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ORIGINAL ARTICLE

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Antimetastatic effect of a lipophilic ascorbic acid derivative with antioxidation through inhibition of tumor invasion

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Abstract *Purpose*: Ascorbic acid (AA), the natural antioxidant, has been demonstrated to exert an antimetastatic action; however, AA is quite unstable in physiological condition. The aim of the present study is to investigate the stability, the antioxidation and the antimetastatic effects of three lipophilic AA derivatives in vitro as well as in vivo. *Methods*: The 95D cells were treated with ascorbic acid-2-*O*-phosphate-6-*O*-laureate (AA2P6L), ascorbic acid-2-*O*-phosphate-6-*O*-myristate (AA2P6M) and ascorbic acid-2-*O*-phosphate-6-*O*-stearate (AA2P6S). AA derivatives' stability in medium under cell culture condition, in the presence and in the absence of 95D cells, was assessed by high-performance liquid chromatography assay. Cell viability and intracellular oxidative stress were measured by MTT assay and CDCFH assay, respectively. Wound healing assay and cell adhesion assay were used to investigate the antimetastatic activities against 95D cells in vitro, and the C57BL/6 mice model was used to evaluate the antimetastatic action in vivo. *Results*: All the three AA derivatives exhibited excellent stability, significantly different from AA. Results of MTT assay showed that IC_{50} values of the cytotoxicity of those AA derivatives, namely AA2P6L, AA2P6M and AA2P6S, were 38.46, 28 and 22.97 μg/ml, while the CDCFH assay indicated that EC_{50} values of antioxidant effects on 95D cells were 31.12, 33.51 and 38.31 μg/ml, respectively. Through the ratio of IC_{50} vs EC_{50} for AA derivatives, AA2P6L was demonstrated to be the most effective AA derivative, which retained the antioxidant ability as well as low cytotoxicity. AA2P6L dose-dependently inhibited 95D cells' migration and adhesion, by 50% at the concentration of 20 and 57 μg/ml, respectively. In the animal experiment, intraperitoneal administration of 75 mg/kg AA2P6L decreased the number of metastatic nodules by 62% and elevated the survival rate of C57BL/6 mice about onefold compared to the control group. *Conclusion*: AA2P6L, a lipophilic AA derivative with antioxidation, is shown to be a potent antimetastatic agent through the inhibition of tumor invasion. These results support future investigations on the feasibility of cancer chemotherapy with AA2P6L.

Keywords Ascorbic acid · Antioxidation · Metastasis · Tumor · Derivative · Cytotoxicity

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Introduction

Metastasis is one of the major causes of mortality in cancer patients [15, 18]. The inhibition of invasion and metastasis of cancer cells is of great significance in cancer treatment. However, the treatment towards metastasis is still far from satisfactory. Lack of effective drugs is responsible for this worrisome phenomenon. So it is critical to find new potent drugs with low cytotoxicity and high efficiency to fight metastasis [9, 19, 23].

The effects of reactive oxygen species (ROS) on tumor invasion and metastasis have increasingly drawn people's attention. Recently, some reports have pointed out that ROS plays an important role in cellular DNA synthesis and proliferation of cancer cells [2, 13, 14]. ROS is produced by human cancer cells themselves [36]. Tumor metastasis may be correlated to ROS [32, 34]. The superoxide anion released from cancer cells is possibly vital in basement membrane degradation [34]. Therefore, the inhibition of ROS generation by cancer cells themselves may inhibit invasion and metastasis [11, 12, 28].

Ascorbic acid (AA), the natural antioxidant, has been suggested to exert an antitumor action [4, 22], inhibit

DNA synthesis of cancer cells [1, 17], enhance the tumoricidal action of another antitumor agent [39] and control metastatic colony formation [38]. Moreover, the antitumor effect of AA is closely related to its antioxidation [10].

Nevertheless, previous studies have emphatically demonstrated that AA is not stable, which hindered its use for therapy of cancer patients [7]. Therefore, three new AA derivatives were obtained through chemical modifications based on AA. Among the three AA derivatives, we seek to establish the most effective one having antioxidant ability coupled with high stability and low cytotoxicity, and further to evaluate its anti-metastatic activities in vitro as well as in vivo.

Materials and methods

Materials

Ascorbic acid-2-*O*-phosphate-6-*O*-laureate (AA2P6L), ascorbic acid-2-*O*-phosphate-6-*O*-myristate (AA2P6M) and ascorbic acid-2-*O*-phosphate-6-*O*-stearate (AA2P6S) were obtained from the Institute of Bioengineering, East China University of Science and Technology. AA was purchased from Sigma (St. Louis, MO, USA). AA derivatives were added from an absolute DMSO stock solution, and the control cells were treated with the same amount of vehicle. The final DMSO concentration never exceeded 0.5% (v/v). Chemical structures of AA and AA derivatives are shown in Fig. 1.

