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Inhibition of human cancer cell line growth and human umbilical vein endothelial cell angiogenesis by artemisinin derivatives in vitro

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Abstract

Artemisinin derivatives artesunate (ART) and dihydroartemisinin are remarkable anti-malarial drugs with low toxicity to humans. In the present investigation, we find they also inhibited tumor cell growth and suppressed angiogenesis in vitro. The anti-cancer activity was demonstrated by inhibition (IC_{50}) of four human cancer cell lines: cervical cancer Hela, uterus chorion cancer JAR, embryo transversal cancer RD and ovarian cancer HO-8910 cell lines growth by the MTT assay. IC_{50} values ranged from 15.4 to 49.7 μM or from 8.5 to 32.9 μM after treatment with ART or dihydroartemisinin for 48 h, indicating that dihydroartemisinin was more effective than ART in inhibiting cancer cell lines. The anti-angiogenic activities were tested on in vitro models of angiogenesis, namely, proliferation, migration and tube formation of human umbilical vein endothelial (HUVE) cells. We investigated the inhibitory effects of ART and dihydroartemisinin on HUVE cells proliferation by cell counting, migration into the scratch wounded area in HUVE cell monolayers and microvessel tube-like formation on collagen gel. The results showed ART and dihydroartemisinin significantly inhibited angiogenisis in a dose-dependent form in range of 12.5–50 μM and 2.5–50 μM, respectively. They indicated that dihydroartemisinin was more effective than ART in inhibiting angiogenesis either. These results and the known low toxicity are clues that ART and dihydroartemisinin may be promising novel candidates for cancer chemotherapy.

Keywords: Artesunate; Dihydroartemisinin; Tumor; Angiogenesis; Tube formation

1. Introduction

Artemisinin and its derivatives such as artesunate (ART) and dihydroartemisinin distinguish themselves as a new generation of anti-malarial drugs with low toxicity. Having been used for the treatment of more than one million cases of malarial infection, artemisinin and its analogs are considered as safe drugs with no obvious adverse reaction or noticeable side effects [1]. Especially, artemisinin derivatives exert remarkable activity against otherwise drug-resistant plasmodium falciparum and plasmodium vivax strains [2–4]. Thus, they are gaining increasing importance in the treatment of malarial infection.

Recently, it is reported that the anti-malarial artemisinin derivatives are also active against tumor cells. Some investigators found artemisinin drugs had inhibitory effects on cancer cell growth such as lung, malanomas, breast, renal, prostate, CNS cancer cells including many drug-resistant cancer cells [5,6]. Moreover, they also have suppressive effects on the growth of human tumor xenografts in rat, mice and nude mice [7,8]. These studies indicated that artemisinin derivatives have anti-cancer activities in vitro and in vivo.

It is known that tumors are angiogenesis dependent and can elicit the production by a new capillary endothelium from the host by themselves [9]. Angiogenesis, the proliferation and migration of endothelial cells (ECs) resulting in the formation of new blood vessels, is a vital process for the progression of all solid tumors from a small, localized focus to an enlarging tumor with the capability to metastasize [10,11]. Consequently, inhibition of angiogenesis may lead to control of tumor growth and metastasis [12]. In cancer therapy, cancer inhibitors which have the anti-angiogenic activity as well as anti-cancer activity may kill cancer much more effectively. Artemisinin derivatives have been suggested to have anti-tumor activity. However, anti-angiogenic activity has not yet been demonstrated.

In the present study, we have investigated whether artemisinin derivatives ART and dihydroartemisinin have the anti-angiogenic activity as well as the anti-tumor activity. We tested the inhibitory effects of ART and dihydroartemisinin on human cervical cancer Hela, uterus chorion cancer

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JAR, embryo transversal cancer RD and ovarian cancer HO-8910 cell lines growth and the proliferation, migration, tube formation of human umbilical vein endothelial cells (HUVECs).

2. Materials and methods

2.1. Materials

ART was purchased from Guiling Pharmaceutical Co. (Guangxi, China) and dihydroartemisinin was a gift from the Engineer, Liuxu of Guiling Pharmaceutical Co. Collagen (Type I) was purchased from Sigma (Bornem, Belgium), RPMI 1640 medium, Dulbecco's modified eagle's medium (DMEM) were supplied by Gibco (BRL, Merelbeke, Belgium). HEPES, DMSO, penicillin, streptomycin, MTT and EC growth factor (VEGF) were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Four human cancer cell lines: cervical cancer Hela, uterus chorion cancer JAR, embryo transversal cancer RD, ovarian cancer HO-8910 cell lines and fibroblast cell line NIH-3T3 were purchased from Shanghai Institutes for Biological Sciences (Shanghai, China). HUVECs were obtained from the American Type Culture Collection (ATCC, Rockville, MD).

