C was obtained from Shanghai Hanhong Chemicals and Technology Company, Ltd. (Shanghai, China). Caprylic acid [$CH_3(CH_2)_6COOH$], decanoic acid [$CH_3(CH_2)_8$-COOH], lauric acid [$CH_3(CH_2)_{10}$-COOH], myristic acid [$CH_3(CH_2)_{12}$-COOH], and palmitic acid [$CH_3(CH_2)_{14}$-COOH] were purchased from Beijing Chaoyang District Xudong Chemical Plant (Beijing, China). All amino acids were of L-configuration and were obtained from Sichuan Sangao Biochemical Company Ltd. (Chengdu, Sichuan, China). Dicyclohexylcarbodiimide (DCC), 1-ethyle-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl), 1-hydroxybenzotriazole (HOBt), N-alpha-*tert*-butyloxycarbonyl-N-gamma-nitro-L-arginine [Boc–Arg(NO_2)OH], H–Gly–OH, H–Asp($OBzl)_2$, and Boc–Val–OH were from GL Biochem Ltd. (Shanghai,

China). Acetonitrile (chromatography grade) was from the Fisher Scientific China (Beijing, China). Water was triple distilled. Sodium chloride solution for injection and sodium carboxymethyl cellulose (CMC-Da) were from Qidu Pharmaceuticals Company, Ltd. (Zibo, Shandong, China). Egg lecithin (molecular weight = 750 Dalton) was purchased from Acros Organics (Geel, Belgium). Cholesterol was purchased from Beijing Aoboxing Biotech Company Ltd. (Beijing, China).

Laborota 4003 rotary evaporator was from Heidolph Instruments (Schwabach, Germany). Water circulating vacuum pump SHB-III was from Zhenzhou Great Wall Scientific Industrial and Trade Company Ltd. (Henan, China). Transmission electron microscope (TEM), JEM-1230, was purchased from JEOL (Tokyo, Japan). Zetasizer Nano ZS-90 with DTS v.4.00 software was purchased from Malvern Inc. (Worcestershire, United Kingdom). Waters 2695 HPLC (Milford, Massachusetts) with C18 (4.6 × 250 mm^2, 5 μm; Capcell Pak (Shiseido, Tokyo, Japan) was used for quantitative analysis and Waters 600 Multisolvent Delivery System with C18 (20 × 250 mm^2, 5 μm; Capcell Pak (Shiseido, Tokyo, Japan) was used for preparation purposes. Magnetic stirrer S21-2 was from Shanghai Sile Instrument Company, Ltd. (Shanghai, China). Nuclear magnetic resonance (NMR) was recorded using an Advance II 500 NMR instrument (Bruker, Fällanden, Switzerland) and mass spectrometry (MS) data were obtained using a Waters Micromass Quattro Micro 2000 API LC–MS–MS system (Milford, Massachusetts). Melting point was determined using a microscopic melting point apparatus (XT5) from Beijing Keyi Electro-optic Instrument Plant (Beijing, China). Optical rotation was measured using a P-1020 Polarimeter from Jasco (Tokyo, Japan). Disposable 1.0 mL sterile syringes were purchased from Shanghai Kangshou Medical Devices Company, Ltd (Shanghai, China).

Animal experiments were carried out according to a protocol approved by the Experimental Animal Care Committee of Capital Medical University, Beijing, China. Male Kunming mice (18–22 g) from the Animal Services of Capital Medical University were supplied with food and water *ad libitum*.

Synthesis and Characterization of $C_nH_{2n+1}CO$–RGDV–Ara-C

According to a previously reported method,[26,31] $CH_3(CH_2)_nCO$–RGD and H–V–Ara-C were first synthesized, and the two parts were then condensed to produce $CH_3(CH_2)_nCO$–RGDV–Ara-C as illustrated in Figure 3. Using dimethylformamide (DMF) as solvent, Boc–Arg(NO_2)–OH reacted with glycyl–benzyl ester (H–Gly–OBzl) to yield Boc–Arg(NO_2)–Gly–OBzl in the presence of DCC and HOBt. After selective deprotection, N-gamma-nitro–L-argininyl–glycyl–benzyl ester [H–Arg(NO_2)–Gly–OBzyl] was obtained. $C_nH_{2n+1}COOH$ and Arg(NO_2)–CO–Gly–COOBzl were condensed in the presence of DCC, HOBt, and N-methylmorpholine (NMM) to yield $C_nH_{2n+1}CO$–Arg(NO_2)–CO–Gly–COOBzl, which was subsequently subject to partial deprotection to remove the benzyl group to obtain $C_nH_{2n+1}CO$–Arg(NO_2)–Gly–COOH. $C_nH_{2n+1}CO$–Arg(NO_2)–Gly–COOH was then condensed with H–Asp(COOBzl)–COOBzl in the presence of DCC, HOBt, and NMM to yield $C_nH_{2n+1}CO$–Arg–Gly–Asp(COOBzl)–COOBzl. The protective benzyl ester groups in $C_nH_{2n+1}CO$–Arg–Gly–Asp(COOBzl)–COOBzl were removed via hydrogenation (Pd/C) to yield $C_nH_{2n+1}CO$–Arg–Gly–Asp(COOH)–COOH (or $C_nH_{2n+1}CO$RGD).