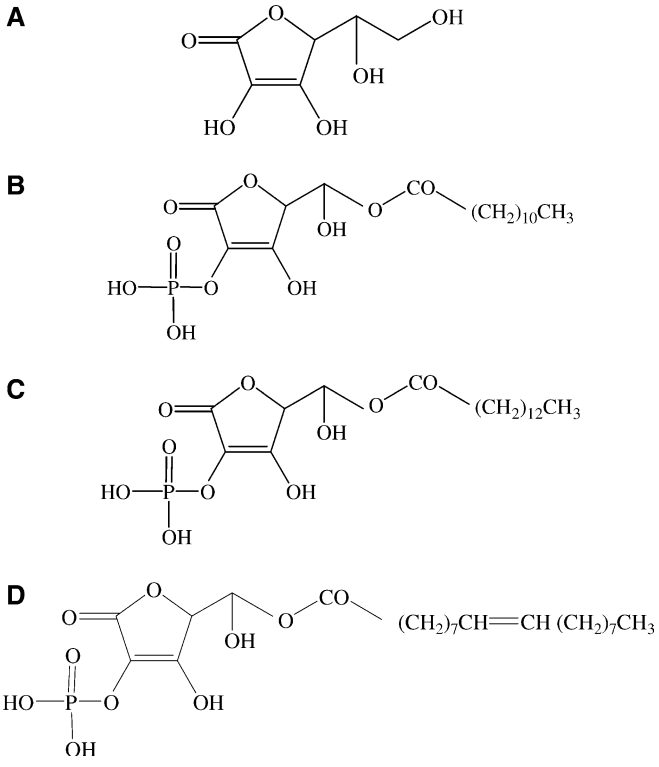

Fig. 1 **(a)** Ascorbic acid (*AA*); **(b)** ascorbic acid-2-*O*-phosphate-6-*O*-laureate (*AA2P6L*); **(c)** ascorbic acid-2-*O*-phosphate-6-*O*-myristate (*AA2P6M*); **(d)** ascorbic acid-2-*O*-phosphate-6-*O*-stearate (*AA2P6S*)

Cells and animals

Highly metastatic human lung carcinoma 95D cells and the highly metastatic substrain B16BL6 of mouse melanoma B16 cells were obtained from the Cell Bank of the Chinese Academy of Sciences, then were cultured in RPMI 1640 medium (GIBCO BRL, Grand Island, NY, USA) and 10% dialyzed heat-inactivated fetal bovine serum (FBS) (GIBCO BRL) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Male C57BL/6 mice (6 weeks old) were purchased from the Animal Center of the Chinese Academy of Sciences and maintained on standard chow and water.

Analysis of AA derivatives' stability in medium

AA and AA derivatives (10 μg/ml) were incubated in serum-free RPMI 1640 medium with 10% FBS at 37°C, 95% humidity and 5% CO_2. At different time points, aliquots were taken and used for a high-performance liquid chromatography (HPLC) assay with a reversed phase column (ZORBAX, Eclipse XDB-C_{18}, 5 μm, 4.6×250 mm, Agilent, USA). Chromatography was carried out with isocratic flow program. The flow rate of mobile phase (A, methanol; B, triethylamine; A/B = 40:60, v/v) was kept constant at 0.75 ml/min and the peaks were detected at 254 nm.

Intracellular AA derivatives concentration

A total of 2×10^4 95D cells were placed in culture dish (6 cm), and treated with and without AA derivatives (10 μg/ml) for 24 h. Then the culture medium was removed and cells collected with cell-police. After rinsing three times with PBS, the cells were crushed by ultrasonication for 30 min. After centrifugation (14,000 *g*, 30 min), the supernatant was used for the HPLC assay, in a similar manner to analysis of AA derivatives' stability in medium. The penetration ratio is defined as the ratio of a derivative's intracellar concentration vs its extracellar concentration.

Cell viability assay

Cell viability was determined by the MTT assay [31]. Briefly, 100 μl cells (10^5 cells/ml) were incubated in 96-well culture plates, with and without increasing amounts of AA derivatives. After 24 h of incubation, 10 mM of MTT (diluted in PBS) was added to each well for an additional 4 h. The blue MTT formazan precipitate was

dissolved in 100 μl of DMSO and measured on a microplate reader model 550 (Bio-Rad, CA, USA) at the absorbance wavelength of 570 nm and reference wavelength of 630 nm.

In situ detection of intracellular oxidative stress

Treatment of 95D cells with AA derivatives was carried out in the same manner as was done for cell viability assay. Cells in the presence and in the absence of AA derivatives were rinsed twice by PBS and were replaced by phenol red-free RPMI 1640 medium containing 10 μM 6-carboxy-2′,7′-dichlorodihydrofluorescein (CDCFH). After 50 min of incubation, the supernatant was removed, and phenol red-free fresh medium was added to the plate well for measurement of the intracellular oxidative stress. The fluorescence intensity was measured by a fluorescence plate reader CytoFluor2350 (Millipore, Bedford, MA, USA) with excitation and emission wavelengths of 485 and 538 nm, respectively.

