Organoarsenic Roxarsone Promotes Angiogenesis In Vivo

Yumei Zhang1,2, Yujing Wang1, Qianqian Lu1, Wenfang Xin1, Weibo Cui1 and Jiaqiao Zhu1,2

1Department of Veterinary Pharmacology and Toxicology, College of Veterinary Medicine, Yangzhou University, Yangzhou Jiangsu, China and 2Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou, China

(Received 19 May 2015; Accepted 25 September 2015)

Abstract: Roxarsone, an organoarsenic feed additive, is widely used worldwide to promote animal growth. It has been found to exhibit a higher angiogenic index than AsIII at lower concentrations and to promote angiogenic phenotype in human endothelial cell in vitro. Little research has focused on the potential angiogenic effect of roxarsone in vitro or in vivo. Here, we investigated the pro-angiogenic effect of roxarsone in vivo. The effects of 0.1–10.0 μM roxarsone were tested in the rat endothelial cell Matrigel plug assay, chicken chorioallantoic membrane (CAM) model and MCF-7 cell xenograft tumour model; 10 ng/mL vascular endothelial growth factor (VEGF) was used as a positive control and PBS as a negative control. Roxarsone significantly increased the volume, weight and haemoglobin content of the Matrigel plugs compared to PBS group (p < 0.05); 1.0 μM roxarsone exerted the most significant effects. H&E staining and CD31 immunohistochemistry revealed obviously more new vessels or capillary-like structures in the plugs of the roxarsone and VEGF groups. Roxarsone significantly increased the numbers of primary/secondary vessels and area of vessels in the CAM assay and obviously increased tumour weight and volume in the xenograft model compared to PBS (p < 0.05). Histochemistry indicated local necrosis was observed at the centre of the xenograft tumours in the PBS and roxarsone groups, with less necrosis apparent in the VEGF-treated tumours. The growth of endothelial cells and VEGF level was obviously affected at blockade of VEGF and its receptor Flt-1/Flk-1 by SU5416 or its antibody in vitro. This study demonstrates roxarsone promotes angiogenesis in vivo, and a VEGF/VEGFR mechanism may be involved.

Angiogenesis and vessel remodelling are fundamental to the pathogenesis of a number of diseases associated with environmental exposure to arsenic, including tumourigenesis and cardiovascular diseases [1,2]. Angiogenesis has a pivotal role in oncogenesis. Epidemiological studies have revealed an association between chronic exposure to arsenic and an increased risk of human cancer in multiple organs, including the skin, bladder, kidney and lung [3–5]. Nanomolar or low micromolar concentrations of arsenic (AsIII) have been shown to stimulate angiogenesis in a variety of in vivo and in vitro models [6,7] and can lead to vascular remodelling that may promote vascular diseases and tumourigenesis [8–11]. In addition to enhancing tumour growth in mice implanted with B16-F10 tumour cells [12,13] and MCF-7 breast cancer cells [14], increased angiogenesis may contribute to the overall growth potential and increase tissue pigmentation.

Some organoarsenic compounds, in particular 4-hydroxy-3-nitrobenzenearsenic acid (roxarsone), are used worldwide in animal production to enhance weight gain and improve feed efficiency although roxarsone is forbidden within the European Union. These compounds are non-toxic to domestic livestock and are excreted unchanged from the animals [15,16]. However, these compounds can be introduced into the environment from litter stored in weather-exposed locations or by field application as a fertilizer or minor residues in meat [17] can directly reach the consumer. For example, roxarsone can easily leach from poultry litter [18–22]. The impact of roxarsone on human health has not been well studied, and the mechanisms underlying its biological effects in mammalian tissues are unknown. However, it has been suggested that the biological effects of organoarsenic compounds are dependent on metabolism to inorganic AsIII [23]. The growth potentiation and enhanced tissue pigmentation observed in broiler chickens [24,25] and meat-strain ducks [26] as a growth promoter suggest that low levels of roxarsone may have an angiogenic effect similar to that of inorganic arsenite (As III).

