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Antiangiogenic effect of alpha-anordrin *in vitro* and *in vivo*[1]

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KEY WORDS α-anordrin; angiogenesis factor; choriocarcinoma; umbilical veins; vascular endothelium; nitric oxide

ABSTRACT

AIM: To study the antiangiogenic effect of α-anordrin (α-Ano), a partial antagonist of estrogen receptor. **METHODS**: The *in vivo* inhibitory effect of α-Ano on angiogenesis was determined by microvascular density (MVD) in tumors and the chicken chorioallantoic membrane (CAM) model. The *in vitro* effects of α-Ano on proliferation, migration, and attachment of human umbilical vein endothelial cells (HUVEC) were assessed by trypan blue exclusion, wound-induced two-dimensional migration model, and their ability to adhere to type I collagen, respectively. The possible involvement of nitric oxide (NO) in α-Ano antiangiogenic effect was determined by measuring NO content using fluorescent assay. **RESULTS**: α-Ano significantly inhibited the MVD in Lewis lung carcinoma model and this effect was correlated with its inhibition of the tumor growth. α-Ano also showed an inhibitory effect on the angiogenesis of CAM with the inhibitory rate of 53 % and such action of α-Ano could not be blocked by simultaneous administration of 17β-estrodiol, a typical agonist of estrogen receptor. *In vitro* studies showed that α-ANO obviously suppressed the proliferation and migration of HUVEC, but had no obvious effect on the attachment of HUVEC to the type I collagen. Moreover, α-Ano significantly reduced the level of NO released by HUVEC in a dose- and time-dependent manner. **CONCLUSION**: α-Ano possesses an antiangiogenic effect, and this effect is mediated, at least in part, by reducing the NO content and subsequently inhibiting the proliferation and migration of endothelial cells.

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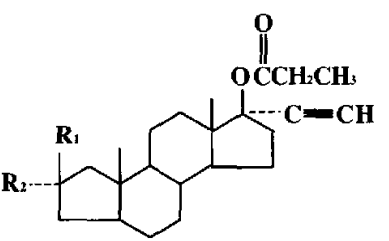
INTRODUCTION

Angiogenesis, the formation of new blood vessels from pre-existing vessels, is necessary for both growth and metastasis of solid tumors[1-3]. Thus inhibition of angiogenesis becomes a promising therapeutic approach for the control of tumor growth, progression, and metastasis[4]. Anordrin, usually containing alpha- and beta-isomers, is a contraceptive drug developed in China[5] which possesses partial antiestrogenic property[6]. Previous studies in our laboratory demonstrated that the alpha isomer of anordrin had potent antitumor activity[7]. However, its antitumor mechanism is still unclear. The present study was undertaken to investigate the antiangiogenic activity of α-Ano.



α R_1: C≡CH R_2: $OCOC_2H_5$ β R_1: $OCOC_2H_5$ R_2: C≡CH

Anordrin

MATERIALS AND METHODS

Reagents Ano was purchased from Shanghai 19th Pharmaceutical Factory and its alpha isomer was isolated by low pressure column chromatography in our institute. Anti-human von Willebrand factor antibody was purchased from Daka Co. Medium 199 was obtained from Gibco/BRL. All-*trans* retinoic acid (ATRA), 17β-estrodiol and 2,3-diaminonaphthalene were purchased from Sigma Co. Fertilised chicken eggs were from Shanghai Qibao Farm. All the other reagents were of analytical grade.

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Tumor transplantation and drug administration Female C57 BL/6 mice (weighing 20 g ± 2 g) were supplied by Shanghai Experimental Animal Centre, Chinese Academy of Sciences (Grade Ⅱ, Certificate № 005). Lewis lung carcinoma was maintained by serial transplantation into C57 BL/6 mice in our laboratory. Tumor cell suspensions (10^9 to 10^{10}/L) were prepared by gently suspending 5 g of tumor tissue in 20 mL 0.85 % NaCl solution (pH 7.4) and 0.2 mL of this cell suspension was sc injected into each mouse. Mice were randomly divided into three groups (n = 10). α-Ano was *po* administered the day after tumor transplantation with the dosage as shown in Tab 1 once a day. On d 11, mice were killed and the tumors were removed, and tumor wet weight was immediately weighed. Then the tumors were finely minced and fixed in 4 % buffered formalin for further immunohistochemical examination.