Wound healing assay

For the wound healing assay, 95D cells were seeded into 24-well culture plates. A plastic pipette tip was drawn across the center of the well to produce a clean 1-mm-wide wound area after cells had reached confluence. After a 24 h culturing at 37°C in RPMI 1640 medium containing 10% FBS and different concentrations of AA2P6L, the migration distances between the leading edge of the migrating cells and the edge of the wound were compared. Migration rate = (migration distances of drug-treated cells/migration distances of untreated cells)×100%.

Cell adhesion assay

The 95D cells were pretreated with and without different concentrations of AA2P6L for 24 h. The cells were suspended in serum-free RPMI 1640 medium to form a single-cell suspension, then 200 μl cells (10^5 cells/ml) were seeded into 96-well culture plates, which had been pre-coated with matrigel (Collaborative Research, Bedford, MA, USA). After a 45 min incubation at 37°C, the wells were washed three times with PBS to remove non-adherent cells. Then 10 μM of MTT was added to each well for an additional 4 h. The blue MTT formazan precipitate was dissolved in 100 μl of DMSO and the absorbance at 570/630 nm was measured on a microplate reader model 550 (Bio-Rad).

Assay of tumor metastasis in an animal model

For injection into mice, cultured B16BL6 cells were detached with trypsin solution, washed twice with PBS, and made up to the cell density of 5×10^5 cells/ml in PBS. A portion (0.2 ml) was inoculated into 6-week-old male C57BL/6 mice through the tail vein. Next day, the drug treatment was started. The animals were divided into five groups of seven animals each. Drugs were dissolved in normal saline containing 0.4% Tween-80. AA2P6L was injected intraperitoneally (i.p.) into mice once a day for 2 weeks at a dose of 25, 50 or 75 mg/kg. The positive control was i.p. administrated vinblastine at a dose of 2 mg/kg, which was commonly used in patients with metastatic cancer [16, 26, 30]. The control group was i.p. administrated vehicle alone. After three weeks, the mice were sacrificed and their lungs were excised, rinsed and fixed in Bouin's solution. The total number of visible nodules on the lung surface per mice was counted.

Statistical analysis

Experimental values are expressed as the mean ± SD. The scientific statistical software GraphPad Instat version 2.04 was used to evaluate the significance of differences between groups with statistical significance considered as $^{*}\ p<0.05$, $^{**}\ p<0.01$ or $^{***}\ p<0.001$ and comparable variances were tested for significance by Student's t test.

Results

Analysis of AA derivatives' stability and intracellular concentration

The stability of AA derivatives under cell culture conditions was analyzed (Fig. 2a). In RPMI 1640 medium at 37°C and 5% CO_2, all three AA derivatives were quite stable and their amounts were still more than 80% of the original ones at the end of this experiment, while AA was unstable with a half-life <24 h and totally disappeared within 72 h.

For the analysis of intracellular concentration (Fig. 2b), penetration ratio of different AA derivatives (AA2P6L, AA2P6M and AA2P6S) into 95D cells were similar: 27.3, 26.4 and 27.7%, respectively; however, it was only 4.1% for AA.

From the results above, it is obvious that AA derivatives (AA2P6L, AA2P6M and AA2P6S) are of higher stability than AA, which makes it clear that the chemical modification on structures of AA is highly successful.

Effects of AA derivatives on viability and intracellular ROS of 95D cells

Effects of AA derivatives on the viability of 95D cells are presented in Fig. 3a. The data indicated that the cytotoxicity of AA derivatives was dose-dependent. As a result, the half inhibitory concentrations (IC_{50}) values of