In a separate experiment, the N-terminus protected valine (Boc–Val–COOH) was conjugated with Ara-C in the presence of EDC, HOBt, and NMM in anhydrous DMF as solvent to yield Boc–Val–CO–Ara-C).[26] The protective Boc group was then removed to yield H–Val–CO–Ara-C.

Finally, $C_nH_{2n+1}CO$–Arg–Gly–Asp(COOH)–COOH was conjugated with H–Val–CO–Ara-C to yield $C_nH_{2n+1}CO$–RGDV–Ara-C. Because there are two COOH groups on aspartate (D), two series of isomers for each of $C_nH_{2n+1}CO$–RGDV–Ara-C compounds as illustrated in Figure 4 were obtained.

The two corresponding isomers for each of the $C_nH_{2n+1}CO$–RGDV–Ara-C compounds synthesized (A and B series) were separated using high-performance liquid chromatography (HPLC). As an example, Figure 5 shows the HPLC chromatogram of $C_9H_{19}CO$–RGDV–Ara-C isomers (peak 1: A series and peak 2: B series) detected at 251 nm and using acetonitrile–water (7:13) as mobile phase with a flow rate of 0.4 mL/min. The purity of the purified isomers was determined to be at least 95%.

The $C_nH_{2n+1}CO$–RGDV–Ara-C compounds synthesized were characterized by their melting points, optical rotation characteristics, and MS and NMR results. Detailed chemistry can be found in the Supplementary Information.

Preparation and Characterization of Liposomal Preparations Containing $C_nH_{2n+1}CO$–RGDV–Ara-C Compounds Synthesized

Liposomal preparations containing $C_nH_{2n+1}CO$–RGDV–Ara-C compounds synthesized were prepared according to a previously reported method[32–34] with minor modifications. In brief, egg lecithin and $C_nH_{2n+1}CO$–RGDV–Ara-C (n = 7, 9, 11, 13, and 15) (molar ratio: 1:1) were dissolved in chloroform in a flask. Chloroform was evaporated under reduced pressure at 35°C to form a film, which was subsequently mixed with phosphate buffer (pH 7.4). The mixture was vortexed, and the fully hydrated