Immunohistochemistry examination and microvascular density (MVD) quantification Two samples were taken from each animal for immunohistochemistry examination. Five μm sections of tumors that had been formalin-fixed and paraffin-embedded were deparaffinized, followed by hydration through graded ethanol series, and soaking in Tris-buffered saline (TBS, 0.05 mol/L Tris-HCl containing 0.15 mol/L NaCl, pH 7.4). The endogenous peroxidase activity was blocked by treating with methanol containing 0.3 % H_2O_2 at room temperature for 20 min. Non-specific reactions were blocked by incubating with the normal horse serum. The section samples were then incubated with rabbit anti-human von Willebrand factor antibody (1∶400) at 4 ℃ for 18 h. After a thorough wash with TBS, sections were incubated with biotinylated secondary antibody for 45 min followed by a 45-min incubation with avidin-biotin-peroxidase complex at 37 ℃ with thorough TBS washes between steps. The antigen-antibody complex was visualized with 3,3′-diaminobenzidine/H_2O_2. The sections were counterstained with methyl green, dehydrated, cleared, and mounted.

MVD of tumors was determined by the method of Maeda *et al* with minor modifications[8]. Sections were examined under low power microscope to identify the region of highest vessel density (the so-called hot spot). Two hot spots in each section were chosen and the vessels in 200 × field were counted in each hot spot. Large vessels with thick muscular walls or lumina greater than the sum of approximately eight red blood cells were not included. Single cell or cell clusters were counted. Counts were expressed as average number of micro vessels per × 200 field.

Chicken chorioallantoic membrane (CAM) assay[9] The chicken eggs were placed in a humidified CO_2 incubator (3 % CO_2/air) at 37 ℃. On d 4 of development, methylcellulose disks (10 μL of 0.45 % methylcellulose containing 2 % dimethyl sulfoxide and the testing compounds was air dried on a metal tray) were placed on the chorioallantoic membrane. The zone around the disks was examined 48 h after disk placement. Angiosuppression was indicated by an avascular zone of ⩾3 mm diameter around the disk. The results were expressed as the percentage of eggs showing inhibition of angiogenesis.

Human umbilical vein endothelial cells (HUVEC) culture HUVEC were isolated from fresh umbilical cords[10] and cultured in Medium 199 containing 20 % heat-inactivated calf serum, benzylpenicillin 100 ku/L, streptomycin 100 mg/L, heparin 90 ku/L, glutamine 2 mmol·L^{-1} and endothelial cell growth factor extracts 500 μg/L (prepared from bovine brain according to the method of Maciag *et al*[11]). At confluence, >95 % of the cells were endothelial cells determined by their distinctive morphology and the presence of von Willebrand factor on the cell surface. HUVEC from serial passage 2 were used in the following assays.

Cell proliferation assays Exponentially growing HUVEC ($2 \times 10^8 \cdot L^{-1}$) were exposed to α-Ano 15 – 135 μmol/L for 2 – 7 d. Cell number was determined by trypan blue exclusion method.

Cell adhesion assay[12] Twenty-four-well plates were coated with 5 μg type Ⅰ collagen(rat tail collagen) and dried at room temperature overnight. HUVEC 5×10^4 which were either nonpretreated or pretreated for 48 h with 15 or 45 μmol/L α-Ano were seeded in each well. Then α-Ano was added to each well at corresponding concentration. After 2-h incubation in 5 % CO_2 at 37 ℃, the culture medium was removed. The plates were washed with PBS, and the washings were combined with the medium removed. The cells in the mixture were counted to determine the number of unattached cells. The washed plates were trypsinized and the number of attached cells was determined. The results were expressed as the percentage of attached cells.

Cell migration assay[13] The effect of α-Ano on the HUVEC migration activity was examined on the wound-induced two-dimensional migration model. Confluent monolayers of HUVEC were wounded with the sterile tip of a pipette, then washed twice. The culture

medium containing α-Ano was added. After 18-h culture, the total number of cells which had crossed the original wound edge in marked field was counted under phase contrast microscope. Results were expressed as the percentage of cells crossing the wound edge in treated groups in comparison with that in the control group.

