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ORIGINAL ARTICLE

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Angiostatin potentiates cyclophosphamide treatment of metastatic disease

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Abstract *Purpose:* We examined the interaction between cyclophosphamide (CPA) and angiostatin (AS) on the growth of primary Lewis lung carcinoma (LLC) tumors and on the development of LLC pulmonary metastases. We studied the effects of AS and CPA on the stages of angiogenesis employing in vitro assays. *Methods:* Primary tumor growth and pulmonary metastases were measured to evaluate the effects of treatment with AS alone, CPA alone or the combination of CPA and AS. We examined the effects of CPA plus AS on endothelial cell (HUVEC) survival, migration and tube formation. *Results:* Combined treatment with CPA and AS did not significantly affect primary tumor growth when compared with CPA treatment alone. However, a significant decrease in the number of pulmonary metastases was observed following CPA plus AS treatment when compared with CPA treatment alone ($P < 0.001$). AS did not enhance CPA-mediated HUVEC cytotoxicity, and CPA failed to enhance AS-mediated inhibition of migration. However, tube formation was inhibited following combined treatment with CPA and AS when compared with either treatment alone. *Conclusions:* AS enhanced the antimetastatic effects of CPA without significantly influencing the effects of CPA on primary tumor growth. CPA plus AS inhibited tube formation, suggesting that interrupting specific steps in the angiogenesis process might be an effective approach to the treatment of subclinical distant metastases.

Keywords Angiostatin · Cyclophosphamide · Lewis lung carcinoma · Metastasis

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Introduction

Many patients with cancer have subclinical metastatic disease at the time of diagnosis of the primary tumor and eventually die of distant metastases. The use of adjuvant chemotherapy and/or hormonal therapy in the absence of detectable disease has been shown to be effective in delaying or preventing the development of metastases. Adjuvant chemotherapy has led to important improvements in cure rates for some adult cancers, such as colon and breast, as well as a variety of pediatric cancers. However, in spite of adjuvant chemotherapy, many patients with advanced localized colon cancer and breast cancer still develop distant disease. Currently, no effective adjuvant chemotherapy is available to treat certain other cancers, including lung and prostate cancer. Therefore, improvement in adjuvant treatment would represent a major advance in cancer treatment.

For a tumor to grow into a solid mass, it must develop a blood supply [1]. Tumor vessels are derived from preexisting vessels in the surrounding tissue, in which the normally quiescent host vasculature responds to an angiogenic signal released by the tumor cells [2].

Steps in tumor angiogenesis include invasion of the basement membrane, proliferation and migration of endothelial cells, and coalescence of endothelial cells into tube-like structures [3, 4, 5, 6]. Folkman and colleagues hypothesized that tumors secrete a variety of proteins that enhance tumor blood vessel growth as well as a variety of antiangiogenic proteins [7, 8]. One of the first antiangiogenic proteins isolated was angiostatin (AS), a 38-kDa cleavage product of plasminogen [9]. The identification of AS led to the hypothesis that antiangiogenic proteins could be used as antitumor agents. In numerous studies, recombinant AS or AS purified from plasma has demonstrated antitumor effects. In animal experiments, these effects range from complete elimination of tumors [10] to slowing of tumor growth [11, 12, 13]. Several experimental systems have shown that AS interacts with radiation therapy to mediate synergistic antitumor effects by enhancing radiation cytotoxicity of tumor vessels [14]. However, when AS was administered alone at doses ranging from 25 to 50 mg/kg per day, primary tumor growth was slowed but tumor regression was not observed [14, 15].

Lewis lung carcinoma (LLC) is a widely employed tumor model for studying the effects of treatment with cytotoxins and antiangiogenic agents. Based on our previous results employing AS and ionizing radiation [14], we hypothesized that combined treatment with AS and alkylating agents, such as cyclophosphamide (CPA), would produce antitumor effects. In contrast to the AS/ionizing radiation studies, combined treatment with AS and CPA did not slow the growth of primary LLC tumors. However, a reduction in the number of pulmonary metastases was observed. We investigated potential mechanisms for the observed reduction in pulmonary metastases employing in vitro angiogenesis assays. Combined treatment with AS and CPA inhibited tube formation, a critical component of blood vessel formation.