Basu et al. [27] demonstrated that low concentrations of roxarsone had a higher angiogenic index than As III in human aortic endothelial cells and lung microvascular endothelial cells. Additionally, increased endothelial nitric oxide synthase (eNOS) activity was observed for roxarsone-induced angiogenesis, but not for As III-induced angiogenesis. In our previous studies, we reported that roxarsone promoted angiogenesis in vitro in rat endothelial cells and ex vivo based on the Matrigel-induced angiogenic tube formation assay and rat aortic ring assay [28]. Additionally, 0.01–10.0 μM roxarsone significantly promoted cell viability and capillary-like tube formation, accompanied by enhanced secretion of vascular endothelial growth factor (VEGF) in rat endothelial cells and rat aortic ring model in our previous studies [28,29]. Here, we investigated the angiogenic potential of roxarsone in vivo using the murine Matrigel plug implantation assay, chicken embryo chorioallantoic membrane (CAM) model and xenograft model of MCF-7 cells in nude mice for knowledge of potential carcinogenic or tumour-promoting risk of roxarsone used in animal production.
Materials and Methods

Experimental animals and chemicals. Male Wistar rats (250 g), male Kunming mice (24–26 g) and female BALB/c athymic nude mice (8–9 weeks old) were obtained from the Center of Comparative Medicine, Yangzhou University, China. Mice and rats were housed at 22°C and relative humidity under a regular 12-hr light/dark schedule. Food and water were available ad libitum.

Roxarsone from Sigma-Aldrich (St. Louis, MO, USA) was dissolved in 5 mL methanol and then diluted to 50 mL with deionized water to obtain a 1.0 mM stock solution. The work solution of 0.01–10.0 μM roxarsone was further diluted from the stock solution with incubation medium. Sodium heparin and trypsin were purchased from Sigma-Aldrich. Dulbecco’s modified Eagle medium (DMEM), penicillin/streptomycin and foetal bovine serum (FBS) were purchased from Gibco (Invitrogen, Carlsbad, CA, USA). Recombinant rat VEGF165 was purchased from Peprotech Co. (Rocky Hill, NJ, USA). Matrigel™ basement membrane matrix was purchased from BD Biosciences (San Jose, CA, USA). Rabbit polyclonal VEGF antibody (at-VEGF) and Flk-1 antibody (at-Flk-1) were purchased from Abcam (Cambridge, MA, USA); rabbit polyclonal Flt-1 antibody (at-Flt-1) and inhibitor of VEGF receptor SU5416 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Tissue culture plates were obtained from Falcon (Becton Dickinson Labware, Franklin Lakes, NJ, USA).

Isolation of rat thoracic aorta and primary endothelial cell culture. Wistar rats were anaesthetized using 2% thiopental sodium and humanely killed, and endothelial cells were isolated from the thoracic aorta as described in [28] and cultured in DMEM supplemented with 15% (v/v) foetal bovine serum, 2 mM L-glutamine, 100 μg/mL sodium heparin, 4 ng/mL VEGF and 100 U penicillin/streptomycin at 37°C in 5% CO₂. All protocols were reviewed by the Committee on the Ethics of Animal Experiments of Yangzhou University.

Fig. 1. Pro-angiogenic effect of roxarsone in the Matrigel plug assay. (A) Matrigel was mixed with different concentrations of roxarsone, vascular endothelial growth factor (VEGF) or PBS as a control. The Matrigel plugs were subcutaneously injected into the mid-ventral region of Kunming mice. Ten days later, the plugs were harvested. Representative images (of at least 5 mice per group) are shown in (a–e) for the PBS, 0.1, 1.0 and 10.0 μM roxarsone and 10 ng/mL VEGF groups, respectively. (B) Excised plugs were embedded in paraffin and stained with H&E to visualize blood vessels in (a–e) for the PBS, 0.1, 1.0 and 10.0 μM roxarsone and 10 ng/mL VEGF groups, respectively (×200) and (f–j) as in (a–e); ×400. (C) Plugs were embedded in paraffin and subjected to immunohistochemical staining for CD31 to visualize blood vessels as shown in (a–f) for the PBS, 0.1, 1.0 and 10.0 μM roxarsone, 10 ng/mL VEGF groups and negative control without first antibody. (D) The relative area of vessels in CD31 immunofluorescence calculated by Leica QWin Pro V 3.5.1 software in five visual fields randomly for each mouse. Values marked with different letters are significantly different; p < 0.05 by Kruskal–Wallis test. Arrow indicates vessel or capillary-like structure.