Nitric oxide (NO) level measurement NO level of the HUVEC culture medium was indirectly determined by quantifying the nitrite content by the method of Misko *et al*[14]. One millilitre of HUVEC culture medium was mixed with 100 μL freshly prepared 2,3-diaminonaphthalene (0.05 g/L in 0.62 mol/L HCl). After a 10-min incubation at room temperature, the reaction was terminated with 50 μL of 2.8 mol/L NaOH. The content of the fluorescent product 2,3-diaminonaphthotriazole was determined with a fluorescence spectrophotometer with excitation wave at 365 nm and emission wave at 450 nm. The standard curve was made with sodium nitrite. The cell number was determined by trypan blue exclusion method. Results were represented by the nitrite content per 10^6 vital cells.

Statistics Data were expressed as $\bar{x} \pm s$ and compared with t test or Mamn-Whitney test.

RESULTS

Effects of α-Ano on MVD in Lewis lung carcinoma samples MVD in tumors is a convincing indication for evaluating the antiangiogenic effect of drugs and Lewis lung carcinoma model in mice is a frequently used for this purpose. After treatment with α-Ano 20 mg/kg, the MVD in drug-treated tumor samples was significantly reduced, and the inhibitory rate reached 55.4 % which was comparable with the inhibitory rate of 61.1 % on tumor growth by the same dosage treatment (Tab 1). The results suggest that inhibition of angiogenesis might be an important mechanism for α-Ano inducing inhibition of tumor growth.

Tab 1. Effects of α-Ano on microvascular density (MVD) ($n = 40$) and growth of Lewis lung carcinoma in mice ($n = 10$). $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Dose/ mg·kg^{-1}	MVD	MVD inhibition/%	Tumor weight/g	Growth inhibition/%
Control	56 ± 19	–	2.2 ± 0.8	–
10	36 ± 12	35.7^b	1.3 ± 0.6	42.3^b
20	25 ± 13	55.4^c	0.9 ± 0.3	61.1^c

Effect of α-Ano on angiogenesis in CAM model Inhibition of angiogenesis by α-Ano was further confirmed in CAM model. A marked inhibition of angiogenesis in CAM was observed after α-Ano treatment for 48 h. Interestingly, this effect of α-Ano on angiogenesis could not be blocked by 17β-estrodiol, a typical agonist of estrogen receptor, although previous research indicates that α-Ano displays antiestrogenic activity, suggesting that α-Ano inhibition of angiogenesis is not mediated via estrogen receptor. 17β-Estrodiol alone had no obvious inhibition on angiogenesis at the dosages of 50 μg/disk and 100 μg/disk (Tab 2).

Tab 2. Inhibitory effects of α-Ano on the angiogenesis of chicken chorioallantoic membrane.

Drugs/μg per disk	Number of eggs*/total	Inhibition rate/%
Control	1/20	–
α-Ano		
25	3/15	20
50	5/14	36
100	8/15	53
17β-estrodiol		
50	1/12	8
100	1/11	9
200	0/14	0
α-Ano + 17β-estrodiol		
50 + 50	4/10	40
50 + 100	5/15	33
100 + 200	9/16	56

*Number of positive eggs.

Effect of α-Ano on proliferation, migration, and attachment of HUVEC The proliferation, migration, and attachment of endothelial cells are critical steps in the development of new vessels. So, the effect of α-Ano on these processes was investigated using HUVEC as a model. After exposure of HUVEC to α-Ano 15 – 135 μmol/L for 2 – 7 d, the cell proliferation was inhibited in a dose- and time-dependent manner (Fig 1). On the 7th day, the growth of HUVEC was inhibited by 51 % by α-Ano 135 μmol/L. Only a few dyed cells existed in trypan blue exclusion assay following α-Ano treatment, suggesting that α-Ano may be having a cytostatic action but may not be cytotoxic to HUVEC.