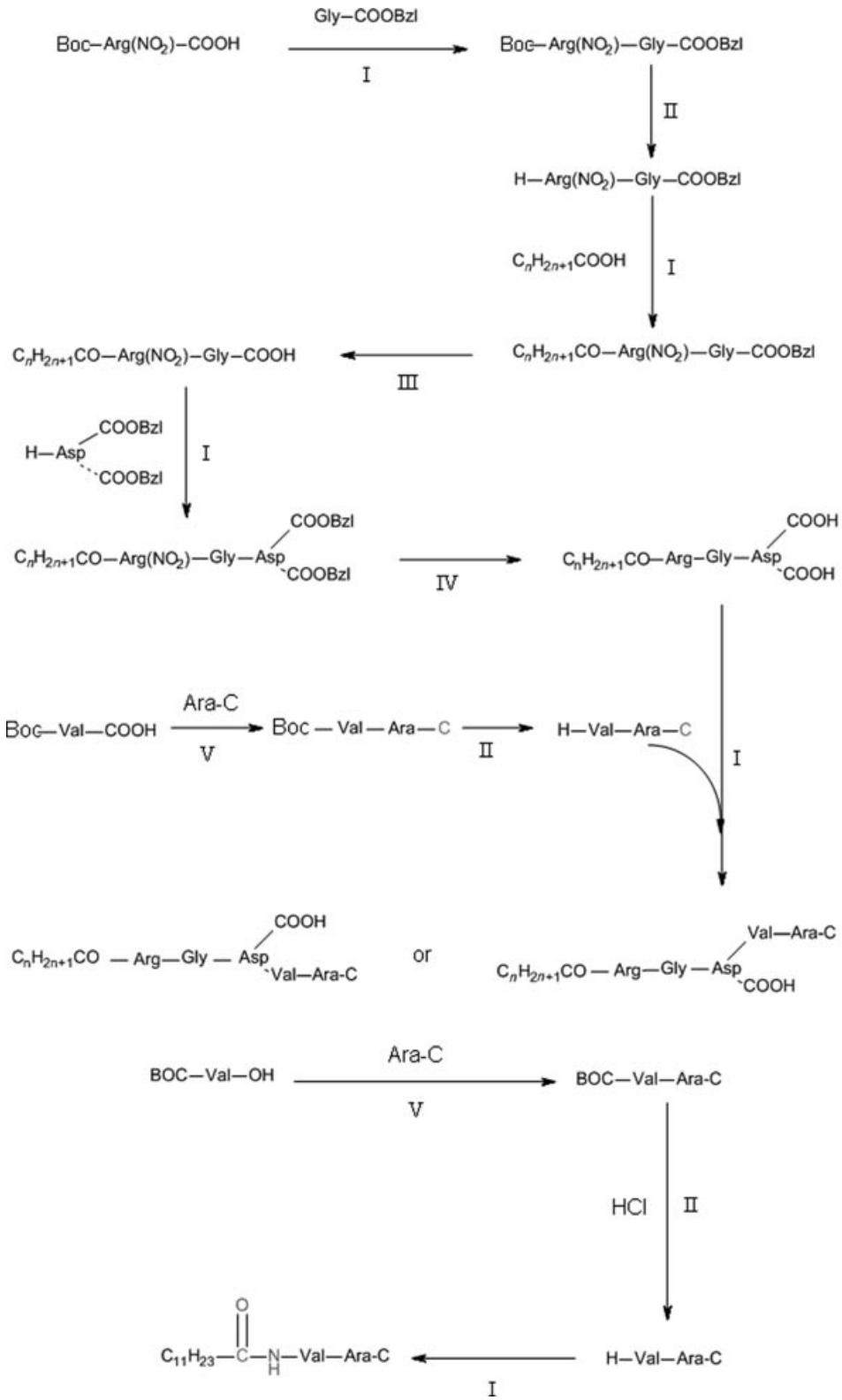

Figure 3. Synthesis of $C_nH_{2n+1}CO$–RGDV–Ara-C and $C_{11}H_{25}CO$–V–Ara-C (I) DCC, HOBt, NMM/anhydrous DMF, and THF; (II) 4N HCl-EtOAc; (III) 2N NaOH/ice bath; (IV) 5% H_2 (0.02 Mba) Pd/C/ethanol; and (V) EDC, HOBt, and NMM/anhydrous DMF. *Abbreviations:* DCC, dicyclohexylcarbodiimide; EDC, 1-ethyle-3-(3-dimethylaminopropyl)carbodiimide; HOBt, 1-hydroxybenzotriazole; NMM, N-methylmorpholine.