© 2015 Nordic Association for the Publication of BCPT (former Nordic Pharmacological Society)
Fig. 1. continued
University and carried out in accordance with the EU Directive 2010/63/EU for animal experiments.

The cells were subcultured once they had formed a monolayer (after approximately 6 days of incubation). For the Matrigel plug assays, the endothelial cells were digested with 2% trypsin, briefly centrifuged at 1000 rpm for 10 min. and resuspended at the required density in DMEM. The experiments were performed at least three times in independent experiments.

Murine Matrigel plug implantation assay of rat endothelial cells. The Matrigel plug implantation assay was based on the method of Hidenori et al. [30]. Male Kunming mice were anaesthetized using 1% thiopental sodium, and 500 lL liquid Matrigel mixture at 4°C was subcutaneously injected into the dorsum using a 25-G needle. Each Matrigel mixture contained 10⁶ primary endothelial cells, 100 µg/mL heparin, and either PBS, 10 ng/mL VEGF, or 0.01 µM, 0.1 µM, 1.0 µM or 10.0 µM roxarsone in final concentration. The liquid Matrigel mixture solidified within 5–10 min. of inoculation.

After 10 days, the mice were killed by cervical dislocation, and the Matrigel plugs were excised, weighed and photographed. The haemoglobin (Hb) content of the harvested plugs was measured using a Hemoglobin test kit (Wako, Osaka, Japan) and normalized to the plug weight. Briefly, the Matrigel plugs were divided and a portion was homogenized in 10 mM PBS (pH 7.4), centrifuged at 8000 × g for 10 min., the supernatant was mixed with haemoglobin colouring reagent and incubated for 3 min. at room temperature, and absorbance was measured at 540 nm. The total haemoglobin concentration was estimated from a standard curve using haemoglobin standard reagent.
**Table 1.**
The size, haemoglobin and vascular endothelial growth factor (VEGF) content of the Matrigel plugs implanted with rat endothelial cells and treated with roxarsone or VEGF.

| Group          | Body-weight (g) | Volume of plugs (cm³) | Weight of plugs (g) | Relative Hb ratio | VEGF content (pg/mL) |
|----------------|-----------------|-----------------------|---------------------|-------------------|----------------------|
| PBS            | 20.25 ± 0.55 a  | 0.05859 ± 0.0610 d    | 0.2648 ± 0.2163 d   | 1.000 ± 0.308 d   | 3.2 ± 0.1 d          |
| Roxarsone 0.1 μM| 20.82 ± 0.48 a  | 0.1225 ± 0.09052 b   | 0.3846 ± 0.0092 c   | 1.272 ± 0.752 c   | 4.5 ± 0.2 c          |
| Roxarsone 1.0 μM| 20.12 ± 0.51 a  | 0.1564 ± 0.1213 a    | 0.4825 ± 0.0074 b   | 1.374 ± 0.099 c   | 5.8 ± 0.2 b          |
| Roxarsone 10.0 μM| 19.89 ± 0.46 a  | 0.06384 ± 0.0447 d   | 0.3471 ± 0.2003 d   | 1.582 ± 0.251 b   | 5.4 ± 0.3 b          |
| VEGF 10.0 ng/mL| 20.08 ± 0.56 a  | 0.0927 ± 0.0524 c    | 0.6556 ± 0.6642 a   | 2.448 ± 0.597 a   | 6.2 ± 0.3 a          |

Different letters in the same column denote significant differences between groups ($p < 0.05$; one-way ANOVA).

Additionally, a tiny fraction of the plugs were fixed with 10% formalin in 0.1 M phosphate buffer, and 5-μm-thick paraffin sections were prepared and stained with haematoxylin–eosin.