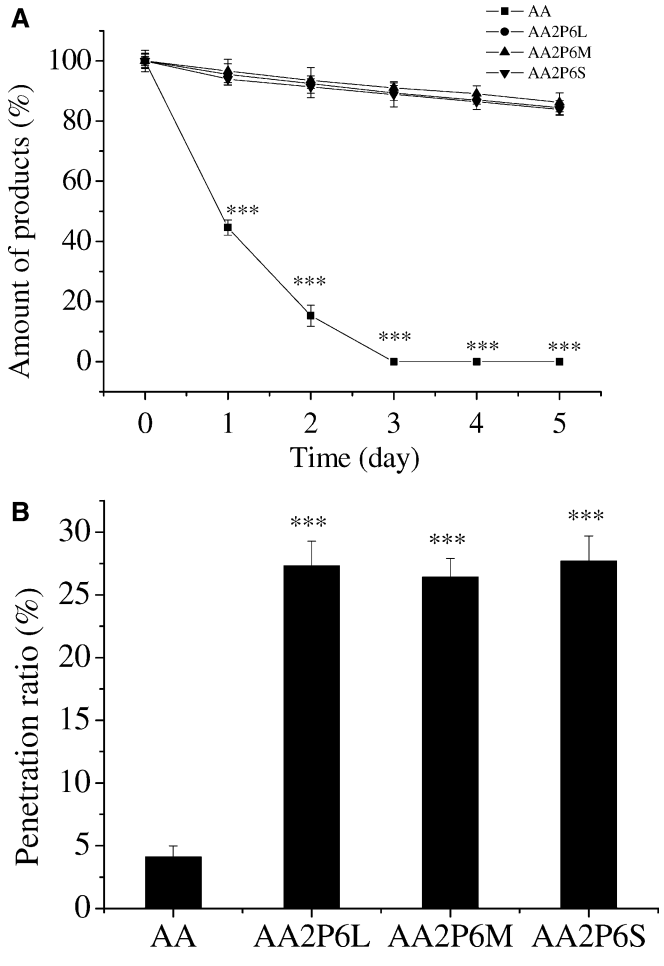

Fig. 2 Analysis of AA and AA derivatives' stability and intracellular concentration. **a** AA derivatives were incubated in serum-free RPMI 1640 medium with 10% FBS at 37°C, 95% humidity and 5% CO_2. At different time points, aliquots were taken and used for HPLC assay. **b** 95D cells were exposed to different concentrations of AA and AA derivatives for 24 h. Intracellular contents of AA and AA derivatives or their metabolites were quantified by HPLC. Data were presented as the mean ± SD of five parallel samples. **a** Significantly different from three AA derivatives groups: $^{***}p < 0.001$. **b** Significantly different from the AA group: $^{***}p < 0.001$

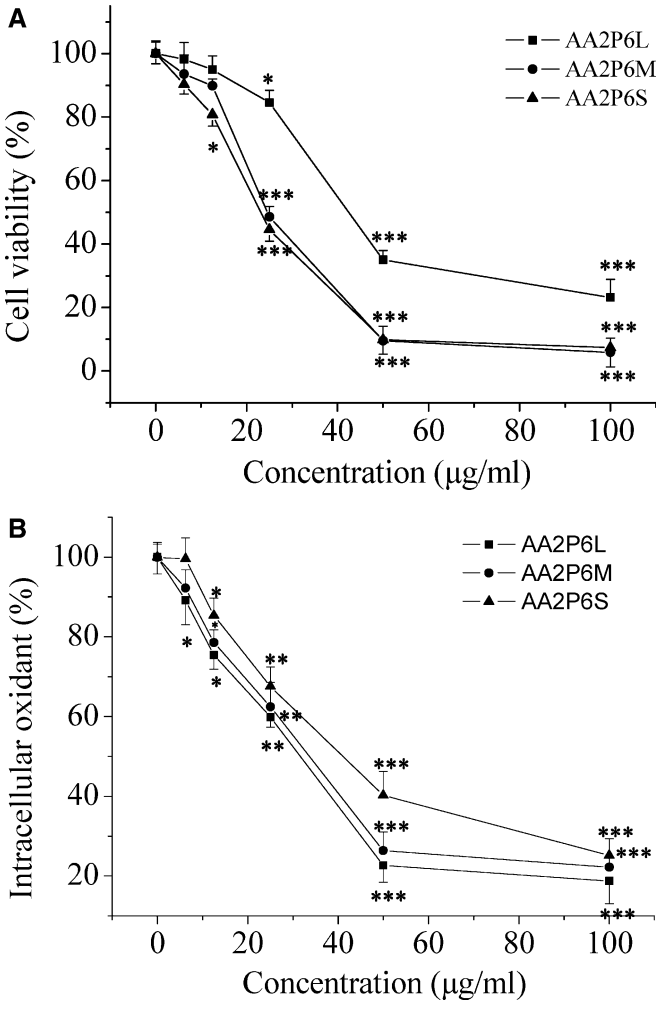

Fig. 3 Effects of AA derivatives on viability and intracellular ROS of 95D cells. **a** Cells were treated for 24 h in the presence and in the absence of AA derivatives in RMPI 1640 medium with 10% FBS at 37°C, 95% humidity and 5% CO_2. Cell viability was then determined by the MTT assay. **b** Cells were pretreated with and without different concentration of AA derivatives for 24 h, given CDCFH-DA, and incubated for 60 min, followed by measurement of absorbance at excitation and emission wavelengths of 485 and 530 nm. Data were presented as the mean ± SD of five parallel samples. Significantly different from the untreated group: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

AA2P6L, AA2P6M and AA2P6S were 38.46, 28 and 22.97 μg/ml, respectively.

According to the graph (Fig. 3b), the level of intracellular ROS in 95D cells was reduced with treatment of different AA derivatives in a dose-dependent way. The half effective concentration (EC_{50}) values of AA2P6L, AA2P6M and AA2P6S were as follows: 31.12, 33.51 and 38.31 μg/ml.

When counting up the above-mentioned results, the ratios of IC_{50} vs EC_{50} for AA derivatives (AA2P6L, AA2P6M and AA2P6S) were 1.236, 0.836 and 0.6, respectively, and followed the order: AA2P6L > AA2P6M > AA2P6S, successively.

