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# Synthesis and evaluation of anti-tumor activities of $N^4$ fatty acyl amino acid derivatives of 1-β-arabinofuranosylcytosine

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ABSTRACT

1-β-D-Arabinofuranosylcytosine (Ara-C, Cytarabine) is one of the drugs used for acute nonlymphocytic leukemia (ANLL). However, the bioavailability of Ara-C is relatively low due to its low lipophilicity. In order to improve the lipophilicity and bioavailability of Ara-C, a series of $N^4$ derivatives of Ara-C, i.e., (fatty acid)–(amino acid)–Ara-C analogues, were prepared. The 15 derivatives synthesized were characterized by their melting points, optical rotations and partition coefficients. It was found that the Ara-C derivatives synthesized in this study were more lipophilic than Ara-C as determined by their partition coefficients. Their *in vitro* cytotoxicity and *in vivo* anti-tumor activity were determined and compared with that of Ara-C. It was found that the derivatives were more active than Ara-C in Hela cells, but not in HL-60 cells. The *in vivo* results showed that some of the derivatives were more effective than Ara-C in mice bearing $S_{180}$ tumor while others showed a decreased activity in comparison with Ara-C.



## 1. Introduction

1-β-D-Arabinofuranosylcytosine (Ara-C, Cytarabine), a pyrimidine nucleotide analogue, is primarily used in the treatment of various leukemias in conjunction with anthracycline antibiotics. This cytidine-based antimetabolite undergoes initial phosphorylation by deoxycytidine kinase to monophosphate with subsequent phosphorylations catalyzed by pyrimidine monophosphate and diphosphate kinases. The active form, triphosphorylated Ara-C, exhibits its anticancer activity via the inhibition of DNA polymerase and/or DNA chain elongation [1]. It is one of the drugs used for acute nonlymphocytic leukemia (ANLL). However, Ara-C is associated with relatively low lipophilicity and low bioavailability. It is also known that Ara-C is quickly deactivated to uracil arabinoside (Ara-U) by cytidine deaminase. Even its monophosphorylated form, Ara-cytidine monophosphate, can be catabolized into uracil arabinotide by deoxycytidylate deaminase, resulting in the loss of activity. In addition, Ara-C is known to be associated with side effects including nausea, vomiting, inhibition of bone marrow and loss of body weight [2,3]. The side effects following long term uses are of a special concern for pediatric patients [4]. Finally, drug resistance is likely another reason for the limited success observed with Ara-C clinically [5–7]. It has been reported that Ara-C is not effective in solid tumors [8].

The low lipophilicity of Ara-C is likely due to the presence of an $-NH_2$ and multiple –OH groups in its structure as shown in Fig. 1. Therefore, there have been many reports where modifications at the $N^4$ and 3′,5′-OH positions [9–15] were made. Enocitabine and $N^4$-palmitoyl-Ara-C are two examples of derivatives with enhanced and prolonged activities. Both of them are fatty acid derivatives of Ara-C and are converted to Ara-C via metabolic biotransformation [16].

There have been many other reports on various $N^4$ amide derivatives of Ara-C. However, they seem to suffer from instability problems. Previous reports by Aoshima et al. [17] and Bergman et al. [18] on some of the $N^4$ fatty acid derivatives of Ara-C suggested that the length of fatty acids on the $N^4$ position seemed to affect the anti-tumor activity observed and found that Ara-C derivatives with fatty acids of $C_{18}$–$C_{22}$ at the $N^4$ position were most effective. As the length of fatty acids further increased, their anti-tumor activity decreased. They also found that the Ara-C derivatives showed prolonged activities compared to Ara-C as the derivatives of Ara-C gradually released Ara-C upon hydrolysis.