The VEGF level in the plugs was also measured by VEGF immunoassay kit (VEGF DuoSet®; R&D Systems, Minneapolis, MN, USA), which recognizes the soluble isoforms VEGF121 and VEGF165 with a sensitivity of 2 pg/mL. The plug was cut up and 1.0 mL PBS was added in 0.2 g sample with ultrasonic oscillation for 5 min. and centrifuged at 1430 g for 5 min. Two hundred microlitres of supernatant was used for VEGF ELISA assay. The absorbance was measured at 450 nm using a microplate reader (Lab Systems, Mumbai, India), and VEGF concentrations were calculated using a standard curve based on the OD value at 450 nm.

Formation of microvessels in the plugs was also assessed by CD31 immunostaining of 5-μm-thick paraffin sections. After deparaffinization and blocking, the sections were incubated with rabbit polyclonal antmouse CD31 antibody (Abcam, Cambridge, UK) at 4 μg/mL followed by fluorescein isothiocyanate (FITC)-labelled secondary antibodies goat anti-rabbit IgG (Abcam) for 30 min. After rinsing, the plugs were examined by fluorescence microscopy. The same concentration of normal rabbit IgG was used to prepare negative control slides. Angiogenesis (neovascularization) was evaluated by observing invasion of the endothelial cells and blood vessels into the Matrigel plug based on the H&E staining histochemistry and CD31 immunostaining analysis [30]. The total areas of intact loops or more than half of loops with FITC staining were determined in five random, visual fields for each mouse by Leica QWin Pro V 3.5.1 software (Leica, Rockville, MD, USA).

The relative area of vessels was normalized to that of PBS group with five repeat every group.

**Chicken embryo chorioallantoic membrane model.** White Leghorn chicken eggs were obtained from Yangzhou Poultry Company (Jiangsu, China). The fertilized eggs were incubated under conditions of constant humidity (65–70%) at a temperature of 37°C. On the seventh day of incubation, a square window was created in the egg (Jiangsu, China). The fertilized eggs were incubated under conditions of the seventh day of incubation with 10 ng/mL VEGF or 0.01 μM roxarsone or VEGF.

Table 2.

| Group          | No. of eggs | No. of live embryos | Survival rate (%) |
|----------------|-------------|---------------------|-------------------|
| PBS            | 30          | 27                  | 90.00             |
| Roxarsone 0.1 μM| 30          | 27                  | 90.00             |
| Roxarsone 1.0 μM| 30          | 24                  | 80.00             |
| Roxarsone 10.0 μM| 30         | 24                  | 80.00             |
| VEGF 10.0 ng/mL| 30          | 26                  | 86.67             |

© 2015 Nordic Association for the Publication of BCPT (former Nordic Pharmacological Society)
incubated for 6 hr. The wells were treated with PBS, roxarsone (0.10, 1.0 or 10.0 \( \mu \)M), 10 ng/mL VEGF, 1.0 \( \mu \)M roxarsone with 10 ng/mL at-VEGF antibody (at-VEGF) or VEGF receptors antibody (at-Flt-1 or at-Flk-1) or 15 \( \mu \)M SU5416 cotreatment, and 10 ng/mL at-VEGF, at-Flt-1, at-Flk-1, 15 \( \mu \)M SU5416 or PBS in DMEM complete medium for 24–48 hr, with six wells in each treatment and triplicate repeat. MTT assays were performed to evaluate cell growth. VEGF concentrations in the culture supernatants of conditioned medium were measured using the above VEGF immunoassay kit. The relative VEGF content was measured by dividing the VEGF concentrations of the treatment groups by that of the PBS control.

Data analysis. The mean ± S.D. values are presented. One-way analysis of variance (ANOVA) followed by the Tukey post hoc test was used for comparisons among treatment groups; Kruskal–Wallis was used for nonparametric test; \( p < 0.05 \) was considered statistically significant.

Fig. 2. Pro-angiogenic effect of roxarsone in the chicken embryo chorioallantoic membrane model. Chicken embryo chorioallantoic membranes (CAM) were treated on day 7 of incubation with 20 \( \mu \)L of the test materials in 6-mm gelatin sponges. CAMs were examined daily until day 10. (A) Image of the CAM arteriovenous system at day 10 in the (a-f) PBS, 0.1, 1.0, 10.0 \( \mu \)M roxarsone and 10 ng/mL VEGF groups, respectively. (B) The relative area of vessels in 5 mm around gelatin sponge in images of the CAMs was determined based on the rate of vessel area of PBS group (table 3). Values marked with different letters in the same column are significantly different; \( p < 0.05 \).
Results

Roxarsone promotes angiogenesis in the murine Matrigel plug assay of rat endothelial cells.