We used the wound-induced migration model to investigate the effect of α-Ano on migration of endothelial cells. A great number of cells were observed in the wound region of control group at 6 h after wound treat-

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ment, and the wound region was completely filled again with HUVEC at 24 h after treatment. In the treatment groups, there were no or only a few cells in the wound region at 6 h, and markedly decreased number of cells entered the wound region at 24 h (Fig 2), indicating that the wound-induced two-dimensional migration of HUVEC was inhibited by α-Ano.

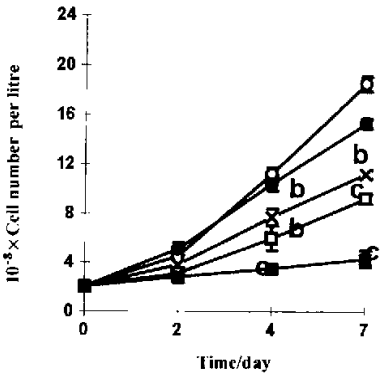

Fig 1. Inhibitory effect of α-Ano 0 (○), 15 (●), 45 (×), 135 (□) μmol/L and ATRA 1 μmol/L (■) for 2-7 d on the growth of HUVEC. $n = 3$ experiments. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control group.

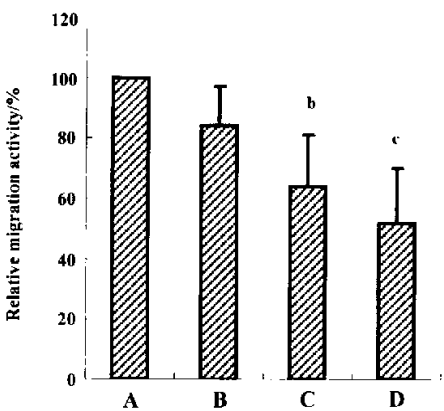

Fig 2. Inhibition of α-Ano 0 μmol/L(A), 15 μmol/L (B) or 45 μmol/L(C) and ATRA 0.1 μmol/L (D) for 24 h on the wound-induced migration of HUVEC. Cell relative migration activity was expressed as the percentage of cells crossing the wound edge in treated groups in comparison with that in the control group. $n = 3$ experiments. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control group.

In contrast to the effect of α-Ano on proliferation and migration, α-Ano had no obvious effect on the adhesion activity of HUVEC. About 78 % of cells attached to collagen matrix after 2-h incubation in control group, but the percentage of attached cells was not obviously increased in α-Ano treatment groups (Fig 3).

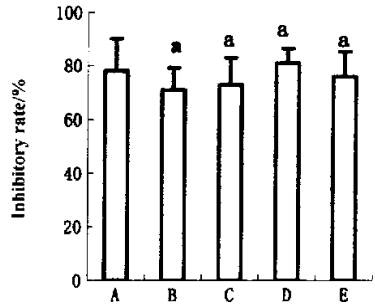

Fig 3. Effects of α-Ano 0 μmol/L (A), 15 μmol/L (B, D) or 45 μmol/L (C, E) for 2 h on the adhesion activity of HUVEC. HUVEC in A, B, and C groups were not pretreated, in D and E groups were pretreated by α-Ano for 48 h. $n = 3$ experiments. $\bar{x} \pm s$. $^aP > 0.05$ *vs* control group.

Effect of α-Ano on the production of NO NO is a potent stimulator for the proliferation and migration of HUVEC. We examined the effect of α-Ano on NO production by HUVEC. The level of NO (reflected by nitrite level) released by HUVEC was elevated on the 2th and 4th day, but diminished gradually thereafter (Fig 4). After incubation with α-Ano, a dose- and time-dependent decrease in NO level was observed (Fig 4).

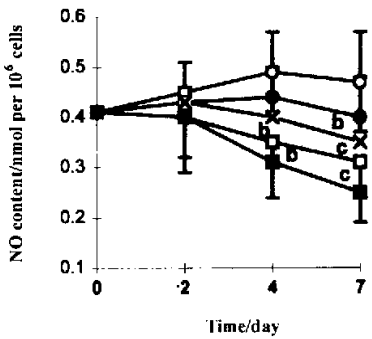

Fig 4. Inhibitory effect of α-Ano 0 (○), 15 (●), 45 (×), 135 (□) μmol/L and ATRA 1 μmol/L (■) for 2, 4, 7 d on the production of NO by HUVEC. $n = 3$ experiments. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control group.

