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Safrole oxide inhibits angiogenesis by inducing apoptosis

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Abstract

Our previous studies indicate that 3, 4-(methylenedioxy)-1-(2′, 3′-epoxypropyl)-benzene (safrole oxide), a newly synthesized compound, induces apoptosis in vascular endothelial cells (VECs) and A549 lung cancer cells. To our knowledge, the inhibition of angiogenesis by safrole oxide has not been reported yet. We report here that cultured rat aorta treated with safrole oxide exhibited a significant microvessel reduction as determined by counting the number of microvessels in a phase contrast microscope. There were more microvessels formed in the presence of A549 lung cancer cells in rat aorta model, while a dramatic inhibition of angiogenesis was obtained by adding 220–450 μmol l^{-1} of safrole oxide to the growth medium ($P<.01$). The culture of rat aorta treated with safrole oxide produced only some abortive endothelial cells but not microvessels. Furthermore, safrole oxide induced antiangiogenic effect in the chorioallantoic membranes (CAM) as a dose dependent manner. Eggs treated with 2–11 μmol 100 μl^{-1} per egg of the safrole oxide for 48 h exhibited a significant reduction in blood vessel area of the CAM, a process likely mediated by apoptosis as demonstrated by DNA fragmentation. Our results suggest that safrole oxide has antiangiogenic activity and this effect might occur by induction of cellular apoptosis.

Keywords: Safrole oxide; Angiogenesis; Apoptosis

1. Introduction

Angiogenesis actively participates in cancer progression and metastasis. Accordingly, the current review focuses on the utilization of a new research engine, chemical genomics, to discover novel antiangiogenic agents and their targets, and to validate their biological relevancy and provide new insights into the biological role of targets in angiogenesis as well as advance the development of new antiangiogenic and anti-cancer agents (Carmeliet and Jain, 2000; Kwon, 2003). Safrole oxide, formed by the epoxidation of safrole (Swanson et al., 1981), has been documented for a long time (Noller and Kneeland, 1964). Safrole oxide is a substrate of microsomal epoxide hydrolases, and can be rapidly inactivated through metabolism (Swanson et al., 1981; Luo and Guenthner, 1995). Although safrole oxide can covalently bind to DNA in vitro, no corresponding adducts are formed with liver DNA when whole animals are exposed to safrole oxide, or safrole itself. We recently have shown that safrole oxide induces apoptosis both in vascular endothelial cells and A549 lung cancer cells (Miao et al., 2002; Du et al., 2004). We observed the potent effects of safrole oxide on VEC apoptosis as well as its antitumor effects in vitro (Miao et al., 2002; Du et al., 2004). When VEC cells were exposed to safrole oxide 280–570 μmol l^{-1}, the detachment and DNA fragmentation of these cells were promoted. The cell cycle was blocked at G_2-M phase by safrole oxide 570 μmol l^{-1}. 220–450 μmol l^{-1} of safrole oxide could promote apoptosis of A549 lung cancer cells. The cell cycle was blocked at G_1-S phase by safrole oxide 220 μmol l^{-1}, at the same time the expression of P53 was increased. However, the effect of safrole oxide in angiogenesis has not yet been reported. To our knowledge, our data provide the first evidence that safrole oxide inhibits

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angiogenesis through apoptosis. Our data suggest that safrole oxide is a potent inhibitor for angiogenesis. Herein, we reported the inhibition of safrole oxide on angiogenesis in vitro and in vivo.

2. Materials and methods

2.1. Materials

Safrole [98% (GC)] was purchased from Fluka and 3-chloroperoxybenzoic acid from ACROS. Safrole oxide [3,4-(methylenedioxy)-1-(20,30-epoxypropyl)-benzene] was synthesized by the reaction of safrole with 3-chloroperoxybenzoic acid and purified by silica gel column chromatography (Zhao et al., 2003). It was dissolved in ethanol and used at the final concentration of ethanol of 10% in the CAMs and below 0.01% in culture medium (v/v) (Ethanol at these final concentrations did not affect the viability of the cells or the chicken embryo). Fertilized chicken eggs were obtained from the agriculture scientific department of Shandong province in China. All other reagents were analytical reagent (AR) grade.

2.2. Cell cultures

A549 lung cancer cells were cultured in RPMI 1640 medium, supplemented with 10% (v/v) bovine calf serum at 37 °C in 5% CO_2 and 95% air. Two days before the culture of rat aorta, A549 lung cancer cells were seeded onto 100×20 mm culture dishes at a density of 500 000 cells per dish.