Materials and methods

Reagents

Murine AS, a gift from Dr. Vikas Sukhatme (Beth Israel Deaconess Medical Center, Boston, Mass.), was cloned and expressed using a yeast expression system as previously described [14]. For in vivo experiments, CPA was purchased from Pharmacia & Upjohn (Kalamazoo, Mich.). For in vitro experiments, 4-hydroperoxycyclophosphamide (4-HC) was obtained as a gift from Dr. Susan M. Ludeman (Duke University Medical Center, Durham, N.C.).

Cell culture

LLC-LM tumor cells, a gift from Dr. Judah Folkman, were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics and maintained in EGM-2 medium (Clonetics Corporation, San Diego, Calif.).

Clonogenic assay

HUVEC (200 to 2×10^4) were plated in 100-mm tissue culture dishes. The culture medium was the same as above except Clonetics VEGF was replaced with 10 ng/ml VEGF from R&D Systems (Minneapolis, Minn.). 4-HC at 0.1, 0.2, 0.3, 0.5, 0.7, or 1.0 μg/ml was added 18 h after plating. For combined treatment, 1.0 or 100 ng/ml AS was added 4 h prior to the addition of 4-HC. Cultures were incubated for 14–17 days, after which they were stained with crystal violet, colonies were counted, and the surviving fractions were determined. Colonies containing >50 cells were scored as positive.

Endothelial cell migration assay

Transwell 6.5-mm inserts with 5-μm pore size membranes (Corning, Corning, N.Y.) were coated overnight with 100 μg/ml collagen type I (Collaborative Biomedical Products, Bedford, Mass.) in 0.2 *N* acetic acid. The inserts were rinsed with phosphate-buffered saline (PBS) and allowed to dry 24 h before the assay, HUVEC growth medium was changed to EBM-2 (Clonetics) plus 0.1% bovine serum albumin (BSA) plus 10% of the normal quantities of serum and growth factors. The medium was changed to EBM-2 plus 0.1% BSA 2 h prior to the addition of AS, and 100 ng/ml AS was added 4 h prior to subculture. The HUVEC were subcultured and resuspended at 3×10^6 cells/ml in EBM-2 plus 0.1% BSA containing 100 ng/ml AS, 0.2 μg/ml 4-HC, or the combination. Cell suspension (100 μl) was added to the Tran swell insert, and 600 μl EBM-2 plus 0.1% BSA plus 20 ng/ml VEGF (R&D Systems) was added to the well. Transwells were incubated in a humidified chamber at 37°C for 16 h. The inserts were rinsed with PBS and fixed and stained with a Diff-Quick Stain set (Dade International, Miami, Fl.) and allowed to dry. The membranes were removed and mounted on microscope slides with Permount. The slides were scored by counting the number of migrated cells per ten high-power fields per treatment group.

Tube formation assay

The tube formation assay was performed as previously described [16]. Briefly, 250 μl Matrigel (Collaborative Biomedical Products, Bedford, Mass.) was added per well of a 24-well dish and allowed to polymerize for at least 30 min at 37°C. AS-treated cultures of HUVEC were incubated with 100 ng/ml AS 4 h prior to subculture. After subculture, 50,000 HUVEC per well were added in EGM-2 with 1% fetal bovine serum, 3 ng/ml VEGF (R&D Systems), 100 ng/ml AS, 0.5 μg/ml 4-HC, or the combination, and incubated overnight at 37°C in a humidified incubator in an atmosphere containing 7% CO_2. The final volume per well was 1.25 ml including the Matrigel. Negative control medium was EGM-2 with all additives except fetal bovine serum and VEGF. After incubating for 18 h, the cells were examined using a Zeiss inverted microscope and photographed using Tmax 400 film (Kodak). Tube formation was scored by blind analysis of the photographs by two individual investigators.