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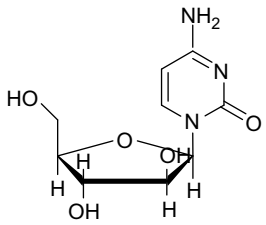

**Fig. 1.** The structure of Ara-C.

The purpose of this work was to introduce various fatty acyl amino acids onto the $N^4$ position (Fig. 2). Such a modification was aimed to reduce the deamination associated with Ara-C in addition to increased lipophilicity. Fatty acids of $C_{10}$, $C_{14}$ and $C_{18}$ were used and were connected to the $N^4$ position of Ara-C via five different amino acids, valine, methionine, tyrosine, arginine and glutamic acid. The selected amino acids are associated with varied physicochemical properties. Valine and methionine are non-polar while tyrosine is polar. Glutamic acid is acidic and arginine is alkaline. The *in vitro* and *in vivo* anti-tumor activities of the derivatives synthesized were evaluated and compared with that of Ara-C.

## 2. Experimental procedures

### 2.1. Materials

Ara-C (pharmaceutical grade) was obtained from Peking University Pharmaceutical Ltd. (Beijing, China), while myristic acid was obtained from Beijing Chaoyang Xudong Chemicals Inc. (Beijing, China). Stearic acid and *n*-capric acid were purchased from Tianjin Fuchen Chemicals Ltd. (Tianjin, China). All amino acids were of L-configuration and were from Sichuan Gaosheng Inc. Ltd. Dicyclohexylcarbodiimide (DCC), 1-hydroxybenzotriazole (HOBt) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)·HCl were from GL Biochem Ltd. (Shanghai, China). NaCl for injection and CMC-Na were purchased from Shandong Qidu Pharmaceutical Co. Ltd. (Zibo, China). All reagents were of chemical grade unless otherwise specified.

Laborota 4000 rotary evaporator was from Heidolph Instruments (Schwabach, Germany). Circulative multifunctional vacuum pump (SHB IIIS) was a product of Zhengzhou Great Wall Industrial Trade Ltd. (Zhenzhou, Henan, China). Hot plate with magnetic stirrer, S21-2, was from Shanghai Si-le Instrument Ltd. (Shanghai, China). Avance II 300 and 500 NMR spectrometers were purchased from Bruker Biospin AG (Fällanden, Switzerland). Quattro Micro 2000 mass spectrometer was from Waters (Milford, MA, USA). Melting point apparatus, XT5, was purchased from Beijing Keyi Electro-optical Instrument Ltd. (Beijing, China). Optical rotation was determined using a polarimeter, P-1020, from Jasco (Tokyo, Japan). Microplate reader, BIO-RAD Model 680, was purchased from Bio-Rad (Hercules, CA, USA). Solutions/suspensions used for injection were autoclaved using a VARIOKLAV® steam sterilizer manufactured by H + P Labortechnik AG (Oberschleissheim, Germany). OLYMPUS IX71 inverted microscope was purchased from Olympus Co. (Tokyo, Japan).

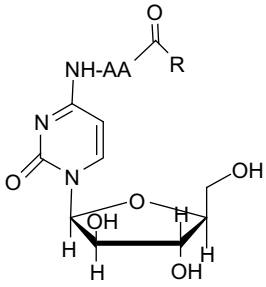

**Fig. 2.** The structures of $N^4$ derivatives of Ara-C synthesized. R: $-(CH_2)_8CH_3$; $-(CH_2)_{12}CH_3$; $-(CH_2)_{16}CH_3$. AA: Val, Met, Arg, Tyr, Glu.

Cell culture media, RPMI 1640 and DMEM, were products of GIBCO (Grand Island, NY, USA). Fetal bovine serum and trypsin were purchased from HyClone (Logan, UT, USA). Penicillin, streptomycin and MTT were products of Sigma, and DMSO was purchased from ACROS ORGANICS (Geel, Belgium).

Human leukemia cell line, HL-60, obtained from the School of Pharmacy of Peking University Medical Center, was maintained in RPMI 1640 containing 10% of fetal bovine serum, penicillin (100 units/mL) and streptomycin (100 units/mL). Hela cells were maintained in DMEM containing 10% of fetal bovine serum, penicillin (100 units/mL) and streptomycin (100 units/mL).

Male Kunming mice purchased from the Animal Services, Peking University Medical Center were supplied with food and water *ad libitum*.