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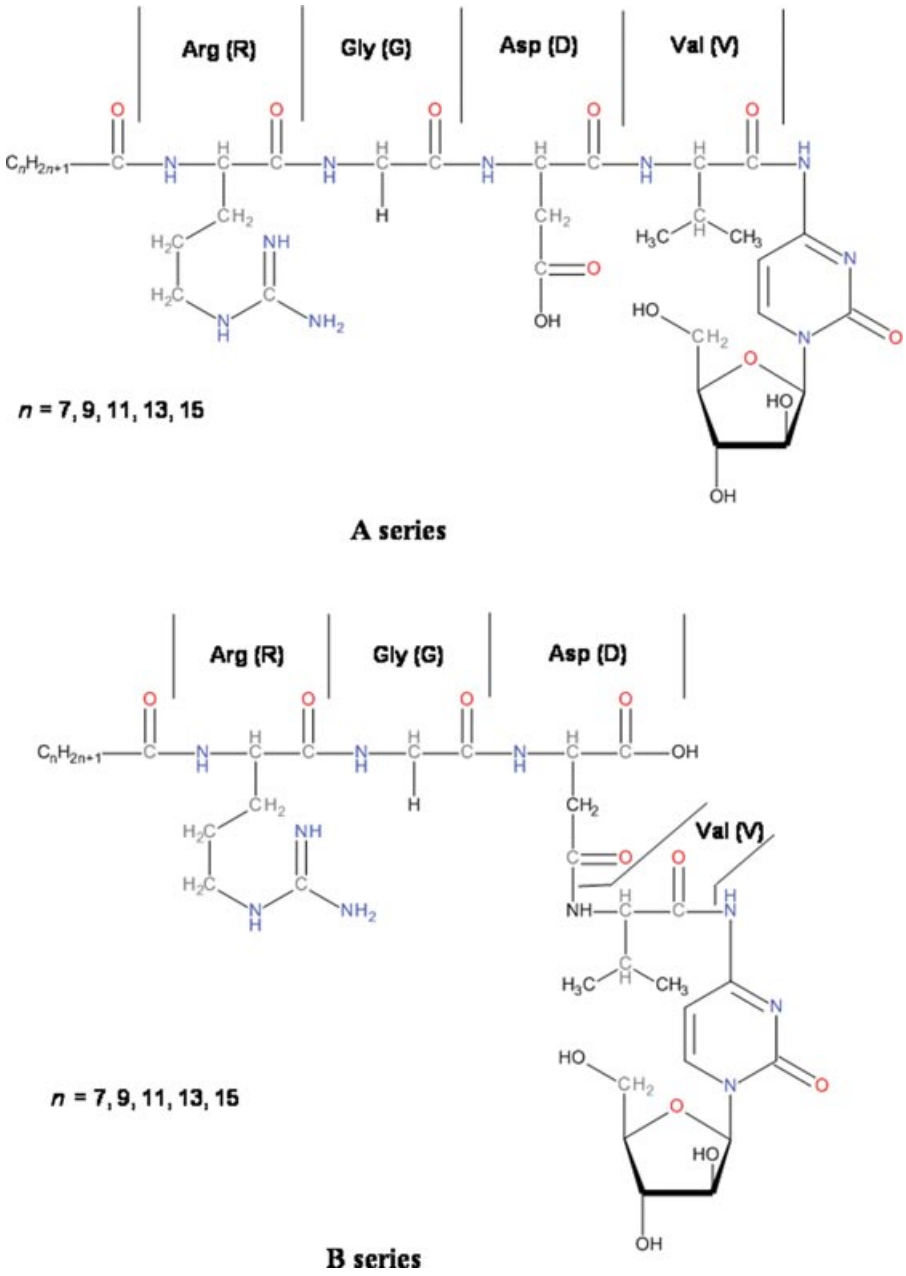

Figure 4. The structures of $C_nH_{2n+1}CO$–RGDV–Ara-C isomers.

mixture was sonicated for 20 min to obtain an opaque liposomal preparation.

Liposomal preparations containing $C_nH_{2n+1}CO$–RGDV–Ara-C compounds at 3.58 mmol/mL were characterized by their particle size and zeta potential at 25°C. Polydispersity index (PI) was also determined as a measure of the breadth of the particle size distribution.[35,36] Results were averages from three measurements.

The morphology of the liposomal preparations was examined. About 5 μL of the preparation was placed on a filter paper supported by a copper grid to allow the sample to dry before being viewed under TEM (80 kV). Images were obtained.

Biological Evaluation of $C_nH_{2n+1}CO$–RGDV–Ara-C Compounds

Animal model: Biological evaluation was determined using male Kunming mice (18–22 g) inoculated with S_{180} sarcoma. Mice were maintained in accordance with institutional guidelines. S_{180} tumor cells passaged in Kunming mice abdomen were harvested on the eighth day and suspended in about 3 mL of sterilized saline in a 15 mL centrifuge tube. The final volume was adjusted to 10 mL using sterilized saline and a homogenous mixture was obtained, which was then centrifuged at 1000 rpm (rotor diameter 15 cm, 83xg) for 5 min. Supernatant was removed and precipitate was mixed with saline, and mixture was