In vivo experiments

C57Bl/6 mice were injected subcutaneously (s.c.) in the right hind limb with 1×10^6 LLC tumor cells in 100 μl serum-free medium. After 3 days, mice were assigned to four experimental groups. Control animals ($n=8$) were injected with 400 μl PBS (divided into two equal doses of 200 μl) intraperitoneally (i.p.) daily for 7 days. Animals treated with CPA ($n=22$) were injected i.p. with 75 mg/kg per day (200 μl) on days 4–7 to a total dose of 300 mg/kg. Mice in the AS group ($n=17$) were injected i.p. with 5 mg/kg per day (200 μl) on days 3–9 to a total dose of 35 mg/kg. Animals in the combined treatment group (CPA plus AS, $n=24$) were injected with CPA and AS according to the same schedules.

Tumor volumes were determined every other day as previously described [17]. Mice were killed on days 10, 12, 14, 17, 19 and 21.

Table 1. Mean volume of primary LLC tumors on day of death following treatment with AS and CPA. C57Bl/6 mice were injected s.c. into the right hind limb with 1×10^6 LLC tumor cells in 100 µl serum-free medium. Treatment with AS (5 mg/kg i.p. on days 3–9) and CPA (75 mg/kg i.p. on days 4–7) was initiated 3 days after cells were injected. Control animals were injected with PBS. Tumor volumes were determined by direct measurement with calipers. A generalized linear model was fitted to the tumor volumes using a logarithmic link function and gamma variance function as described in Methods. Data are reported as mean ± SD tumor volumes (mm^3) and the results of two separate experiments are shown

Experiment	Day	Treatment group			
		PBS	AS	CPA	AS plus CPA
A	17	3102.0 ± 810.3 ($n=2$)	2378.0 ± 1078.4 ($n=3$)	1171.0 ± 277.2 ($n=2$)	1115.3 ± 411.7 ($n=3$)
	19		3464.7 ± 319.4 ($n=3$)	2118.0 ± 277.2 ($n=2$)	1709.7 ± 130.3 ($n=3$)
	21		4322.2 ± 478.9 ($n=4$)	3221.0 ± 1373.2 ($n=2$)	3229.0 ± 258.7 ($n=3$)
B	17	5120.5 ± 235.5 ($n=2$)	3084.0 ± 1284.1 ($n=2$)	923.3 ± 496.5 ($n=3$)	861.7 ± 555.5 ($n=3$)
	19	4342.5 ± 1376.7 ($n=2$)	5676.0 ± 1551.4 ($n=3$)	1721.3 ± 350.6 ($n=2$)	1732.7 ± 215.0 ($n=3$)
	21	1452.0 ± 834.4 ($n=2$)	3240.0 ± 1959.3 ($n=3$)	1837.1 ± 593.5 ($n=7$)	3229.0 ± 1025.8 ($n=6$)

Both lungs were removed and placed in neutral buffered formalin. Paraffin-embedded tissues were sectioned at 6 µm and stained with hematoxylin and eosin. Total numbers of metastases in both lungs were determined using light microscopy at ×100 magnification by an investigator blinded to the treatment groups. The care and treatment of animals was in accordance with the institutional guidelines established by the Animal Care and Use Committee at the University of Chicago.

Statistical analysis

A generalized linear model [18] was fitted to the primary tumor volumes. A logarithmic link function and gamma variance function were used. Since each animal had multiple volume measurements (every other day), the model was fitted using the Generalized Estimating Equations (GEE) approach with an exchangeable correlation structure [19]. Standard deviations were obtained using the sandwich (robust) variance estimator. Covariates included indicator variables for experiment, treatment group, and day following injection of tumor cells.

A generalized linear model was also fitted to the number of lung metastases, using a logarithmic link function and gamma variance function as above. The primary evaluation of the treatment effects was based on a model including indicator variables for experiment, treatment group, and day. The log weight of the primary tumor was then added to the model (via a separate linear term for each day of death) to determine how this affected the estimated differences between treatment groups.

Computations were performed using the Stata statistical software package, release 7.0 (Stata Corporation, College Station, Tx.). All reported *P*-values are two-sided.