The potential pro-angiogenic effect of roxarsone in rat endothelial cells was assessed using a murine Matrigel plug assay. Compared to the PBS controls, VEGF and roxarsone induced neovascularization in the Matrigel plugs, as demonstrated by the visible formation of blood vessels (fig. 1) and assessment of the size, haemoglobin and VEGF content in the Matrigel plugs (table 1). At approximately 5–6 days after implantation, the Matrigel plugs began to clearly protrude in the VEGF and roxarsone groups, whereas protrudes appearing in the PBS control were delayed about 2 days. Although the size or areas of the plugs were not visually different between control and treatment groups, the redness and depth of the plugs in the VEGF and roxarsone groups were markedly more obvious on gross examination at 10 days (time of harvesting; fig. 1A). The mean volumes and weights of the plugs in the 0.1, 1.0 and 10.0 μM roxarsone groups were significantly higher than that of the PBS group, with the highest volume and weight observed in the 1.0 μM roxarsone group. However, the mean volumes and weights of the Matrigel plugs in the roxarsone groups were all significantly lower than those of the 10 ng/mL VEGF group. The haemoglobin content of the roxarsone groups was significantly higher than that of the PBS control, with the highest haemoglobin content observed in the 10.0 μM roxarsone group. The VEGF contents in plugs treated by different concentration of roxarsone were all clearly higher than that of the control; those in the 1.0 and 10.0 μM roxarsone groups were higher than that of 0.1 μM roxarsone (table 1). H&E staining and CD31 immunohistochemistry revealed obviously higher numbers of vessel or capillary-like structures in the Matrigel plugs of the roxarsone and VEGF-treated groups compared to the PBS controls (fig. 1B,C). Compared with the PBS group, the vessel areas in 0.1–10.0 μM roxarsone and 10 ng/mL VEGF groups were clearly enhanced (fig. 1D), with the order of the VEGF group >1.0 μM roxarsone >0.1 or 10.0 μM roxarsone.

Table 3. Angiogenic effect of roxarsone in the chicken embryo chorioallantoic membrane assay.

| Group          | No. of embryos | No. of primary vessels | No. of secondary vessels | Rate of vessel area (%) |
|----------------|----------------|------------------------|--------------------------|-------------------------|
| PBS            | 25             | 9.00 ± 3.31 e          | 13.6 ± 3.79 d            | 44.06 ± 5.14 c          |
| Roxarsone 0.1 μM | 22             | 23.70 ± 5.60 e         | 36.80 ± 7.29 b           | 57.80 ± 7.08 b          |
| Roxarsone 1.0 μM | 23             | 29.30 ± 7.83 b         | 51.00 ± 15.07 a          | 77.90 ± 11.22 a         |
| Roxarsone 10.0 μM | 24             | 16.87 ± 5.03 d         | 29.83 ± 9.70 c           | 64.27 ± 9.38 b          |
| VEGF 10.0 ng/mL | 23             | 33.70 ± 10.40 a        | 55.20 ± 15.48 a          | 84.00 ± 10.60 a         |

Mean ± S.E.M. values were determined on the seventh day of incubation after treatment. Different letters in the same column denote significant differences between groups (p < 0.05; one-way ANOVA).
tight structure and MCF-7 cells cluster growth inward. Immunohistochemical analysis for CD31 demonstrated the PBS control xenograft tumours contained a low number of vessels, especially large diameter vessels, whereas the xenografts in the roxarsone and VEGF groups contained high numbers of vessels and more large diameter vessels (fig. 3B,C).

Roxarsone promotes rat endothelial cell viability via a VEGF/VEGFR mechanism.

The effect of roxarsone and blockade of VEGF or VEGFR on rat endothelial cells with 24- to 48-hr incubation by MTT assay are shown in fig. 4. The cell survival rates in 0.1, 1.0, 10.0 μM roxarsone, 1.0 μM roxarsone cotreated with at-VEGF, at-Flk-1 and at-Flt-1 treatment were significantly different.