### 2.2. Chemistry

The Ara-C derivatives shown in Fig. 2 were synthesized according to the scheme in Fig. 3. The respective fatty acids (**1a**–**c**) with different carbon chain lengths (C-10, C-14 or C-18) were first connected to the C-terminus protected amino acid chosen (Val, Met, Arg, or Tyr) in the presence of DCC, HOBt and *N*-methylmorpholine (NMM) to yield the C-terminus protected fatty acyl amino acid 1-methyl esters (**3a**–**l**). The de-protected fatty acyl amino acids (**4a**–**l**) were then conjugated with Ara-C using EDC/HOBt as the condensing agent in pyridine at 45 °C to yield derivatives, **6a**–**l**, as shown in Fig. 3.

For glutamic acid containing derivatives, *N-tert*-butoxycarbonyl-L-glutamic acid 5-benzyl ester (Boc-Glu-OBzl) was first conjugated with $N^4$-amine of Ara-C to yield **5′**. After selective de-protection of N-Boc (**5″**), the respective fatty acids (**la**–**c**) with different carbon chain lengths (C-10, C-14, C-18) were connected to yield the intermediates, **5m**–**o**. The 5-benzyl protection was then removed to obtain the glutamate derivatives (**6m**–**o**) as shown in Fig. 3.

### 2.3. Supplementary data [Details of synthetic experiments]

The data are available at the end of manuscript and are free of charge via the Internet at http://www.sciencedirect.com.

### 2.4. Characterization of Ara-C derivatives synthesized

The structures of the derivatives synthesized, **6a**–**o**, were confirmed with mass-spectrometry (MS) and nuclear magnetic resonance (NMR). Their melting points and optical rotations were measured. The purity of the final compounds, **6a**–**o**, were verified by high pressure liquid chromatography (HPLC) using a C18 SunFire™ column (Waters). Analytical HPLC was performed on a Waters 2695 system equipped with an UV detector set at 248 nm. Compounds were dissolved in MeOH and injected through a 50 μL loop. The eluent systems, A ($H_2O/CH_3CN$, 20:80) and B (pure $CH_3CN$ 100%), were used. HPLC retention times ($t_R$) were obtained, at a flow rate of 0.5 mL/min with 100% eluent A for the first 20 min followed by gradient increase to 100% eluent B over the next 20 min. The partition coefficients of **6a**–**o** were determined in a water/1-octanol system by the shake-flask method. Briefly, about 1 mg of each of the compounds were dissolved in 5 mL of water-saturated 1-octanol. An aliquot of this solution was diluted with chloroform and the absorbance ($A_0$) of the resultant solution at