Results

CPA but not AS inhibits primary tumor growth

In both experiments, treatment was initiated 3 days following the injection of tumor cells into the hind limb. Treatment was initiated prior to the appearance of a palpable tumor, and no attempt was made to size-match primary tumors in the four treatment groups. Mean tumor volumes were determined on the day of death. At the end of the treatment period (day 10), mean tumor volumes in the CPA-alone groups were 61% smaller ($P<0.001$) than in the PBS control groups while mean tumor volume in the AS-alone group was 12% smaller than in the control group. Combined treatment with CPA and AS did not lead to a significant reduction in mean primary tumor volume compared with CPA alone ($P=0.538$) on days 17, 19 and 21 (Table 1). These findings suggest that AS does not significantly enhance the antitumor effects of CPA on the growth of primary LLC tumors.

AS combined with CPA reduces the mean number of pulmonary metastases compared with CPA alone

We next examined the effects of treatment on the development of LLC pulmonary metastases. From day 10 to day 21 following the initiation of treatment, the mean number of lung metastases across all four treatment groups increased by 52% per day ($P<0.001$) in the first experiment (Fig. 1, experiment A). In the second experiment (Fig. 1, experiment B), the mean number of lung metastases increased by 14% per day ($P=0.138$). During this period, the mean number of metastases was 82% lower in the CPA-alone groups ($P=0.001$) compared with the PBS control groups. Treatment with AS alone did not significantly reduce the number of metastases when compared with control. Importantly, the mean number of metastases in the groups treated with CPA and AS was 83% lower ($P<0.001$) than in the CPA-alone groups. The number of pulmonary metastases was positively correlated with the volume of the primary tumor. However, controlling for the volume of the primary tumor on the day of death indicated that treatment with CPA alone should produce only a 37% reduction in the number of metastases compared with PBS. The 82% reduction in the number of metastases following CPA treatment suggests that the effects of treatment with CPA alone on the number of pulmonary metastases reflects the antitumor effects of CPA on the growth of primary tumors.

Exposure to AS does not enhance 4-HC-mediated cytotoxicity of HUVEC

Because treatment with AS in vivo was associated with a reduction in the number of pulmonary metastases, we evaluated the effects of AS and CPA on the various

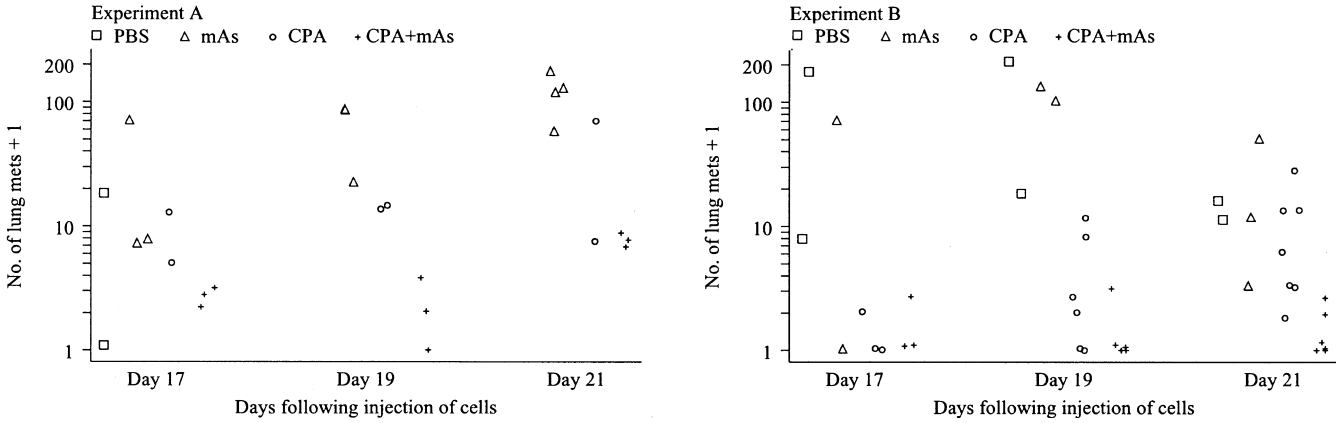

Fig. 1. The number of LLC pulmonary metastases for experiments A and B. Mice were killed at the indicated time points. Both lungs were removed, placed in neutral buffered formalin, paraffin-embedded, sectioned and stained with hematoxylin and eosin. Total numbers of metastases were determined at ×100 by a blinded investigator. A generalized linear model was fitted to the tumor volumes using a logarithmic link function and gamma variance function