Table 4. Effect of roxarsone in a xenograft tumour model of MCF-7 cells in nude mice.

| Group          | No. of mice | No. of tumour-bearing mice | Rate of tumour formation (%) | Tumour weight (g)  | Tumour volume (cm³) |
|---------------|-------------|----------------------------|-----------------------------|--------------------|---------------------|
| PBS           | 15          | 11                         | 73.33                       | 0.0578 ± 0.0571 c  | 0.0586 ± 0.0610 d  |
| Roxarsone 0.1 μM | 15          | 11                         | 73.33                       | 0.0733 ± 0.0558 c  | 0.1225 ± 0.0805 c  |
| Roxarsone 1.0 μM | 15          | 12                         | 80.00                       | 0.1435 ± 0.1355 b  | 0.2864 ± 0.1133 a  |
| Roxarsone 10.0 μM | 15          | 12                         | 80.00                       | 0.0764 ± 0.0656 c  | 0.0730 ± 0.0522 d  |
| VEGF 10.0 ng/mL | 15          | 13                         | 86.67                       | 0.2340 ± 0.1530 a  | 0.1937 ± 0.1008 b  |

Different letters in the same column denote significant differences between groups (p < 0.05; one-way ANOVA).

Fig. 3. Pro-angiogenic effect of roxarsone in a xenograft model of MCF-7 tumour cells in nude mice. The xenograft tumours were excised and paraffin sections were subjected to (A) haematoxylin and eosin staining and histological analysis (100×) or (B) CD31 immunochemical analysis (400×). Representative images (of at least 5 mice per group) are shown for the (a) PBS control; (b–d) 0.1, 1.0 and 10.0 μM roxarsone; and (e) 10 ng/mL vascular endothelial growth factor (VEGF) groups. Local necrotic area such as cell shrinkage, cell lysis and eosinophilic staining (arrow indicated) was observed in the control and roxarsone groups. (C) The relative area of vessels in CD31 immunofluorescence calculated by Leica QWin Pro V 3.5.1 software in five visual fields randomly for each mouse. Values marked with different letters are significantly different; p < 0.05 by Kruskal–Wallis test.
higher than that of PBS, those in at-VEGF and at-Flk-1 were clearly lower than that of control. At the blockage of VEGFR by SU5416, the relative rates in 1.0 μM roxarsone cotreated with SU5416 or SU5416 alone were obviously lower than that of control ($p < 0.01$). There was extreme difference between the 1.0 μM roxarsone and the 1.0 μM roxarsone cotreated with SU5416 group ($p < 0.01$). The relative viability in at-VEGF and at-Flk-1 was clearly lower than that of control ($p < 0.05$), but there was no difference within at-Flt-1 and control ($p > 0.05$). Compared with 1.0 μM roxarsone, the cell viability in 1.0 μM roxarsone cotreated with at-Flk-1 and at-VEGF was extremely lower ($p < 0.01$), whereas that in 1.0 μM roxarsone cotreated with at-Flt-1 was obviously lower ($p < 0.05$).

The VEGF contents in culture supernatant of endothelial cells treated by roxarsone and blockage of VEGFR were determined by ELISA assay (fig. 5). The relative VEGF contents in 0.1, 1.0, 10.0 μM roxarsone, 1.0 μM roxarsone...
cotreated with at-Flk-1 and at-Flt-1, at-Flk-1 treatment were significantly higher than that of PBS control ($p < 0.05$). There was no difference within at-Flt-1 and control ($p > 0.05$). At the inhibition of SU5416, the VEGF contents in 1.0 $\mu M$ roxarsone cotreated with SU5416 or SU5416 alone were obviously lower than that of control ($p < 0.01$). There was an extreme difference between the 1.0 $\mu M$ roxarsone and 1.0 $\mu M$ roxarsone cotreated with SU5416 group ($p < 0.01$). Compared with 1.0 $\mu M$ roxarsone, the VEGF content in 1.0 $\mu M$ roxarsone cotreated with at-Flk-1 was clearly higher ($p < 0.05$), and there was no difference in 1.0 $\mu M$ roxarsone cotreated with at-Flt-1 ($p > 0.05$).