2.3. Rat aortic assay

2.3.1. Preparation of agarose culture wells

The agarose wells (10 and 17 mm in diameter and 3 mm in height) were made with sterile 1.5% solution of agarose.

2.3.2. Preparation of collagen solution

Collagen was purified from rat tail tendon as described by Elsdale and Bard (1972). The final collagen solution was obtained by mixing 7 volumes of 4.3 mg ml^{-1} collagen in 1/10 DMEM, pH 4, with 2 volumes of $NaHCO_3$ 11.7 mg ml^{-1} and 1 volume of $10 \times$ DMEM. Collagen gelation occurred at 37 °C.

2.3.3. Culture of Rat Aorta

Rat Aorta was cultured as described previously with slight modifications (Nicosia and Ottinetti, 1990). Briefly, Male Sprague–Dawley rats (230 g) were killed by decapitation, their thoracic aorta was rapidly excised and placed in phosphate buffered saline (PBS) supplemented with 100 U ml^{-1} penicillin and 100 U ml^{-1} streptomycin. Under sterile conditions, adventitia was removed and the aorta was rinsed three times with PBS and cut into 1 mm rings. Before embedding the aortic rings in collagen gel, the bottom of each agarose well was coated with 2 drops of clotting collagen solution. After the collagen gel had formed, an additional 5 drops of clotting collagen solution was added in the agarose well. At this point, the aortic ring was transferred into the agarose well, allowed to sink to the bottom and properly positioned in the center of the gelling solution. At 37 °C, collagen gelation was formed after about thirty minutes. Then the aortic rings embedded in collagen gels were transferred to dishes containing A549 lung cancer cells and cultured with fresh serum-free MCDB 131 medium. Three aortic rings in the collagen gel were housed in each dish. During the experiment two-thirds of the growth medium was replaced every other day. For the rat aortic experiment, 220–450 μmol l^{-1} of safrole oxide was added to each culture. Control group was cultured without safrole oxide. The newly formed microvessels were counted on the sixth day following the adding of safrole oxide.

2.3.4. Quantitation of angiogenesis

Curves of microvascular growth were counted under a phase contrast microscope as described by Nicosia and Ottinetti (1990). Then the rat aorta was stained with acridinorange for 5 min, the nuclears reflecting the newly formed microvasculars were observed under laser scanning confocal microcopy (Zeiss, LSM510).

2.4. CAM bioassay

2.4.1. Incubation of chicken eggs

The chorioallantoic membranes (CAM) obtained from 6 day-old fertilized white Leghorn chicken eggs were incubated at 37.8 °C, 60% relative humidity.

2.4.2. Exposure of the CAM

On the sixth day, the apex of the egg was cleaned with 70% ethanol, a hole was drilled through the eggshell at the narrow end of the egg by using the false air sac technique, a part of the CAM was exposed by opening a round window of about 2 cm diameter.

2.4.3. The tumor model of the chick embryo chorioallantoic membrane (CAM)

The tumor model of chick embryo chorioallantoic membrane (CAM) was set up by seeding $5 \times 10^6 \cdot 100$ μl^{-1} A549 lung cancer cells on the CAMs of the embryos. The window was covered with cellophane tape and the eggs were returned to the incubator. After 24 h, sterile filter paper disks were placed on the CAMs, and safrole oxide or 10% ethanol in PBS was added on the disk. The windows were then covered, and the eggs were further incubated at 37 °C for 48 h.

2.4.4. Light microscopy

CAMs were fixed in situ in 10% (v/v) neutral buffered formalin for 30 min before carefully isolated. CAM vessels

in 10 viable embryos of each treatment group were counted under a light microscope (Olympus, Tokyo, Japan) and the neovascular zones of the CAM under the disks were photographed and analyzed by the Microsoft of Jieda, China.

2.4.5. Analysis of DNA fragmentation

CAM tissue directly below the safrole oxide saturated filter disks was resected at 48 h after adding the safrole oxide on the CAM. Tissues were washed three times with sterile PBS and were finely minced, resuspended in 0.1% bacterial collagenase and incubated for 90 min at 37 °C with occasional vortexing. DNA was extracted from equal numbers of CAM cells (Brooks et al., 1994). DNA extractions were performed as previously described (Zhao et al., 2004). In brief, cells (1×10^6) were incubated in a digestion buffer that contained 0.2 mg ml^{-1} proteinase K at 50 °C overnight. The cellular DNA was extracted once with phenol and once with a mixture of phenol, chloroform and 3-methyl-1-butanol (25:24:1, v/v). After digestion with RNase (final concentration, 0.6 mg ml^{-1}) at 37 °C for 30 min, the sample was subjected to electrophoresis on a 2% agarose gel in Tris-acetate buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.0). The gel was then stained with ethidium bromide and photographed on a UV transilluminator.