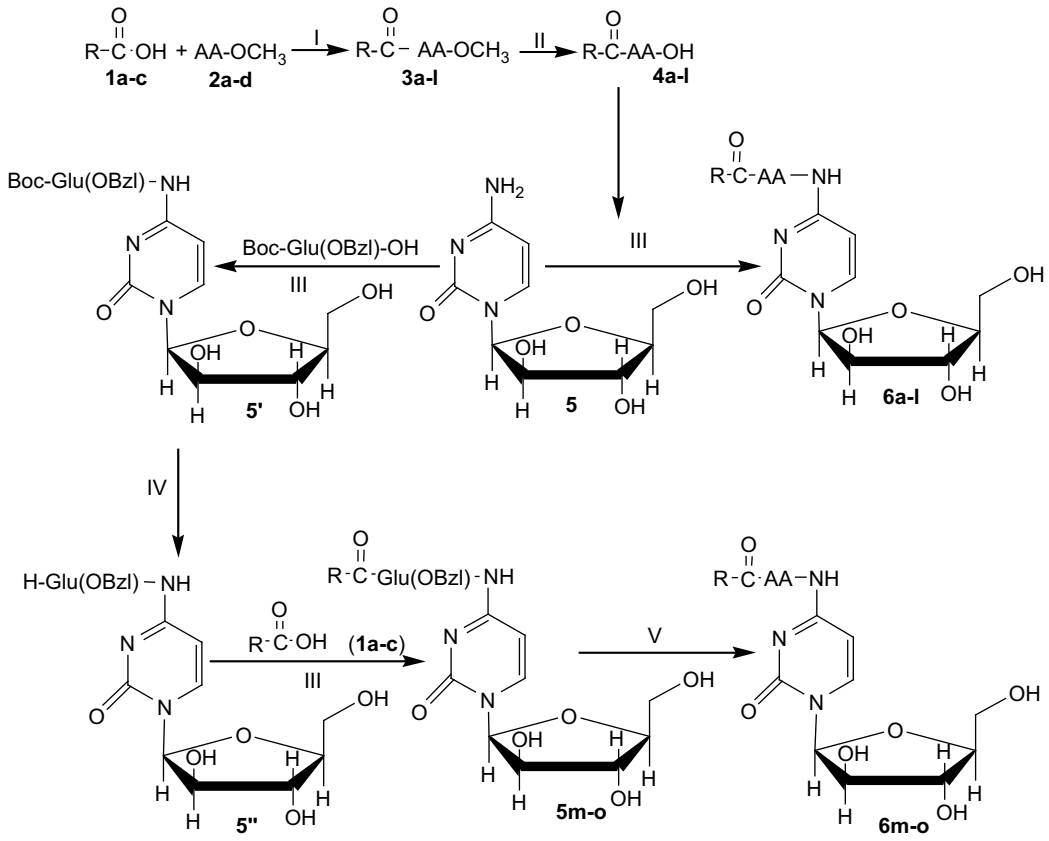


**Fig. 3.** Synthesis of (fatty acid)–(amino acid)–Ara-C derivatives. I. DCC, HOBt and NMM; II. 2 N NaOH; III. EDC, HOBt and pyridine; IV. 4 N HCl-EtOAc; V. $H_2$, Pd/C. R = $-(CH_2)_8CH_3$, $-(CH_2)_{12}CH_3$, $-(CH_2)_{16}CH_3$ in **1a–c**, **3a–l**, **4a–l**, **5m–o**, **6a–l**, **6m–o**; AA = Val, Met, Tyr or Arg in **2a–d**, **3a–l**, **4a–l**, **6a–l**; AA = Glu in **6m–o**.

280 nm was determined using an UV-spectrophotometer. Five milliliters of the above solution was mixed with 5 mL of 1-octanol-saturated water in a 15 mL conical tube. Sample tubes were vigorously shaken for 2 h at 25 °C following which the mixture was allowed to stand for 30 min. The 1-octanol layer was removed and diluted in chloroform to determine the absorbance at 280 nm ($A_x$).

The partition coefficient was calculated as

$$p = \frac{n_X A_X V_X}{n_O A_O V_O - n_X A_X V_X}$$

where $n_x$ is the dilution factor of the 1-octanol layer prior to measuring absorbance following partitioning; $V_x$ is the aliquot amount taken for the above dilution; and $A_x$ is the absorbance of the diluted 1-octanol layer following partitioning, while $n_0$ is the dilution factor of the 1-octanol sample prior to measuring absorbance before partitioning; $A_0$ is the absorbance of the diluted 1-octanol sample before partitioning and $V_0$ is the aliquot volume of the 1-octanol sample used for dilution before partitioning.

### 2.5. *Determination of* in vitro *cytotoxicity of Ara-C derivatives*

The *in vitro* cytotoxicity of Ara-C derivatives synthesized was evaluated in HL-60 and Hela cells, and was compared with that of Ara-C.

HL-60 or Hela cells were suspended at $5 \times 10^4$/mL and 100 μL of the cell suspension were placed in each well of the 96-well culture plate. Cells were incubated with various concentrations (0.01, 0.05, 0.10, 0.50 and 1.00 μg/mL for HL-60 cells, and 0.5, 1.0, 5.0, 10 and 50 μg/mL for Hela cells) of test compounds in PBS containing 4% DMSO and tests were in triplicates. Incubation was carried out at 37 °C in an incubator supplied with 5% $CO_2$ for 48 h. Following incubation, 25 μL of 5 mg/mL of MTT were added to each well and the plate was put back in the incubator for another 4 h at 37 °C. The plate was then subjected to centrifugation at 1000 r/min for 10 min in the case of HL-60 cells, while no centrifugation was needed for Hela cells. Supernatant was discarded, and 100 μL of DMSO were added to each well and the plate was vortexed for 10 min to dissolve the crystals formed. The OD values were obtained using a microplate reader at 570 nm with a reference wavelength of 630 nm. The percentage of cell survival was calculated as OD value of cells treated with test compound − OD value of culture medium/(OD value of control cells − OD value of culture medium) × 100% and $IC_{50}$ was calculated as the concentration which resulted in 50% of cell death.