Discussion

Roxarsone is widely used as an animal growth promoter to improve weight gain, feed efficiency and pigmentation in chicken or pigs. It has been demonstrated that the exposure of human beings to arsenic has increased due to the levels of arsenic present in meat products or released into the environment from excrement and urine, in which roxarsone is almost unmetabolized. Basu et al. [27] demonstrated that low concentrations of roxarsone had a higher angiogenic index than $\text{As}^{\text{III}}$ in human aortic endothelial cells and lung microvascular endothelial cells. Our previous studies clearly demonstrated the potential of roxarsone to promote angiogenesis in vitro in

---

**Fig. 4.** MTT assay of rat aortic endothelial cells after 24- to 48-hr treatment with roxarsone (ROX) and/or blockade of vascular endothelial growth factor (VEGF) (at-VEGF) and VEGFR (at-VEGFR). MTT assays were performed to determine the viability of cells treated with PBS (negative control) or 0.1–10.0 $\mu M$ roxarsone and/or 15 $\mu M$ SU5416, 10 ng/mL at-VEGF, 10 ng/mL at-Flt-1 or at-Flk-1 by relative antibodies for 24–48 hr. Results are the mean ± S.D. of the OD value relative to the PBS control and are representative of three independent experiments. *$p < 0.01$, *$p < 0.05$ relative to PBS; **$p < 0.01$, *$p < 0.05$ between two groups as indicated by the connecting lines by ANOVA.

---

**Fig. 5.** Vascular endothelial growth factor (VEGF) ELISA analysis of rat aortic endothelial cells after 24-hr treatment with roxarsone (ROX) and/or blockade of VEGFR. ELISA analysis was performed to measure VEGF levels of culture supernatants from endothelial cells treated with 0.1–10.0 $\mu M$ roxarsone and/or 15 $\mu M$ SU5416, 10 ng/mL at-Flt-1 and Flk-1 by their antibodies for 24 hr. Results are the mean ± S.D. of three independent experiments relative to the PBS control. *$p < 0.01$, relative to PBS; **$p < 0.01$, *$p < 0.05$ between two groups as indicated by the connecting lines by ANOVA.
rat endothelial cells and ex vivo in rat aortic rings [28]. In addition, the mechanism by which roxarsone increases pigmentation is not yet clear; however, increased blood supply (venous stasis) is recognized as a factor that promotes coloration [32,33]. Therefore, we investigated the potential angiogenic effect of roxarsone in vivo using the Matrigel plug assay and chicken embryo CAM model. In both assays, roxarsone (0.01–10.0 μM) promoted obvious vascularization in vivo compared to the PBS control (p < 0.05); however, the maximal pro-angiogenic effect of roxarsone was lower than that of 10 ng/mL VEGF. Additionally, Matrigel is composed of different growth factors for which pro-angiogenic effects have previously been attributed [34]. Therefore, an increase in the number of blood vessels in this system could actually derive from a synergistic interaction of roxarsone with one or more of those growth factors. Study of the angiogenic effect of roxarsone in vivo provides useful information for evaluating the safety of roxarsone as an animal growth promoter.

In the current MTT assay or previous tube formation assay [28], 0.01–1.0 μM roxarsone showed concentration-dependent enhancement effects but not at 10 μM roxarsone. Although the obvious inhibition effect of roxarsone within 10–50 μM on endothelial cells in our previous works was not observed, the promotion effect was indeed weakened. It is interesting whether roxarsone shows the ‘double-edge’ effect on angiogenesis like inorganic arsenic in a chorioallantoic membrane model, which appeared to induce stimulating effects when exposed to low levels (≤1 μM) and suppressive effects when exposed to high levels of arsenic (1–10 μM) [35].