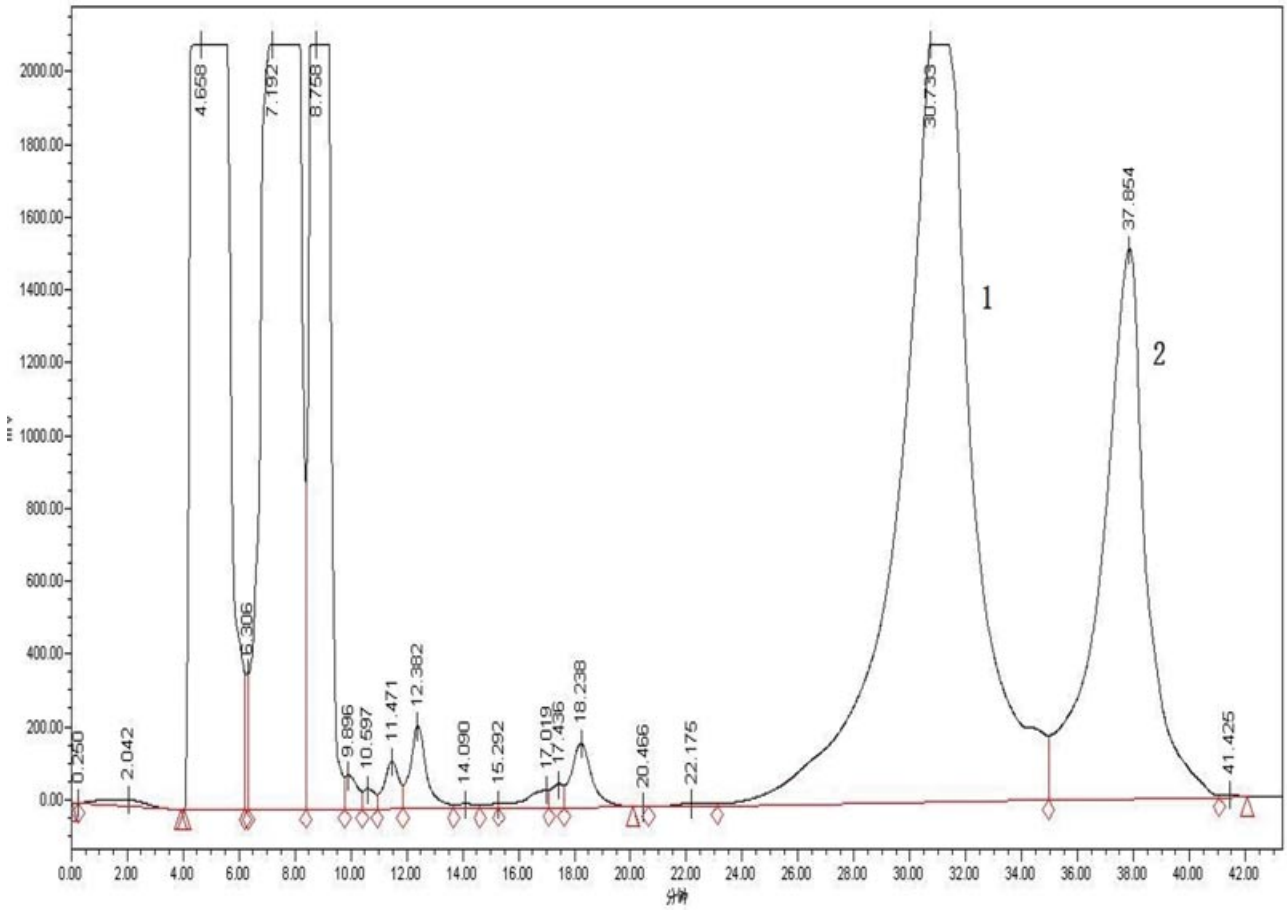

Figure 5. HPLC chromatogram of $C_9H_{19}CO$–RGDV–Ara-C isomers (peak 1: series A, peak 2: series B). Mobile phase: acetonitrile–water, 7:13; flow rate: 0.4 mL/min; UV detection: 251 nm.

recentrifuged. Following centrifugation, precipitate was suspended in about 9 mL of sterilized saline from which 0.1 mL of cell suspension was collected and mixed with 9.9 mL of sterilized saline. Cell suspension thus obtained was stored in ice until use. Cell count was determined by transferring 0.10 mL of the cell suspension obtained above into an Eppendorf tube into which 100 μL of Trypan Blue was added before cell number was counted. Tumor cells harvested were suspended in sterilized saline at 2.0×10^7/mL and used for inoculation by injecting 0.2 mL of the cell suspension into the abdomen of each mouse under aseptic conditions. Mice inoculated with S_{180} tumor cells were randomly divided into 25 groups with 10 mice in each group. Mice were given (via the tail vein) 0.2 mL of 3.58 mmol/L of the respective $C_nH_{2n+1}CO$–RGDV–Ara-C containing liposomal preparations for which liposomal Ara-C preparation of equal molar concentration was used as positive control and blank liposome as negative control, or $C_nH_{2n+1}CO$–RGDV–Ara-C suspensions prepared in 0.5% of CMC-Na for which Ara-C suspension in CMC-Na was used as positive control and CMC-Na solution (0.5%) as negative control. First administration was given 24 h after inoculation, and administration was repeated every other day for a total of four treatments. On day 8, mice were sacrificed, and tumors were removed and weighed.

Antitumor activity was defined by tumor growth inhibition (TGI%), which was calculated as [(tumor weight of negative control − tumor weight of treatment group)/tumor weight of negative control] × 100%. Results are expressed as $X \pm$ SD, and statistical significance was determined using Student's t-test.