Our study pointed out that, in the course of scavenging ROS in 95D cells, AA2P6L was the most effective one among the three AA derivatives, which had both characteristics such as low cytotoxicity and high efficiency of decreasing the ROS level in tumor cells. Since it is acknowledged that the level of ROS in cancer patients was closely interrelated to the metastatic capability of tumor, and the antitumor action of AA chiefly depended on its antioxidation, so next we examined the antimetastatic effect of AA2P6L on 95D cells in vitro and in vivo.

Effects of AA2P6L on migration and adhesion of 95D cells in vitro

To evaluate the antimetastatic activity of AA2P6L, we first assessed the inhibitive effect of AA2P6L on the

migration of 95D cells by the wound healing assay. As shown in Fig. 4a, the cellular motility was evidently controlled in a dose-dependent manner by AA2P6L; AA2P6L at 20 μg/ml significantly inhibited the migration of 95D cells by 50% ($p < 0.001$).

Similarly, the results of the adhesion experiment also displayed that AA2P6L was able to inhibit matrigel-mediated attachment of 95D cells dose-dependently, by 50% at 57 μg/ml AA2P6L in Fig. 4b. In addition, such inhibitory ability of AA2P6L is not attributable to direct cytotoxic action as determined by the trypan blue exclusion assay (data not shown).

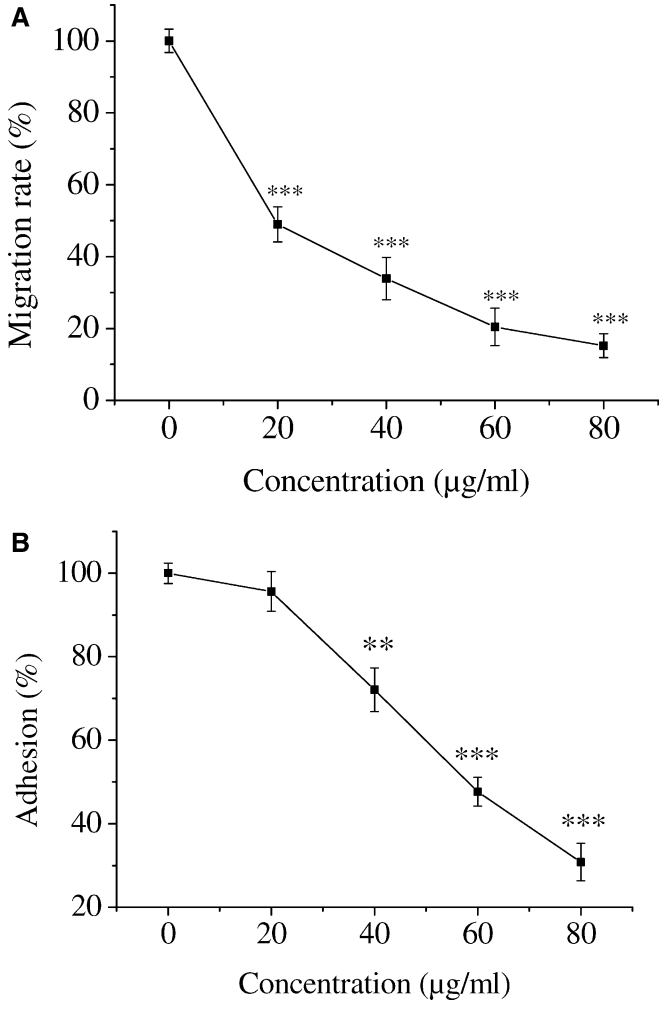

Fig. 4 Effects of AA2P6L on migration and adhesion of 95D cells. **a** Effect of AA2P6L on migration was tested by wound healing assays. A wound was introduced by scraping with a pipette tip when the cells had reached confluence. After 24 h incubation with and without different concentrations of AA2P6L, the migration distances were measured. **b** Effect of AA2P6L on cell adhesion on matrigel. 95D cells were treated with and without different concentrations of AA2P6L for 24 h and placed onto the wells precoated with matrigel. After a 45 min incubation at 37°C, the percentage of adhering cells were determined by MTT method. Data were presented as the mean ± SD of five parallel samples. Significantly different from the untreated group: ** $p < 0.01$, *** $p < 0.001$

On the basis of the wound healing assay and matrigel-mediated adhesion experiment, it can be concluded that AA2P6L could effectively inhibit the migration and invasion of 95D cells.

Effects of AA2P6L on pulmonary metastasis and host survival in vivo

As is known, lung metastasis of mouse melanoma is often a useful animal model on metastasis research, therefore, we chose this to further test the antimetastatic capability of AA2P6L in vivo. After inoculation with mouse melanoma B16BL6 cells, pulmonary metastatic foci were markedly scavenged by i.p. administration with AA2P6L on the experimental animals. And, as can be seen in Table 1, this inhibitory effect on pulmonary metastasis of AA2P6L was distinctly observed in a dose-dependent way. The inhibitory rate of metastatic nodules formation reached 62% in the case of administration with 75 mg/kg AA2P6L. Furthermore, the survival rate of mice was elevated from 43% (group 1, negative control) to 50% (group 2), 67% (group 3) and 83% (group 4) when the experiment was concluded. Meanwhile, AA2P6L did not cause body weight loss in mice during the whole treatment period (data not shown).