### 2.6. In vivo *anti-tumor activity*

Kunming mice were used. Animal care was in accordance with institutional guidelines. $S_{180}$ tumor cells passaged in mice abdomen were harvested on the eighth day and suspended at $2.0 \times 10^7$/mL. The tumor cell preparation (0.2 mL) was injected to each mouse. The mice inoculated with $S_{180}$ tumor cells were randomly divided into 17 different groups with 10 in each group.

Ara-C was dissolved in 0.5% CMC-Na and its derivatives synthesized (**6a–o**) were suspended in 0.5% CMC-Na at 7.8 mmol/L. Mice inoculated with $S_{180}$ tumor cells were injected intraperitoneally with 0.2 mL of the Ara-C solution or suspensions of its derivatives daily for 7 days. At the end of 7 days, mice were sacrificed and tumor was weighed. Tumor inhibition was calculated as: (average tumor weight in the control group − average tumor weight in drug treated group)/average tumor weight in the control group × 100%.

**Table 1**
Characterization of Ara-C and its derivatives.

| Compound | Code | Melting point (°C) | Optical rotation $[\alpha]_D^{25}$, deg (c10, MeOH) | Partition coefficient (*P*) | log *P* |
|---|---|---|---|---|---|
| Ara-C | | 189–195 | 127 | 0.12 | −0.94 |
| $C_{10}$-Val-Ara-C | **6a** | 98–100 | 36.6 | 6.04 | 0.78 |
| $C_{14}$-Val-Ara-C | **6b** | 102–104 | 29.1 | 7.91 | 0.9 |
| $C_{18}$-Val-Ara-C | **6c** | 134–136 | 47.7 | 8.15 | 0.91 |
| $C_{10}$-Met-Ara-C | **6d** | 84–86 | 45.7 | 4.81 | 0.68 |
| $C_{14}$-Met-Ara-C | **6e** | 84–86 | 45.9 | 7.71 | 0.88 |
| $C_{18}$-Met-Ara-C | **6f** | 125–127 | 51.3 | 12.41 | 1.09 |
| $C_{10}$-Tyr-Ara-C | **6g** | 114–116 | 65.4 | 6.5 | 0.81 |
| $C_{14}$-Tyr-Ara-C | **6h** | 156–158 | 52.2 | 7.97 | 0.9 |
| $C_{18}$-Tyr-Ara-C | **6i** | 194–196 | 33.9 | 9.3 | 0.97 |
| $C_{10}$-Arg-Ara-C | **6j** | 98–100 | 61.4 | 5.72 | 0.76 |
| $C_{14}$-Arg-Ara-C | **6k** | 124–126 | 67.8 | 2.805 | 0.45 |
| $C_{18}$-Arg-Ara-C | **6l** | 114–116 | 34.9 | 4.08 | 0.61 |
| $C_{10}$-Glu-Ara-C | **6m** | 77–79 | 64.1 | 0.52 | −0.28 |
| $C_{14}$-Glu-Ara-C | **6n** | 129–131 | 29.1 | 1.78 | 0.25 |
| $C_{18}$-Glu-Ara-C | **6o** | 174–176 | 30.8 | 3.15 | 0.5 |

### 2.7. Statistics

Data are expressed as $X \pm SD$ and statistical significance was determined using unpaired two-sided Student's *t* test.

## 3. Results and discussion

### 3.1. Characterization of the Ara-C derivatives synthesized

Fifteen new derivatives of Ara-C (**6a**–**o**) were synthesized. Analytical HPLC revealed that the purity of **6a**–**o** was in the range of 95.56–99.15%. Details about the purity and retention time of **6a**–**o** are shown in the Supplementary data [Details of synthetic experiments] section. Their melting points, optical rotation and partition coefficients were determined, and the results are summarized in Table 1.