2.2. Cell culture

The four human cancer cell lines: Hela, JAR, RD, HO-8910 and NIH-3T3 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics (100 IU ml^{-1} penicillin and 100 $\mu g\,ml^{-1}$ streptomycin), at 37 °C, 5% CO_2 in air. HUVECs were grown in DMEM medium with 10% FCS, 10 $ng\,ml^{-1}$ VEGF and antibiotics. HUVECs were used within 10 passages. Human endometrium cells were isolated from the uterus of misbirth woman by 0.05% trypsin digestion and cultured in DMEM medium with 15% FCS. Cells were used within seven passages. Human endometrium cells were cultured and checked by the technologist of Shanghai Institutes for Biological Sciences.

2.3. Growth assay of various types of cells

Four cancer cells, NIH-3T3 cells and endometrium cells were plated in 24-well plates at a density of 1×10^4 cells per well. After 24 h of culture in the normal growth medium, cells were exposed to graded concentrations of ART or dihydroartemisinin for 48 h. Cells were then incubated with 5 $mg\,ml^{-1}$ MTT solution for 4 h. One hundred microliter of 10% sodium dodecyl sulfate solution was added to the culture. Absorbance at 570 nm was determined by using an ELISA reader (Bio-Tek instruments, Inc., USA). By the MTT method, cell numbers were obtained as absorbance values. The results were expressed as IC_{50} values (50% inhibitory concentration).

2.4. Growth inhibition assay of HUVECs

HUVECs were seeded at a density of 1×10^4 cells per well into 24-well plates. After 24 h incubation at 37 °C in a 5% CO_2 incubator, ART or dihydroartemisinin at different concentrations were added to the wells and the cells were further cultured for 48 h. The number of cells was counted with a coulter counter. Cell viability was evaluated by the trypan blue exclusion test.

2.5. HUVEC migration assay

5×10^4 HUVECs per well were seeded into 24-well plates and grown to confluence. The 'scratch wound' in the confluence monolayers was made using a razor blade, then each well was rinsed with PBS and 10% FBS–DMEM medium containing ART or dihydroartemisinin were added. The plates were incubated at 37 °C, 5% CO_2 in air for 24 h. The number of cells that had migrated from the edge of the wound in each 250 $\mu m \times 500\,\mu m$ area of 10 randomly chosen fields was counted. Results were expressed as the average number of cells per field.

2.6. Cell microvessel formation assay

HUVEC differentiation was evaluated by using a tube formation method as described previously [13,14] with minor modifications. An 8:1:1 volume of 3 $mg\,ml^{-1}$ Type I collagen, DMEM (10×), 0.1 M NaOH + 0.2 M HEPES + 0.26 M Na_2CO_3 was made and poured into 24-well plates with 750 μl per well at 4 °C. After a collagen gel formed by incubating at 37 °C for 1 h, 1×10^4 HUVECs per well were seeded on the collagen-coated wells and incubated for 24 h. Subsequently, 10% FBS–DMEM supplemented with 10 $ng\,ml^{-1}$ VEGF containing ART or dihydroartemisinin were added into the wells. After incubation for 3 days, microvessel formation was observed using a light microscopy and photographed. The total lengths of the tubular structures in three randomly chosen microscopic fields per well were measured by making use of a curvimeter in the microscope (Olympus, Tokyo, Japan).

2.7. Data analysis

All values are expressed as the mean ± S.D. and the significant levels between two groups were assessed by Student's *t*-test. *P* values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Effect of ART and dihydroartemisinin on the growth of various types of cells

Both ART and dihydroartemisinin inhibited the growth of the four cancer cell lines in a concentration-dependent

Table 1
Inhibition (IC_{50}) of four cancer cell lines by ART and dihydroartemisinin

Substance	Cell line, IC_{50} (μM)			
	Hela	JAR	RD	HO-8910
ART	38.6 ± 4.3	40.4 ± 5.8	15.4 ± 1.0	49.7 ± 6.9
Dihydroartemisinin	15.7 ± 3.7	24.5 ± 5.3	8.5 ± 1.1	32.9 ± 4.7

Various cells were cultured with ART or dihydroartemisinin for 48 h and cell growth was assessed by the MTT colorimetric method. Results of experiments in triplicate are expressed as the IC_{50} values that suggest the inhibitory activity of ART and dihydroartemisinin against four cancer cell lines.

manner by the MTT assay. Treatment with ART and dihydroartemisinin at concentrations greater than 5 μM for 2 days reduced the cell growth of all four lines at different levels. RD cell line was the most sensitive to ART and dihydroartemisinin in this test panel, and can be inhibited 89.7% or 94.5% by 120 μM ART or 120 μM dihydroartemisinin, respectively. The other cancer cell lines can be inhibited above 80% by 120 μM ART or 120 μM dihydroartemisinin (data not shown). The IC_{50} of the four cancer cell lines by ART and dihydroartemisinin was shown at Table 1. We showed that dihydroartemisinin was more effective in inhibiting all the four cell lines growth than ART.