RESULTS AND DISCUSSION

Particle Size and Zeta Potential

As shown in Table 1, the particle size of liposomes containing various $C_nH_{2n+1}CO$–RGDV–Ara-C was found to be approximately 215 nm, which is similar to that of Ara-C liposomes. The zeta potential of the preparations was approximately −30 mV, which is slightly higher than that of Ara-C. The PI values of the preparations are also listed in Table 1. Generally speaking, PI values less than 0.7 indicate a rather homogenous system. The relatively low PI values associated with the liposomal preparations prepared in this study (<0.3) suggested more uniform particles.[35,36] There

Table 1. Particle size, Zeta Potential, and Polydispersity Index (PI) of $C_nH_{2n+1}CO$–RGDV–Ara-C Liposomes

Liposomal Preparation	Particle Size (nm, $X \pm$ SD)	Zeta Potential (mV, $X \pm$ SD)	PI ($X \pm$ SD)
$C_7H_{15}CO$–RGDV–Ara-C(A)	235.0 ± 25	−30.56 ± 4.36	0.283 ± 0.028
$C_7H_{15}CO$–RGDV–Ara-C(B)	236.6 ± 29	−30.99 ± 4.82	0.276 ± 0.023
$C_9H_{19}CO$–RGDV–Ara-C(A)	200.1 ± 22	−31.06 ± 5.21	0.216 ± 0.019
$C_9H_{19}CO$–RGDV–Ara-C(B)	176.2 ± 19	−26.92 ± 3.58	0.255 ± 0.028
$C_{11}H_{23}CO$–RGDV–Ara-C(A)	198.0 ± 15	−28.79 ± 3.65	0.238 ± 0.022
$C_{11}H_{23}CO$–RGDV–Ara-C(B)	210.5 ± 19	−29.81 ± 3.91	0.236 ± 0.19
$C_{13}H_{25}CO$–RGDV–Ara-C(A)	238.2 ± 27	−31.15 ± 5.50	0.219 ± 0.039
$C_{13}H_{25}CO$–RGDV–Ara-C(B)	221.0 ± 26	−27.50 ± 4.42	0.233 ± 0.036
$C_{15}H_{29}CO$–RGDV–Ara-C(A)	216.3 ± 18	−25.16 ± 4.53	0.266 ± 0.028
$C_{15}H_{29}CO$–RGDV–Ara-C(B)	195.6 ± 22	−27.78 ± 3.89	0.286 ± 0.021
$C_{11}H_{23}CO$–V–Ara-C	220.2 ± 21	−25.56 ± 4.51	0.246 ± 0.056
Ara-C	234.5 ± 25	−25.18 ± 3.26	0.334 ± 0.052

$n = 3$.

does not appear to be a relationship between particle sizes/zeta potential and types of fatty acids. The liposomal preparations were stable in a refrigerator for at least 3 weeks. Particle size and zeta potential of preparations administered to animals were monitored to ensure quality.

Evaluation of Antitumor Activity

Inhibition of Tumor Growth

The tumor inhibition results of liposomal preparations and suspension containing $C_nH_{2n+1}CO$–RGDV–Ara-C are provided in Tables 2 and 3.

Table 2. Tumor Growth Inhibition (TGI) of $C_nH_{2n+1}CO$–RGDV–Ara-C Liposomes in S_{180}-Bearing Mice

Liposomal Preparation	Tumor Weight (g, $X \pm$ SD)	TGI (%)
$C_7H_{15}CO$–RGDV–Ara-C(A)	0.4651 ± 0.2428*	56.8
$C_7H_{15}CO$–RGDV–Ara-C(B)	0.4978 ± 0.1406*	53.8
$C_9H_{19}CO$–RGDV–Ara-C(A)	0.3741 ± 0.2049*,**,***	65.3
$C_9H_{19}CO$–RGDV–Ara-C(B)	0.3836 ± 0.1037*,**,***	64.4
$C_{11}H_{23}CO$–RGDV–Ara-C(A)	0.3875 ± 0.2040*,**,***	64.0
$C_{11}H_{23}CO$–RGDV–Ara-C(B)	0.4145 ± 0.2154*,**,***	61.5
$C_{13}H_{25}CO$–RGDV–Ara-C(A)	0.3645 ± 0.1285*,**,***	66.1
$C_{13}H_{25}CO$–RGDV–Ara-C(B)	0.4356 ± 0.2641*,**	59.5
$C_{15}H_{29}CO$–RGDV–Ara-C(A)	0.4103 ± 0.2030*,**,***	61.9
$C_{15}H_{29}CO$–RGDV–Ara-C(B)	0.4904 ± 0.1187*,**	54.5
$C_{11}H_{23}CO$–V–Ara-C	0.7224 ± 0.3085	32.9
Ara-C	0.8173 ± 0.2593	24.1
Blank liposome	1.077 ± 0.2858	0

$n = 8$.
*Significant compared with blank liposome, $p < 0.01$.
**Significant compared with Ara-C liposome, $p < 0.05$.
***Significant compared with C_{12}–V–Ara-C liposome, $p < 0.05$.