components of the angiogenesis process beginning with endothelial cell survival. HUVEC cells were plated and 18 h later exposed to increasing concentrations of 4-HC in the presence or absence of AS. Neither 1 ng/ml nor 100 ng/ml of AS enhanced 4-HC-mediated cytotoxicity of HUVEC cells (Fig. 2). These findings demonstrate that AS does not enhance 4-HC-mediated endothelial cell cytotoxicity and suggest that AS does not interact with 4-HC at the proliferation stage of angiogenesis.

AS but not 4-HC inhibits endothelial cell migration

We next sought to determine whether 4-HC alone, or in combination with AS, inhibited endothelial cell migration. Migration assays were performed in which HUVEC cells were exposed to AS, 4-HC or the combination in the presence of VEGF for 16 h. The number of migrating cells per ten high-power fields in each treatment group was counted using light microscopy. We found that treatment with AS alone inhibited HUVEC migration by 10.6% (compared with the positive control). However, treatment with 4-HC alone did not inhibit migration (Fig. 3). Furthermore, when endothelial cells were exposed to the combination of 4-HC and AS, no further inhibition of HUVEC migration was observed when compared with AS alone. These findings demonstrate that AS, but not 4-HC, can inhibit endothelial cell migration.

Combined treatment with AS and 4-HC inhibits tube formation

We next examined the effects of treatment with AS and 4-HC on the formation of capillary tubes. The effects of each agent were evaluated by light microscopy and compared with positive and negative controls (Fig. 4). Neither AS alone (Fig. 4C) nor 4-HC alone (Fig. 4D) significantly affected tube formation when compared with the positive control (Fig. 4A). However, inhibition of tube formation was observed in HUVEC cultures treated with the combination of AS and 4-HC (Fig. 4E).

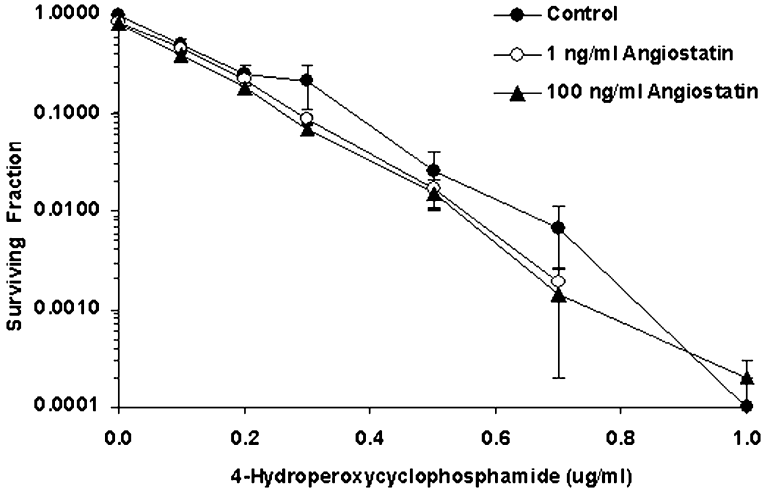

Fig. 2. Clonogenic survival of HUVEC following treatment with AS (angiostatin) and 4-HC (4-hydroperoxycyclophosphamide). HUVEC were plated and exposed to 10 ng/ml VEGF. Cultures were treated with AS 14 h later and 4-HC 18 h later. Cultures were incubated for 14–17 days, stained with crystal violet, colonies counted and surviving fractions determined. The means ± SEM of four experiments are shown