DISCUSSION

In the present study, α-Ano, a partial estrogen antagonist with novel chemical structure, exhibited an inhibitory effect on angiogenesis in tumors and CAM *in vivo* and on proliferation and migration of endothelial cells *in vitro*. The suppression of angiogenesis was accompanied by the growth inhibition of Lewis lung cancer, indicating that the antiangiogenic effect of α-Ano contributes to its therapeutic action. Moreover, the angiosupression induced by α-ANO was not antagonized by 17β-estradiol, a classical pure agonist of estrogen receptor, suggesting that α-Ano inhibits angiogenesis via a mechanism other than inhibiting the estrogen activity, ie, via an estrogen receptor-independent mechanism. This action is consistent with the results previously reported by others[15]. Collins *et al*[15] reported that some partial estrogen antagonists such as clomiphene, tamoxifen, nafoxidine, and some pure estrogen agonists such as ICI 164384 and ICI 182780 displayed antiangiogenic effect and this effect was not antagonized by 17β-estrodiol in CAM model. The chemical structure of α-Ano is different from those mentioned above[15], but they inhibit angiogenesis via a somewhat similar mechanism, ie, in an estrogen receptor-independent manner, so it will be of interest to investigate the antiangiogenic mechanism of antiestrogen compounds.

Angiogenesis is a complex process involving a series of sequential steps[16], including the degradation of the basement membrane of vessels, migration of endothelial cells towards angiogenic stimuli, proliferation of endothelial cells, formation of capillary lumina and maturation of blood vessels. Angiosuppression may be a consequence of inhibition of any one or more of these steps. The *in vitro* results in this study revealed that α-Ano significantly inhibited the growth and migration of HUVEC, and effectively reduced the production of NO by HUVEC. Based on the fact that NO plays an important role in angiogenesis by stimulating the proliferation and migration of endothelial cells as an autocrine and paracrine factor[17-19], we conclude that α-Ano may inhibit the proliferation and migration through diminishing the production of NO, and finally lead to inhibition of angiogenesis. However, we do not exclude that other targets may also be involved in these processes.

In summary, our *in vivo* and *in vitro* results demonstrate that α-Ano possesses an antiangiogenic effect and this effect possibly contributes to its anti-tumor action.

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α-双炔失碳酯体内、外抑制血管生成[1]

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关键词 α-双炔失碳酯；血管生成因子；绒毛膜癌；脐静脉；血管内皮；一氧化氮

目的：研究雌激素受体部分拮抗剂 α-双炔失碳酯(α-Ano)对体内、外肿瘤血管生成的抑制作用. **方法**：在 C57BL/6 小鼠中观察 Lewis 肺癌的生长情况，用免疫组织化学方法观察肿瘤微血管密度(MVD)；在鸡胚绒毛膜尿囊膜(CAM)模型上观察药物对血管生成的影响；用台盼蓝排染法研究药物对人脐静脉内皮细胞(HUVEC)生长的作用；在体外观察了药物对 HUVEC 的由伤口引起的迁移和对胶原基质的粘附活性的影响；用荧光法间接测定一氧化氮(NO)水平. **结果**：α-Ano 显著地抑制 Lewis 肺癌的 MVD，同时抑制小鼠皮下接种的肿瘤生长，MVD 的降低程度与肿瘤生长的抑制程度相关. α-ANO 在 CAM 模型上也显示出对血管生成的抑制活性，抑制率达 53 %，17β-雌二醇对 α-Ano 抑制 CAM 血管生成的活性无明显拮抗作用；α-Ano 对 HUVEC 的增殖和迁移活性有抑制作用，但对 HUVEC 对 I 型胶原的粘附能力无明显影响. 同时，α-Ano 能够抑制 HUVEC 释放 NO 的水平，且具有时间和剂量依赖性. **结论**：α-Ano具有血管生成抑制活性，此作用是通过减少 NO 的释放，随后抑制内皮细胞的增殖和迁移而实现的.

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