2.5. Statistical analyses

Data were expressed as the mean ± SE and accompanied by the number of experiments performed independently, and analyzed by *t*-test. Differences at $P<.05$ were considered statistically significant.

3. Results

3.1. Inhibitory effect of safrole oxide on angiogenesis in vitro

Aortic rings embedded in collagen gels gave rise to microvessels when they were cultured in the presence of serum-free MCDB 131 medium. The first microvessel sprouts appeared after 5 days, the largest number of microvessels formed after 6 days. The microvessels formed a network around the aortic explant, developed lumina and branched. The microvessels on the sixth day were counted and compared. There were more microvessels formed in the presence of A549 lung cancer cells (Fig. 1A). In contrast, a dramatic inhibition of angiogenesis was obtained by adding 220–450 μmol l^{-1} of safrole oxide to the growth medium (Fig. 1B, $P<.01$). The culture treated with safrole oxide produced only some abortive endothelial cells but not microvessels (Fig. 1A). These results showed that safrole oxide has antiangiogenic activity in vitro.

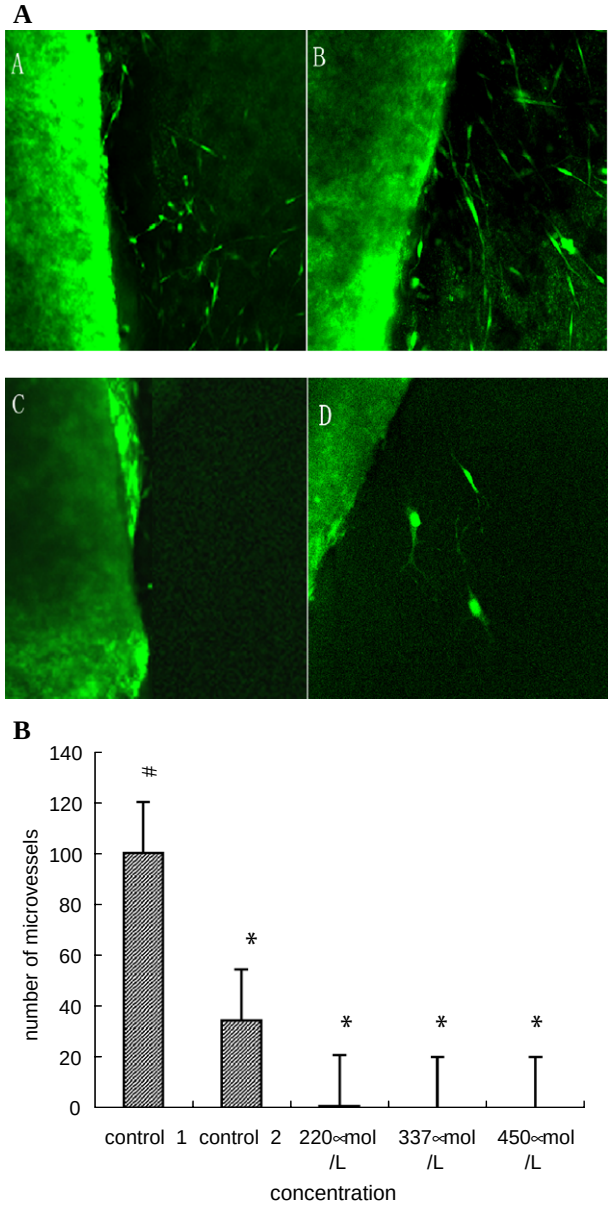

Fig. 1. Effect of safrole oxide on the cultured rat aorta in serum-free medium (MCDB 131). A. The nuclears reflecting the newly formed microvasculars observed under laser scanning confocal microcopy (Zeiss, LSM510). (A) Control, the rat aorta was cultured in the absence of cancer cells and safrole oxide; (B) the rat aorta was cultured in the presence of cancer cells; (C) the rat aorta was treated with safrole oxide in the absence of cancer cells; (D) the rat aorta was treated with safrole oxide in the presence of cancer cells. More microvessels were formed with the function of A549 lung cancer cells. The culture treated with safrole oxide 220 μmol l^{-1} could not produce microvessels in the absence of cancer cells or only produced some abortive endothelial cells but not microvessels in the presence of cancer cells (×400). B. The number of microvessels of rat aorta in collagen gel ($n=5$) in serum-free MCDB 131. Control 1 is with the A549 lung cells; control 2 is without the A549 lung cells. A549 lung cells stimulated angiogenesis markedly ($P<.01$). Angiogenesis is markedly inhibited by 220–450 μmol l^{-1} of safrole oxide ($P<.01$). *, $P<.01$, vs. #.