Table 3. TGI of $C_nH_{2n+1}CO$–RGDV–Ara-C Suspensions in S_{180}-Bearing Mice

Suspension	Tumor Weight (g, $X \pm$ SD)	TGI (100%)
$C_7H_{15}CO$–RGDV–Ara-C(A)	0.5981 ± 0.1756	16.2
$C_7H_{15}CO$–RGDV–Ara-C(B)	0.6065 ± 0.3328	15.0
$C_9H_{19}CO$–RGDV–Ara-C(A)	0.5325 ± 0.1753	25.4
$C_9H_{19}CO$–RGDV–Ara-C(B)	0.5590 ± 0.2220	21.7
$C_{11}H_{23}CO$–RGDV–Ara-C(A)	0.5768 ± 0.2605	19.2
$C_{11}H_{23}CO$–RGDV–Ara-C(B)	0.6246 ± 0.1777	12.5
$C_{13}H_{25}CO$–RGDV–Ara-C(A)	0.5968 ± 0.2282	16.4
$C_{13}H_{25}CO$–RGDV–Ara-C(B)	0.5964 ± 0.2573	16.5
$C_{15}H_{29}CO$–RGDV–Ara-C(A)	0.5531 ± 0.1854	22.5
$C_{15}H_{29}CO$–RGDV–Ara-C(B)	0.5839 ± 0.1508	18.2
$C_{11}H_{23}CO$–V–Ara-C suspension	0.6039 ± 0.1679	15.4
Ara-C suspension	0.6041 ± 0.1592	15.4
Blank CMC-Na solution	0.7139 ± 0.2724	0

$n = 8$.