As shown in Table 1, the log *P* of Ara-C was found to be −0.94 while the log *P* values of the derivatives were found to be higher and were above 0 except for **6m**. The lower lipophilicity associated with **6m** is probably due to the $C_{10}$ fatty acid and the additional hydroxyl group in glutamic acid in the molecule. As expected, as the length of fatty acids increased, the lipophilicity of the resultant derivatives increased. The increased lipophilicity associated with the derivatives may lead to increased permeability across the cell membranes and ultimately improved bioavailability.

**Table 2**
The $IC_{50}$ values of Ara-C and its derivatives in HL-60 and Hela cells.

| Compound | Code | $IC_{50}$ (μmol/L) | |
|---|---|---|---|
| | | HL-60 | Hela |
| Ara-C | Ara-C | 0.118 ± 0.093 | 464.433 ± 10.009 |
| $C_{10}$-Val-Ara-C | **6a** | 2.036 ± 0.160 | 4.993 ± 0.009 |
| $C_{14}$-Val-Ara-C | **6b** | 32.294 ± 3.584 | 26.000 ± 2.000 |
| $C_{18}$-Val-Ara-C | **6c** | 31.875 ± 2.5 | 13.031 ± 1.002 |
| $C_{10}$-Met-Ara-C | **6d** | 0.280 ± 0.032 | 2.996 ± 0.018 |
| $C_{14}$-Met-Ara-C | **6e** | 0.272 ± 0.065 | 8.022 ± 0.122 |
| $C_{18}$-Met-Ara-C | **6f** | 0.803 ± 0.090 | 9.000 ± 1.000 |
| $C_{10}$-Tyr-Ara-C | **6g** | 2.978 ± 0.054 | 1.000 ± 0.036 |
| $C_{14}$-Tyr-Ara-C | **6h** | 1.706 ± 0.018 | 39.057 ± 3.004 |
| $C_{18}$-Tyr-Ara-C | **6i** | 1.147 ± 0.036 | 10.000 ± 2.000 |
| $C_{10}$-Arg-Ara-C | **6j** | 0.584 ± 0.090 | 5.999 ± 0.990 |
| $C_{14}$-Arg-Ara-C | **6k** | 0.405 ± 0.036 | 31.996 ± 5.990 |
| $C_{18}$-Arg-Ara-C | **6l** | 1.703 ± 0.068 | 12.051 ± 7.029 |
| $C_{10}$-Glu-Ara-C | **6m** | 1.860 ± 0.074 | 5.000 ± 1.000 |
| $C_{14}$-Glu-Ara-C | **6n** | 0.502 ± 0.011 | 40.999 ± 1.999 |
| $C_{18}$-Glu-Ara-C | **6o** | 1.136 ± 0.065 | 19.01 ± 9.004 |

**Table 3**
The weight of tumor and percent tumor growth inhibition (%TGI) 7 days after implantation of $S_{180}$.

| Compound | Code | Tumor weight ($X \pm SD$) g | TGI % |
|---|---|---|---|
| $C_{10}$-Val-Ara-C | **6a** | 1.181 ± 0.308 | 7.0 ± 2.42 |
| $C_{14}$-Val-Ara-C | **6b** | 1.163 ± 0.656 | 8.4 ± 5.16 |
| $C_{18}$-Val-Ara-C | **6c** | 0.523 ± 0.095** | 58.9 ± 0.74 |
| $C_{10}$-Met-Ara-C | **6d** | 0.944 ± 0.438 | 25.6 ± 2.42 |
| $C_{14}$-Met-Ara-C | **6e** | 0.793 ± 0.361* | 37.6 ± 3.44 |
| $C_{18}$-Met-Ara-C | **6f** | 0.438 ± 0.102** | 65.5 ± 0.80 |
| $C_{10}$-Tyr-Ara-C | **6g** | 1.028 ± 0.232 | 19.1 ± 1.83 |
| $C_{14}$-Tyr-Ara-C | **6h** | 1.125 ± 0.58 | 11.4 ± 4.56 |
| $C_{18}$-Tyr-Ara-C | **6i** | 1.06 ± 0.689 | 16.5 ± 5.42 |
| $C_{10}$-Arg-Ara-C | **6i** | 1.071 ± 0.402 | 15.7 ± 3.16 |
| $C_{14}$-Arg-Ara-C | **6k** | 0.773 ± 0.263* | 39.2 ± 2.07 |
| $C_{18}$-Arg-Ara-C | **6l** | 1.168 ± 0.406 | 8.1 ± 3.20 |
| $C_{10}$-Glu-Ara-C | **6m** | 1.072 ± 0.451 | 15.6 ± 3.55 |
| $C_{14}$-Glu-Ara-C | **6n** | 0.958 ± 0.226 | 24.6 ± 1.78 |
| $C_{18}$-Glu-Ara-C | **6o** | 1.106 ± 0.439 | 12.9 ± 3.46 |
| Ara-C in CMC-Na | | 0.891 ± 0.272* | 29.9 ± 2.14 |
| CMC-Na | | 1.27 ± 0.3 | |