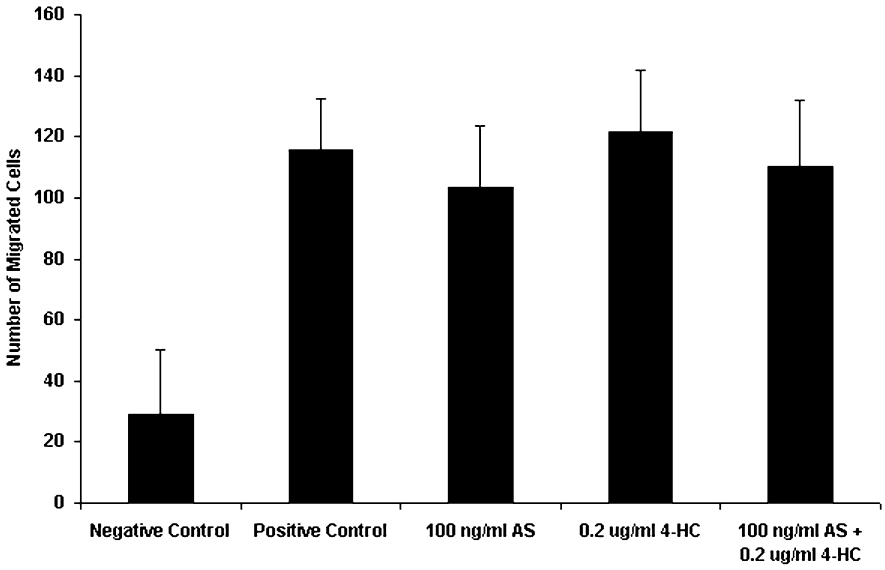

Fig. 3. HUVEC migration following treatment with AS and 4-HC. Migration assays were performed using collagen-coated transwell membranes. HUVEC were exposed to AS (100 ng/ml) and 4-HC (0.2 μg/ml) 4 h prior to subculture. Cell suspension (100 μl) was added to transwell inserts and incubated in medium containing BSA and VEGF at 37°C for 16 h. Inserts were rinsed, fixed and stained. The numbers of migrated cells per ten high-power fields per treatment group are shown

The morphology of the cells treated with 4-HC and AS was similar to the that of the negative control cells not exposed to VEGF (Fig. 4B). These findings suggest that the combination of AS and 4-HC inhibits angiogenesis at the stage of tube formation.

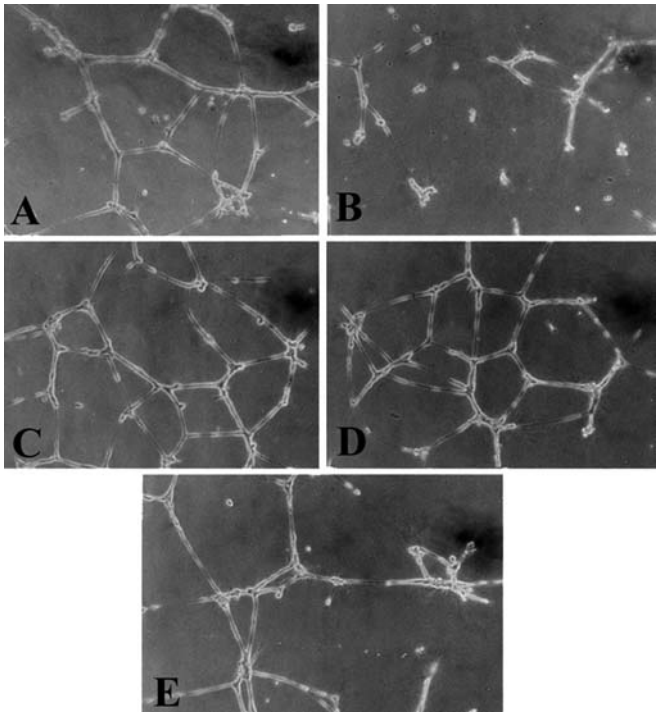

Fig. 4A–E. HUVEC tube formation following treatment with AS and 4-HC. Tube formation assays were performed using Matrigel-coated plates. HUVEC were exposed to AS (100 ng/ml) and 4-HC (0.5 μg/ml) 4 h prior to subculture. After subculture HUVEC were incubated in medium containing BSA and VEGF at 37ºC for 18 h. Cells were examined, photographed and scored using blind analysis of photographs (**A** positive control, **B** negative control, **C** AS treatment, **D** 4-HC treatment, **E** AS plus 4-HC treatment)