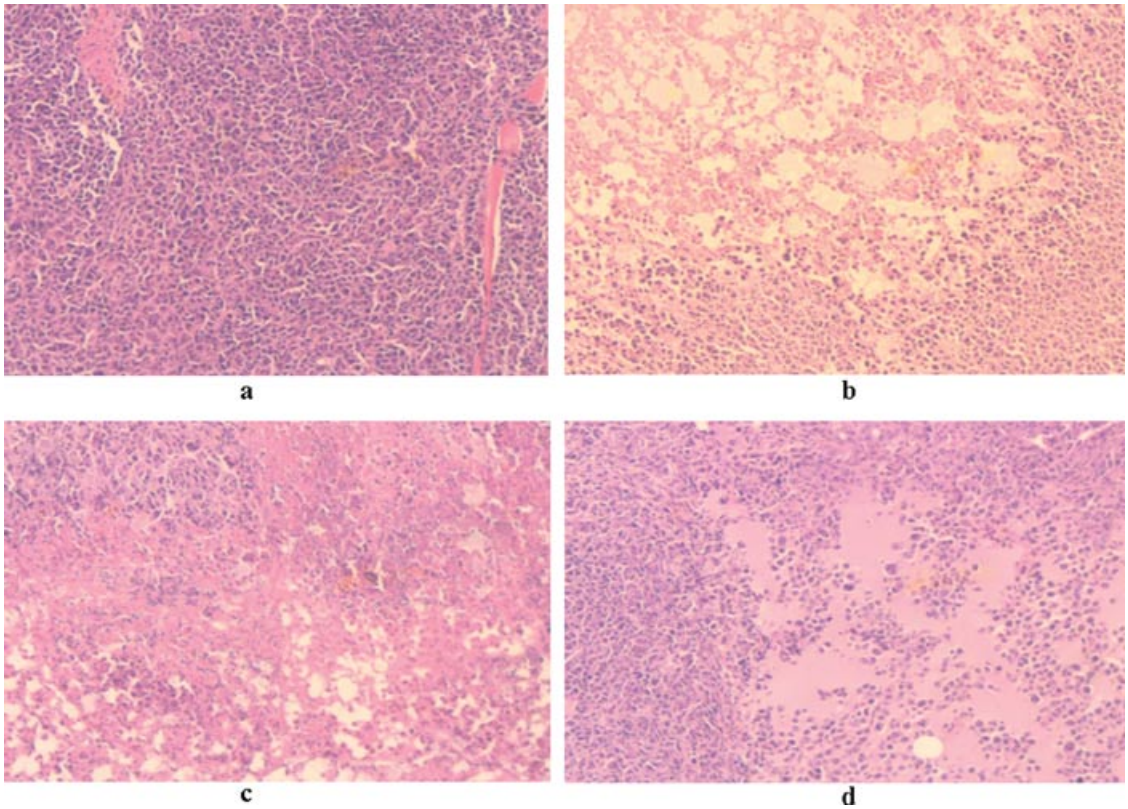

Figure 6. Biopsy results of tumor tissue of S_{180} inoculated mice following tail vein administration of blank liposomes (a), $C_{11}H_{23}CO$–RGDV–Ara-C(A) liposomes (b), Ara-C liposomes (c), and $C_{11}H_{23}CO$–Val–Ara-C liposomes (d) (H&E staining, 10 × 10 magnification).

As shown in Table 2, all $C_nH_{2n+1}CO$–RGDV–Ara-C liposomal preparations were found to be more effective than Ara-C and $C_{11}H_{23}CO$–V–Ara-C in the inhibition of tumor growth in mice, whereas there was little difference between Ara-C and $C_{11}H_{23}CO$–V–Ara-C liposomes. The data in Table 3 showed that there was little difference between $C_nH_{2n+1}CO$–RGDV–Ara-C suspensions and Ara-C suspension or $C_{11}H_{23}CO$–V–Ara-C suspension. The results clearly demonstrated that RGDV was able to improve the antitumor activity of Ara-C in its liposomal formulations, whereas it made little difference in the antitumor activity of Ara-C in its suspensions. It was also found that the length of the fatty acid in $C_nH_{2n+1}CO$–RGDV–Ara-C had little impact on their activity against tumor growth. In addition, there was little difference observed between the two isomers for each of the $C_nH_{2n+1}CO$–RGDV–Ara-C.

Biopsy of Tumor Tissue

In addition to tumor size, pathological examinations are also important in assessing the antitumor potential of cytotoxic agents or formulations. Figure 6 shows the H&E stained images of tumor tissues dissected from mice inoculated with S_{180} sarcoma after treatment with different formulations. Figure 6a is the image of tumor cells treated with blank liposome in which the structure of tumor tissue was not affected and the nuclei of tumor cells were clearly stained indicating viable tumor cells. Following the treatment of $C_{11}H_{23}CO$–RGDV–Ara (Fig. 6b), the structure of the tumor tissue was significantly damaged and cell death was clearly observed, whereas Ara-C and $C_{11}H_{23}CO$–V–Ara-C (A) resulted in localized tumor cell death and cytolysis (Figs. 6c and 6d). However, there were still a significant number of unlysed tumor cells with intact nuclei shown.

CONCLUSIONS

This study focused on the potential of RGD to enhance the antitumor activity of Ara-C by attaching RGD to Ara-C via another amino acid, valine. In addition, fatty acids of various chain lengths were introduced into the structure. A series of $C_nH_{2n+1}CO$–RGDV–Ara-C were synthesized and characterized. They were subsequently prepared into liposomal preparations and suspensions, respectively. The fatty acid moiety in the structure was used to improve the overall lipophilicity of $C_nH_{2n+1}CO$–RGDV–Ara-C so that they can be more readily incorporated in the vesicles of liposome,

whereas RGD was used for its unique specificity for the integrins overexpressed in cancer cells to achieve more selective delivery. $C_nH_{2n+1}CO$–RGDV–Ara-C would undergo hydrolysis to release Ara-C, which is cytotoxic.

As noted earlier, there are two isomers for each of the $C_nH_{2n+1}CO$–RGDV–Ara-C compounds, which were successfully separated using HPLC. The particle size, zeta potential, and PI of the liposomal preparations of $C_nH_{2n+1}CO$–RGDV–Ara-C were found to be approximately 215 nm (diameter), approximately −30 mV, and <0.3, respectively.

The antitumor activities of $C_nH_{2n+1}CO$–RGDV–Ara-C liposomal preparations and suspensions were evaluated using mice inoculated with S_{180} sarcoma. It was found that $C_nH_{2n+1}CO$–RGDV–Ara-C liposomes were more effective (TGI and biopsy results) than Ara-C, suggesting more Ara-C was delivered to the tumor cells likely because of the presence of RGDV. However, there was little difference between $C_nH_{2n+1}CO$–RGDV–Ara-C suspensions and Ara-C suspension. The improved antitumor activity of $C_nH_{2n+1}CO$–RGDV–Ara-C liposomes over their suspension counterparts may be because of the fact that liposomes possess a better penetrating capability through tumor cell membranes.[26] The current study may lead to the therapeutic applications of Ara-C in the treatment of solid tumors.

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