Discussion

Metastasis is one of the important factors related to cancer therapeutic efficacy and prognostic survival [37]. In cancer research, concerted efforts are on to develop novel antimetastatic drugs with low toxicity and high efficiency [9, 19, 23]. Previous studies have shown that AA could inhibit tumor metastasis [38]. However, it is unusable in cancer patients, as it is unstable. In order to enhance AA's stability, we obtained three lipophilic AA derivatives by the modification according to the chemical structure of AA. Through HPLC assay, all of those

Table 1 Effect of AA2P6L on pulmonary metastasis and host survival in vivo

Group	Treatment	Number of lung nodules[b]	Survival rate[a] (%)
1	Control	72.00 ± 16.90	43
2	AA2P6L (25 mg/kg)	64.00 ± 11.70*	50
3	AA2P6L (50 mg/kg)	48.00 ± 13.10**	67
4	AA2P6L (75 mg/kg)	27.50 ± 5.43***	83
5	Vinblastin (2 mg/kg)	15.75 ± 8.98***	86

Seven C57BL/6 mice per group were inoculated i.v. with B16BL6 cells (10^5 cells per mouse) and then injected i.p. with drugs once a day for 2 weeks. Experiments were repeated twice with similar results.

[a] The number indicated the percentage of surviving mice at the end of the experiment.

[b] Mean ± SD for each group of mice significantly different from the control group.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

three lipophilic AA derivatives could keep over 80% of the original concentration after 5 days in cell culture condition, and effectively penetrate across the cell membrane as well. The additional lipophilic structure of AA derivatives may have contributed to the improvement of their stability. Moreover, with the help of action between the lipophilic structure and the lipophilic cell membrane, AA derivatives were capable of penetrating into the tumor cells successfully and exert the antitumor activity more effectively.

AA is known for its antioxidation, which is the main reason for many of the physiological functions of AA [4, 10, 39], including the antimetastatic effect [38]. Whether these AA derivatives could decrease the ROS levels in tumor cells is necessary to be established for confirming their antitumor activities. In the present study, all the three AA derivatives used by us could markedly scavenge ROS of 95D cells. Nowadays, the antimetastatic pharmaceuticals under development are rapidly shifting from tumoricidal toxins to non-cytocidal or carcinostatic natural endogenous compounds [9, 19, 23]. Therefore, we also examined the IC_{50} values of AA derivatives' cytotoxicity on 95D cells. Then by the ratio of IC_{50} vs EC_{50} of antioxidation, AA2P6L was determined as the most effective AA derivative for its lowest cytotoxicity and highest efficiency of reducing the level of ROS in tumor cells. As a result, it is considered worthwhile exploring the antimetastatic capability of AA2P6L in vivo as well as in vitro.

During the complicated multi-step processes of cancer metastasis, tumor cell invasion of the basement membrane is one of the earliest critical steps [24, 35, 42] to occur, and several reports indicated that adhesive interactions of tumor cells with extracellular matrix (ECM) components play a key role in the establishment of metastasis [18, 27, 40]. In our study, cell adhesion assay indicated that AA2P6L could counteract the effect of adhesion on matrigel. At the same time, AA2P6L was also able to demonstrably inhibit migration and invasion of 95D cells in vitro by 50% at 20 μg/ml through the wound healing assay.

The formation of metastasis is the result of a series of complex interactions between the tumor cells and environment in the host [3, 8, 42]. That is to say, the environment in host influences the process of drug getting to its target, too. So taking the influence of host into account, we further tested the antimetastatic activity of AA2P6L in animal model. In line with the results in vitro, AA2P6L also could actively control cancer metastasis in vivo. These results above showed for the first time that AA2P6L has notable antimetastatic and anti-invasive capability in vitro as well as in vivo.

ROS is associated with cancer invasion and metastasis, while suppression of ROS had an inhibitory effect on tumor metastasis and invasiveness [20, 29, 33]. ROS is the critical messenger molecule in downstream signaling pathways, ultimately leading to the induction of invasion-related genes [5, 6, 21]; ROS is involved, at least in part, in the activation of nuclear factors kappa B (NF-κB) and matrix metalloproteinases (MMPs) [25, 34, 41]. According to results of our work in this article, the antimetastatic effect of AA2P6L possibly is because of its antioxidation. Further studies are now in progress in our laboratory to throw light on the antimetastatic mechanisms of AA2P6L, such as MMPs gene expression and the distribution of NF-κB between cytoplasm and nucleus.