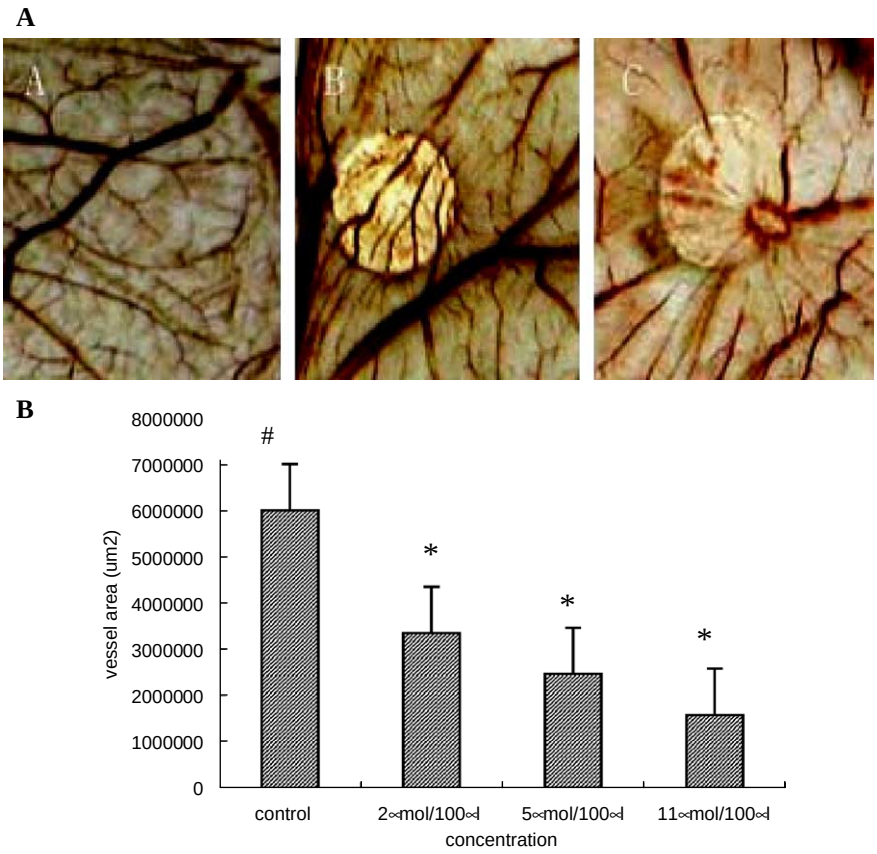

Fig. 2. Effect of safrole oxide on CAM angiogenesis. A. Morphological micrographs. (A) Normal, the blood vessels of untreated CAM; (B) control, the blood vessels of CAM treated with 10% ethanol in PBS; (C) safrole oxide treatment, the blood vessels of CAM treated with 11 μmol·100 μl^{-1} per egg of safrole oxide. B. Vessel area of the CAM. 2–11 μmol·100 μl^{-1} per egg of the safrole oxide induced an antiangiogenic effect in the CAM ($P<.01$). *, $P<.01$, vs. #.

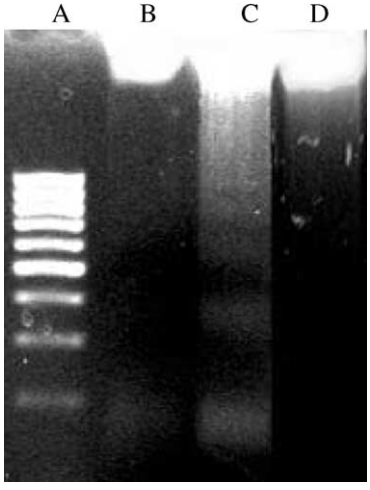

Fig. 3. Soluble fragmented DNA from an equal number of CAM cells were analyzed on a 2% agarose gel as described in experimental procedures. 48 h after the treatment, DNA fragmentation was observed at the concentration of 2 μmol·100 μl^{-1} per egg, however, in the control, DNA fragmentation was not found. (A) DNA Marker; (B) 2 μmol·100 μl^{-1} per egg of safrole oxide treatment; (C) 11 μmol·100 μl^{-1} per egg of safrole oxide treatment; (D) control.