We also tested the effects of ART and dihydroartemisinin on the proliferation of the normal control cells. The IC_{50} values for NIH-3T3 cells and human endometrium cells were 105.77 μM or 69.56 μM for ART or dihydroartemisinin and 139.4 μM or 88.02 μM for ART or dihydroartemisinin, respectively.

3.2. Effects of ART and dihydroartemisinin on proliferation of HUVECs

Effects of ART and dihydroartemisinin on proliferation of HUVECs were observed (Fig. 1). Cell proliferation was inhibited in a concentration-dependent manner. At every concentration >0.5 μM, the groups of treatment with ART and dihydroartemisinin were significantly different when compared with each other ($P < 0.05$). Dihydroartemisinin was more effective than ART in inhibiting the cell growth. At the highest concentration of 50 μM ART and dihydroartemisinin had inhibition rates of about 40 and 50%, respectively.

The above results show that the IC_{50} values for HUVEC and four human cancer cell lines were all lower than those for fibroblast cells and human endometrium cells, indicating that the growth inhibition activity of ART and dihydroartemisinin against HUVEC and the four cancer cell lines was stronger than fibroblast cells and human endometrium cells.

3.3. Effects of ART and dihydroartemisinin on migration of HUVECs

On HUVECs, either ART or dihydroartemisinin induced a dose-dependent decrease in cell migration (Figs. 2 and 3). Compared to the inhibition of cell growth, the effect was evident from lower concentrations. ART and dihydroartemisinin suppressed cell migration slightly at a concentration of 0.5 μM and inhibited it completely at 50 μM. Dihydroartemisinin was more effective than ART ($P < 0.01$).

3.4. Effects of ART and dihydroartemisinin on HUVEC tube formation

We tested the effects of ART and dihydroartemisinin on HUVEC tube formation in vitro. Tubulogenesis was induced in vascular ECs by seeding them on the surface of the collagen (Type I) gel for 24 h. Fig. 4 shows the branching vessel-like structures formed by HUVECs. When ART or dihydroartemisinin was added to the culture, there was a

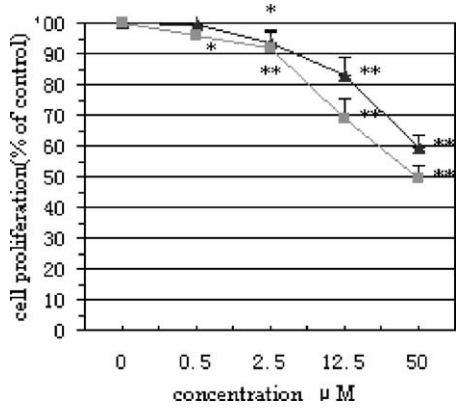

Fig. 1. Quantification of inhibitory effects of ART and dihydroartemisinin on HUVECs. HUVECs were plated in 24-well plates, allowed to attach for 24 h and then treated with different concentrations of ART or dihydroartemisinin for 2 days. Cell proliferation was determined by cell counting. Data represent the average (±S.D.) of three experiments. Symbols indicate ART (■) and dihydroartemisinin (▲). (*) $P < 0.05$; (**) $P < 0.01$, compared to control.

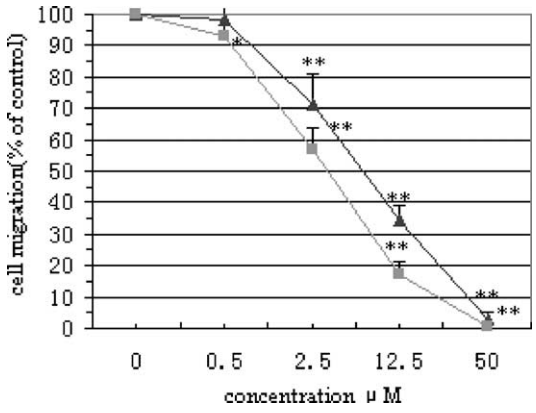

Fig. 2. Quantitative inhibition of HUVECs by ART and dihydroartemisinin. Confluent cultures of HUVECs were wounded with a razor blade. The cells were incubated with ART or dihydroartemisinin at different concentrations for 24 h. The numbers of cells migrated from the edge of the wound within each 125 μm × 500 μm area were counted. Data represent the average (±S.D.) ($n = 3$). Symbols indicate ART (■) and dihydroartemisinin (▲). (*) $P < 0.05$; (**) $P < 0.01$, compared to control.