Discussion

The objective of the present studies was to determine whether antiangiogenic agents could successfully be combined with alkylating agents for adjuvant chemotherapy. LLC was chosen as the model for the present studies because this tumor type has a rapid doubling time (20.4 h) [20] and is relatively resistant to many cancer therapies [21]. Once the primary tumor has reached a volume of 400 mm^3, one to four lung metastases are discernible [22]. It has previously been reported that treatment with CPA produces a 19-day growth delay in LLC tumors [23] and that the addition of antiangiogenic agents to cytotoxic therapy is effective against metastasis development [24]. Teicher et al. have conducted extensive studies combining treatment with various cytotoxins (CPA, Adriamycin, CDDP, BCNU and 5-fluorouracil) and antiangiogenic compounds (TNP-470, minocycline, suramin and genistein) [23]. The conclusion drawn from these studies was that antiangiogenic agents, while ineffective when used alone, could be effective adjuvants when employed in combination with cytotoxic therapies. Importantly, the treatment schedule that was the most effective in slowing the growth of LLC primary tumors was not necessarily the most beneficial course of therapy to reduce the number of pulmonary metastases [21].

In the present studies we randomized experimental animals and began treatment with AS prior to the appearance of a primary tumor in the hind limb. The total amount of CPA given to each mouse (75 mg/kg per day to a total dose of 300 mg/kg) in the present experiments was 33% lower that that employed by Teicher et al. [21, 23]. The CPA dosing schedule employed in the present studies was selected to allow optimal evaluation of a potential interaction between AS and CPA. We observed that treatment with CPA alone significantly

reduced the growth of primary LLC tumors. However, the addition of angiostatin to CPA treatment did not produce further reduction in primary tumor growth. These findings may be associated with the AS dosing schedule employed. We selected the CPA dose based on dose response studies conducted in our laboratory (data not shown) and the AS dose based on our previously published studies [14]. The present findings suggest that AS enhances the antimetastatic effects of CPA without significantly influencing the effects of CPA on primary tumor growth.

In the present studies, AS did not enhance the cytotoxic effects of 4-HC against endothelial cells. The lack of interaction between 4-HC and AS differs from the interactive cytotoxicity observed between AS and ionizing radiation [25]. The differences between the results of the two studies may be related to the doses of ionizing radiation and 4-HC employed, and/or differences in the mechanisms of action of the two agents.

We have previously reported that exposure to 1000 ng/ml of AS produces a 30% inhibition of endothelial cell migration [25]. In the present studies, a lower dose of AS (100 ng/ml) also inhibited HUVEC migration but to a lesser extent (10.6% inhibition). Importantly, when HUVEC cultures were treated with the combination of AS and 4-HC, no further inhibition of migration was observed. These findings confirm that AS inhibits endothelial cell migration and demonstrate that 4-HC does not appear to affect this stage of angiogenesis.

Our in vivo results demonstrating that combined treatment with AS and CPA was associated with a reduction in pulmonary metastases compared with either treatment alone correlate with the results of the tube formation experiments. These in vitro assays demonstrated that combined treatment with AS and 4-HC inhibits tube formation, while neither compound used as a single agent appears to exert an inhibitory effect. Taken together, the in vitro experiments suggested that both AS and CPA exert inhibitory effects on endothelial cells but at different stages of the angiogenesis process. An interaction between AS and CPA was observed during the later stage of angiogenesis involving tube formation. Although in vitro assays of angiogenesis do not necessarily reflect in vivo conditions, information from these assays can be used to evaluate in vivo observations. The combination of CPA and AS may inhibit the ability of tumor cells to attach to the pulmonary vascular endothelium [26], which would result in a reduction in the number of metastases in the lungs of mice in the combined treatment (AS plus CPA) group. The present studies suggest that combining antiangiogenic compounds with different classes of DNA-damaging agents may be useful for the treatment of human cancers.

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