3.2. Safrole oxide suppresses CAM angiogenesis

The CAM assay is particularly suitable for the initial screening of potential inhibitors of angiogenesis in a living organism. The antiangiogenic effect was characterized by the absence of blood vessels in the region directly below the filter paper disks. 2–11 μmol·100 μl^{-1} per egg of the safrole oxide induced a dose dependent antiangiogenic effect in the CAM ($P<.01$). An avascular zone was apparent after a 2-day exposure. The normal growth of the dense capillary network was seen in the control CAM (Fig. 2).

3.3. Safrole oxide suppresses CAM angiogenesis by inducing apoptosis

48 h after the treatment, DNA fragmentation of the CAM tissue was observed at the concentration of 2 μmol·100 μl^{-1} per egg of safrole oxide, however, the DNA fragmentation was not found in the control (Fig. 3).

4. Discussion

Angiogenesis or neovascularization is essential in reproduction, development, and wound repair. However, persis-

tent unregulated angiogenesis might cause tumor growth and metastasis. The targeting of tumors' supportive blood vessel networks could lead to improvements in cancer cure rates (Siemann et al., 2004). Therefore, inhibitors of angiogenesis are promising agents in the treatment of cancer. Safrole oxide has piperonyl and epoxy structures, which are important traits for the compounds in this category to exert their physiological activity (Aguirre and Barrionuevo, 1999). The great potential of safrole oxide as antitumor agents is based on the data showing that safrole oxide can promote apoptosis of A549 lung cancer cells and VECs. In the study reported here, we confirmed the effect of safrole oxide on angiogenesis in vitro and in vivo. All the findings suggested that safrole oxide might be a promising candidate for angiogenesis inhibition. The DNA ladder assay suggested that the loss of the blood vessel might be due to apoptosis.

The rat aortic assay bridges the gap between in vivo and in vitro models combining advantages from both systems. Antagonistic effects of soluble factors or matrix factors on angiogenesis can be evaluated easily by using this model. A549 lung cancer cells promoted the growth of microvessels. The model of co-culture of rat aorta and A549 lung cancer cells reflects the growth of tumor microvessels. The result that 220 μmol l^{-1} of safrole oxide, a low concentration of this drug, inhibits angiogenesis indicated that safrole oxide is a promising angiogenic inhibitor.

The CAM assay in the chick embryo is perhaps the most widely used vessel development assay in vivo. The tumor model of the chick embryo chorioallantoic membrane (CAM) was set up by seeding A549 lung cancer cells on the CAMs of the embryos 24 h before the experiment. Therefore, our findings that safrole oxide inhibited angiogenesis through tumor cells induction provide a potential antiangiogenic treatment.

Since 1960s, safrole has been known as a weak cancer inducer (Long et al., 1963). It also has been thought that safrole oxide, a metabolic product of safrole in liver, might be a harmful compound in vivo (Wislocki et al., 1976). However, more recently, it has been shown that safrole oxide has no actual genotoxicity in the metabolic pathway (Qato and Guenthner, 1995; Guenthner and Luo, 2001). Safrole and its 2′, 3′-oxides were hydroxylated by hepatic microsomes from rats and mice at the 1′-carbon (Swanson et al., 1981). They were substrates of microsomal epoxide hydrolases, with Michaelis constants in the low micromolar range (Luo et al., 1992). The general rate of epoxide hydrolysis is much greater in human liver than in rat liver (Stillwell et al., 1974). The reason for this may be their rapid metabolic inactivation by both glutathione S-transferases and epoxide hydrolases, which occur approximately to the same extent in vitro (Luo and Guenthner, 1995). The LDH assay was performed in A549 lung cancer cells, the results indicated that safrole oxide 449 μmol l^{-1} did not show drug toxicity to the cancer cells (Du et al., 2004). Taken together, these data suggest that safrole oxide does not appear to be of genotoxicity or cytotoxicty.

Here, we report that safrole oxide can inhibit angiogenesis induced by cancer cells in vitro and in vivo. The data suggested that safrole oxide might be a promising angiogenesis inhibitor candidate. Also, in a chemical genetics perspective, cell-permeable safrole oxide can be used as a chemical probe for understanding the functions of target proteins. It could be an exciting tool to advance functional research of genes and proteins in angiogenesis and vascular disease.