The three most widely used assays for studying angiogenesis in vivo are the CAM assay, the rabbit (rat) corneal micro-pocket assay and subcutaneous implant assays such as the Matrigel plug assay [36–38]. A number of CAM angiogenesis assays are based on testing purified factors and intact cells incorporated into filter discs cut from nitrocellulose membrane or Whatman paper, gelatin sponges, or gelated materials such as methylcellulose, Matrigel or sodium alginate [39]. In this study, we tested roxarsone in the CAM assay using either nitrocellulose membranes or gelatin sponges under the same conditions. In our experiments, obvious vessels formed around the gelatin sponges in the roxarsone or VEGF treatments compared to the vehicle control, whereas few vessels formed around the nitrocellulose membrane carriers at same treatments in the CAM assay. One of the major limitations of filter disc angiogenesis assays is that the support material may not enable sufficient retention of the test materials compared to gelatin sponges. Therefore, only the data from the gelatin sponge CAM assay were analysed in this study.

The formation of new blood vessels is essential for tumour growth [40–42]. We aimed to compare the effect of roxarsone and VEGF on xenograft tumour growth. In the MCF-7 xenograft model, roxarsone appeared to promote tumour growth to some degree, but a dose–response relationship was not observed. Nevertheless, the promotion effect of roxarsone on tumour growth was not reported so far, and implying safety risk on the residue of roxarsone in foods derived from animals when used as feed additive. MCF-7 cells were selected for the xenograft model in nude mice as the cells were available in our laboratory. It is possible that these cells may not be optimal for investigating the angiogenic effects of roxarsone in vivo, as the MCF-7 cells were oestrogen responsive so that the growth of breast cancer xenografts may be dependent on the oestrogen levels in the mice tested [43,44]. We plan to investigate the effect of roxarsone on tumour growth using other xenograft models in the future.

The investigation of roxarsone on rat endothelial cells in vitro was crude and slight. But these primary data especially at the blockage of VEGFR by SU5416 or Flk-1 antibody demonstrated that a VEGF/VEGFR mechanism may be involved in the promotion of roxarsone on rat endothelial cells.

Our studies described in this article indicate that roxarsone promoted angiogenesis in vivo by Matrigel plug assay, the chicken embryo chorioallantoic membrane model and MCF-7 xenograft model. Understanding the angiogenesis effect of roxarsone in vivo, especially in tumour growth promotion, would benefit to know the potential safety risks to human beings. We will investigate the effect of roxarsone on other tumour cells or other tumour models to find out whether it promotes tumour growth, and the mechanism of angiogenesis promotion in the future.

Acknowledgements

This work was financially supported by the Priority Academic Program Development of Jiangsu Higher Education Institutions and The Talented Person Project of Yangzhou University. We thank the staff of the Comparative Medicine Center of Yangzhou University for their assistance on animal treatment.

Conflict of interest statement

There is no conflict of interests related to this research.

References

1 Pritha B, Mayukh B, Ashok KG. Role of genomic instability in arsenic-induced carcinogenicity. Environ Int 2013;53:29–40.
2 Medrano MJ, Raquel B, Roberto PB, Margarita P, Javier D, Rebeca R et al. Arsenic in public water supplies and cardiovascular mortality in Spain. Environ Res 2010;110:448–54.
3 Tchounwou PB, Patlolla AK, Conteno JA. Carcinogenic and systemic health effects associated with arsenic exposure – a critical review. Toxicol Pathol 2003;31:575–88.
4 Shohreh FF, Margaret RK, Yu C. In utero and early life arsenic exposure in relation to long-term health and disease. Toxicol Appl Pharmacol 2013;272:384–90.
5 IARC. Some drinking-water disinfectants and contaminants, including arsenic. IARC monographs on the evaluation of carcinogenic risks to humans/World Health Organization. Int Agency Res Cancer 2004;84:1–477.
6 Dan M, Xin W, Qingshan C, Andrew H, Zhuo Z, Mei X et al. Arsenic promotes angiogenesis in vitro via a heme oxygenase-1-dependent mechanism. Toxicol Appl Pharmacol 2010;244:291–9.
7 Fei W, Shengnan L, Shuhua X, Ling Y, Huihui W, Yingli S et al. Arsenic induces the expressions of angiogenesis-related factors through PI3K and MAPK pathways in SV-HUC-1 human uroepithelial cells. Toxicol Lett 2013;222:303–11.