In summary, our results indicated that the adhesive and invasive properties of tumor cells were reduced by AA2P6L derived from AA. These observations may suggest a mechanism for the action of AA2P6L in reducing the rate of lung metastasis in vivo. Based on the results of Fig. 3a and b, AA2P6L could decrease the ROS level of tumor cells, which may play an important part in the antimetastatic capability in this study.

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References

1. Abate C, Patel L, Rauscher RJ, Curran T (1990) Redox regulation of fos and jun DNA-binding activity in vitro. Science 249:1157–1161
2. Amstad P, Crawford D, Muehlematter D, Zbinden I, Larsson R, Cerutti P (1990) Oxidants stress induces the proto-oncogenes c-fos and c-myc in mouse epidermal cells. Bull Cancer 77:501–502
3. Andela VB, Schwarz EM, Puzas JE, O'Keefe RJ, Rosier RN (2000) Tumor metastasis and the reciprocal regulation of prometastatic and antimetastatic factors by nuclear factor κB. Cancer Res 60:6557–6562
4. Bissell MJ, Hatie C, Farson DA, Schawrz RI, Soo WJ (1980) Ascorbic acid inhibits replication and infectivity of avian RNA tumor virus. Proc Natl Acad Sci USA 77:2711–2715
5. Brenneisen P, Briviba K, Wlaschek M, Wenk J, Scharffetter KK (1997) Hydrogen peroxide (H_2O_2) increases the steady-state mRNA levels of collagenase/MMP-1 in human dermal fibroblasts. Free Radic Biol Med 22:515–524
6. Brizel DM, Scully SP, Harrelson JM, Layfield LJ, Bean JM, Prosnitz LR, Dewhirst MW (1996) Tumor oxygenation predicts for the likelihood of distant metastases in human soft tissue sarcoma. Cancer Res 56:941–943
7. Cameron E, Pauling L, Leibovitz B (1979) Ascorbic acid and cancer: a review. Cancer Res 39:663–681
8. Comoglio PM, Boccaccio C (2001) Scatter factors and invasive growth. Semin Cancer Biol 11:153–165
9. Cora NS (2003) Metastatic bladder cancer: role of chemotherapy and new agents. EAU Update Ser 1:108–117
10. Drake IM, Davies MJ, Mapstone MP, Dixon MF, Schorah CJ, White KL, Chalmers DM, Axon AT (1996) Ascorbic acid may protect against human gastric cancer by scavenging mucosal oxygen radicals. Carcinogenesis 17:559–562
11. Eskenazi AE, Pinkas J, Whitin JC, Arguello F, Cohen HJ, Frantz CN (1993) Role of antioxidant enzymes in the induction of increased experimental metastasis by hydroxyurea. J Natl Cancer Inst 85:711–721
12. Guven M, Ozturk B, Sayal A, Ozeturk A, Ulutin T (1999) Lipid peroxidation and antioxidant system in the blood of cancerous patients with metastasis. Cancer Biochem Biophys 17:155–162
13. Hockel M, Schlenger K, Aral B, Mitze M, Schaffer U, Vaupel P (1996) Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. Cancer Res 56:4509–4515