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References

Aguirre, N., Barrionuevo, M., 1999. A-lipoic acid prevents 3,4-methylenedioxy-methamphetamine (MDMA)-induced neurotoxicity. NeuroReport 10, 3675–3680.

Brooks, P.C., Montgomery, A.M., Rosenfeld, M., Reisfeld, R.A., Hu, T., Klier, G., Cheresh, D.A., 1994. Integrin αVβ3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. Cell 79, 1157–1164.

Carmeliet, P., Jain, R.K., 2000. Angiogenesis in cancer and other diseases. Nature (Lond.) 407, 249–257.

Du, A.Y., Zhang, S.L., Miao, J.Y., Zhao, B.X., 2004. Safrole oxide induces apoptosis in A549 human lung cancer cells. Exp. Lung Res. 30, 419–429.

Elsdale, T., Bard, J., 1972. Collagen substrata for studies on cell behavior. J. Cell Biol. 54, 626–637.

Guenthner, T.M., Luo, G., 2001. Investigation of the role of the 2′, 3′-epoxidation pathway in the bioactivation and genotoxicity of dietary allylbenzene analogs. Toxicology 160, 47–58.

Kwon, H.J., 2003. Chemical genomics-based target identification and validation of anti-angiogenic agents. Curr. Med. Chem. 10 (9), 717–736.

Long, E.L., Nelson, A.A., Fitzhugh, O.G., Hansen, W.H., 1963. Liver tumours produced in rats by feeding safrole. Arch. Pathol. 75, 595–604.

Luo, G., Guenthner, T.M., 1995. Metabolism of allylbenzene 2′, 3′-oxide and estragole 2′, 3′-oxide in the isolated perfused rat liver. J. Pharmacol. Exp. Ther. 272, 588–596.

Luo, G., Qato, M.K., Guenthner, T.M., 1992. Hydrolysis of the 2′, 3′-allylic epoxides of allylbenzene, estragole, eugenol, and safrole by both microsomal and cytosolic epoxide hydrolases. Drug Metab. Dispos. 20, 440–445.

Miao, J.Y., Zhao, B.X., Li, H.H., Zhang, S.L., Du, C.Q., 2002. Effect of safrole oxide on vascular endothelial cell growth and apoptosis induced by deprivation of fibroblast growth factor. Acta Pharmacal. Sin. 23, 323–326.

Nicosia, R.F., Ottinetti, A., 1990. Growth of microvessels in serum-free matrix culture of rat aorta. Lab. Invest. 63, 115–122.

Noller, R.C., Kneeland, P.D., 1964. The synthesis of some tertiary amino alcohols related to chelidonine. J. Am. Chem. Soc. 68, 201–202.

Qato, M.K., Guenthner, T.M., 1995. 32P-postlabeling analysis of adducts formed between DNA and 20, 30-epoxide: absence of adduct formation in vivo. Toxicol. Lett. 75, 201–207.

Siemann, D.W., Chaplin, D.J., Horsman, M.R., 2004. Vascular-targeting therapies for treatment of malignant disease. Cancer 100, 2491–2499.

Stillwell, W., Carman, J.K., Bell, L., Horning, M.G., 1974. The metabolism of safrole and 2′, 3′-epoxysafrole in the rat and guinea pig. Drug Metab. Dispos. 2, 489–498.

Swanson, A.B., Miller, E.C., Miller, J.A., 1981. The side-chain epoxidation and hydroxylation of the hepatocarcinogens safrole and estragole and some related compounds by rat and mouse liver microsomes. Biochim. Biophys. Acta 673, 504–516.

Wislocki, P.G., Borchert, P., Miller, J.A., Miller, E.C., 1976. The metabolic activation of the carcinogen 1′-hydroxysafrole in vivo and in vitro and the electrophilic reactivities of possible ultimate carcinogens. Cancer Res. 36, 1686–1695.

Zhao, B.X., Wang, D.W., Zuo, H., Qian, G.T., Miao, J.Y., 2003. Synthesis and bioactivity of 1-alkoxy(phenoxy)-3-piperonyl-2-propanol. Chin. J. Org. Chem. 23, 1026–1028.

Zhao, Q.T., Wang, N., Jia, R., 2004. Integrin β4 is a target of rattlesnake venom during inducing apoptosis of vascular endothelial cells. Vascul. Pharmacol. 41, 1–6.