*Significant compared to control ($P < 0.05$); **Significant compared to Ara-C CMC-Na ($P < 0.05$); $n = 10$.

### 3.2. In vitro *cytotoxicity*

The $IC_{50}$ values of Ara-C and the derivatives synthesized in HL-60 and Hela cells are shown in Table 2. It was found that Ara-C was more effective in HL-60 than in Hela cell. The derivatives were shown to have comparable $IC_{50}$ values as that of Ara-C in HL-60 cells. It was also found that valine containing derivatives (**6a**, **6b**, **6c**) had the lowest activity while derivatives containing methionine as the linker (**6d**, **6e** and **6f**) showed the best results. The length of the fatty acids seemed to play a role in the anti-tumor activity observed as well and $C_{10}$ fatty acid containing derivatives (**6a**, **6d**, **6g**, **6j** and **6m**) appeared to be more active. This is likely due to the more balanced solubility of these derivatives and/or better affinity for membrane attributed to the medium chain fatty acid of $C_{10}$.

It was also found that the $IC_{50}$ values of most of the derivatives synthesized in Hela cells were higher than in HL-60 cells suggesting that HL-60 cells are more sensitive than Hela cells. In this context, previous studies have reported that the fatty acid derivatives of Ara-C were found to be more active than Ara-C in L1210 cells [19,20] while Bergman et al. [18] showed that the activity of fatty acid derivatives of Ara-C increased as the length of fatty acids decreased.

### 3.3. In vivo *anti-tumor activity*

The *in vivo* anti-tumor results are shown in Table 3. Due to the low water solubility of Ara-C derivatives, they were formulated into their respective suspensions for injection. It was found that derivatives **6c** and **6f** were more effective than Ara-C, and derivatives **6e**, **6g** and **6k** showed similar activities as Ara-C. The rest of the derivatives were less effective than Ara-C. The reduced anti-tumor activity observed with most of the derivatives was likely due to their poor water solubility.

## 4. Conclusions

A series of fatty acid–amino acid–Ara-C analogues were synthesized. The fatty acids used were of $C_{10}$, $C_{14}$ and $C_{18}$, and amino acids include valine, methionine, tyrosine, glutamate and arginine. The chemical structures of the derivatives and intermediates were confirmed using NMR and MS. The derivatives were characterized by melting point, light rotation and partition coefficient. It was found that the derivatives synthesized were more lipophilic than Ara-C with log *P* in the range of 0–1. Their

anti-tumor activity determined in HL-60 and Hela cells showed that the derivatives were more active in Hela cells than Ara-C while most of them demonstrated similar activities to Ara-C in HL-60 cells. Among the Ara-C derivatives synthesized, those containing methionine demonstrated the best activity. In addition, the length of fatty acids in the derivatives seemed to have an impact on the activity observed and it was shown that the activity decreased as the chain length of the fatty acid increased. However, their *in vivo* anti-tumor activity determined in mice bearing $S_{180}$ tumor was not conclusive with some of the derivatives being more active while others being less active than Ara-C.

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## Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version, at doi: 10.1016/j.ejmech.2009.02.028.

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