14. Huang P, Feng L, Oldham EA, Keating MJ, Plunkett W (2000) Superoxide dismutase as a target for the selective killing of cancer cells. Nature 407:390–395
15. Jemal A, Thomas A, Murray T, Thun M (2002) Cancer statistics. CA Cancer J Clin 52: 23–47
16. Joan HS, Barry S, Jordan B, Jay W, Martha L, Lisa P, Marilyn L, William B (1996) Amifostine, cisplatin, and vinblastine in metastatic non-small-cell lung cancer: a report of high response rates and prolonged survival. J Clin Oncol 14:1913–1921
17. Kagayama K, Onoyama Y, Otani S, Matsui-Yuasa I, Nagao N, Miwa N (1995) Enhanced inhibitory effects of hyperthermia combined with ascorbic acid on DNA synthesis in Ehrlich ascites tumor cells grown at a low cell density. Cancer Biochem Biophys 14:273–280
18. Kato R, Ishikawa T, Kamiya S, Oguma F, Ueki M, Goto S, Nakamura H, Katayama T, Fukai F (2002) A new type of antimetastatic peptide derived from fibronectin. Clin Cancer Res 8:2455–2462
19. Kohn EC, Liotta LA (1995) Molecular insights into cancer invasion: strategies for prevention and intervention. Cancer Res 55:1856–1862
20. Kozuki Y, Miura Y, Yagasaki K (2000) Inhibitory effects of carotenoids on the invasion of rat ascites liver cancer cells in culture. Cancer Lett 151:111–115
21. Lam EWN, Zwecka R, Seftor EA, Nieva DRC, Davidson BL, Engelhardt JF, Hendrix MJC, Oberley LW (1999) Effects of antioxidant enzyme overexpression on the invasive phenotype of hamster cheek pouch carcinoma cells. Free Radic Biol Med 27:572–579
22. Leung PY, Miyashita K, Young M, Tsao CS (1993) Cytotoxic effect of ascorbate and its derivatives on cultured malignant and nonmalignant cell lines. Anticancer Res 13:475–480
23. Levine E, Raghaven D (1993) MVAC for bladder cancer-time to move forward again. J Clin Oncol 11:387–389
24. Liotta LA, Wewer U, Rao NC, Schi.mann E, Stracke M, Guirguis R, Thorgeirsson U, Muschel R, Sobel M (1988) Biochemical mechanism of tumor invasion and metastasis. Prog Clin Biol Res 256:3–16
25. Liu JW, Nagao N, Kageyama K, Miwa N (1999) Antimetastatic and anti-invasion ability of phospho-ascorbyl palmitate through intracellular ascorbate enrichment and the resultant antioxidant action. Oncol Res 11:479–487
26. Maase HV, Hansen SW, Roberts JT, Dogliotti L, Oliver T, Moore MJ, Bodrogi I, Albers P, Knuth A, Lippert CM, Kerbrat P, Rovira PS, Wersall P, Cleall SP, Roychowdhury DF, Tomlin I, Visseren-Grul CM, Conte PF (2000) Gemcitabine and cisplatin versus methotrexate, vinblastine, doxorubicin, and cisplatin in advanced or metastatic bladder cancer: results of large, randomized, multinational, multicenter, phase III study. J Clin Oncol 17:3068–3077
27. Mareel M, Leroy A (2003) Clinical, cellular, and molecular aspects of cancer invasion. Physiol Rev 83:337–376
28. McCarty MF (1986) An antithrombotic role for nutritional antioxidants: implications for tumor metastasis and other pathologies. Med Hypotheses 19:345–357
29. McMullen CB, Fleming E, Clarke G, Armstrong MA (2000) The role of reactive oxygen intermediates in the regulation of cytokine-induced ICAM-1 surface expression on endothelial cells. Mol Cell Biol Res Commun 3:231–237
30. Michael BA, Jared AG, Jeffrey AS, David FM, Linda T, Patricia S, Robert A, James W (2002) A phase II pilot trial of concurrent biochemotherapy with cisplatin, vinblastine, temozolomide, interleukin 2, and IFN-α2B in patients with metastatic melanoma. Clin Cancer Res 8:3075–3081
31. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63
32. Mukai M, Shinkai K, Tateishi R, Mori Y, Akedo H (1987) Macrophage potentiation of invasive capacity of rat ascites hepatoma cells. Cancer Res 47:2167–2171
33. Nagao N, Etoh T, Yamaoka S, Okamoto T, Miwa N (2000) Enhanced invasion of tax-expressing fibroblasts into the basement membrane is repressed by phosphorylated ascorbate with simultaneous decreases in intracellular oxidative stress and NF-kappa B activation. Antioxid Redox Signal 2:727–738
34. Nonaka Y, Iwagaki H, Kimura T, Fuchimoto S, Orita K (1993) Effect of reactive oxygen intermediates on the in vitro invasive capacity of tumor cells and liver metastasis in mice. Int J Cancer 54:983–986
35. Ohnishi Y, Sakamoto T, Fujii H, Kimura F, Murata J (1997) Characterization of a liver metastatic variant of murine colon 26 carcinoma cells. Tumor Biol 18:113–122
36. Szatrowski TP, Nathan CF (1991) Production of large amounts of hydrogen peroxide by human tumor cells. Cancer Res 51:794–798
37. Sugiyama T, Sadzuka Y (1999) Combination of theanine with doxorubicin inhibits hepatic metastasis of M5076 ovarian sarcoma. Clin Cancer Res 5:413–416
38. Takenaga M, Igarashi R, Nakayama T, Mizushima Y (1999) Lecithinized ascorbic acid effectively inhibits murine pulmonary metastasis. Anticancer Res 19:1085–1091
39. Wells WW, Bocque PA, Xu DP, Meyer EB, Charamella LJ, Dimitrov NV (1995) Ascorbic acid and cell survival of adriamycin resistant and sensitive MCF-7 breast tumor cells. Free Radic Biol Med 18:699–708
40. Yamada KM, Kennedy DW, Yamada SS, Gralnick H, Chen WT, Akiyama SK (1990) Monoclonal antibody and synthetic peptide inhibitors of human tumor cell migration. Cancer Res 50:4485–4496
41. Yang JQ, Zhao W, Duan H, Robbins MEC, Buettner GR, Oberley LW, Doman FE (2001) v-Ha-Ras oncogene upregulate the 92-kDa Type IV collagenase (MMP-9) gene by increasing cellular superoxide production and activating NF-κB. Free Radic Biol Med 31:520–529
42. Zhu ZY, Sanchez-Sweatman O, Huang XJ, Wiltrout R, Khokha R, Zhao Q, Gorelik E (2001) Anoikis and metastatic potential of cloudman S91 melanoma cells. Cancer Res 61:1707–1716