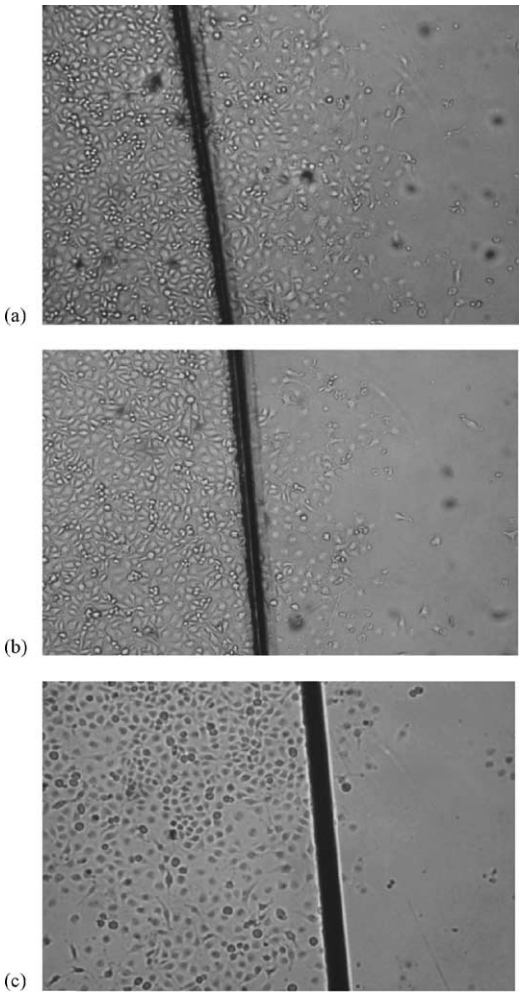

Fig. 3. Effect of dihydroartemisinin on HUVECs migration. Microscopic morphology (200×) of HUVECs treated as in Fig. 2: (a) control; (b) 2.5 μM dihydroartemisinin; (c) 50 μM dihydroartemisinin.

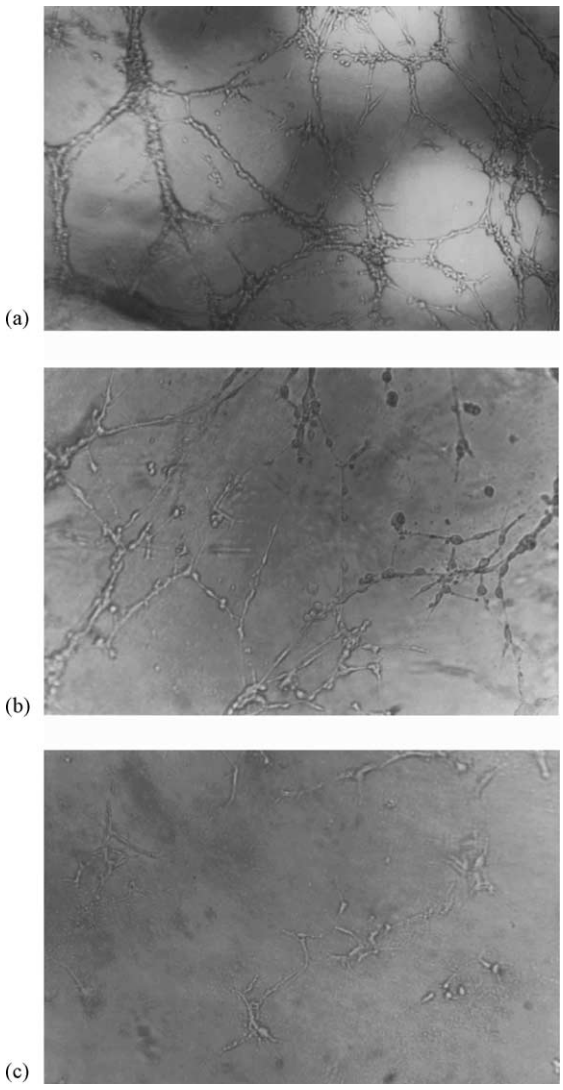

Fig. 4. Effect of dihydroartemisinin on HUVEC tube formation. Microscopic morphology (200×) of HUVECs treated as in Fig. 5: (a) control; (b) 12.5 μM dihydroartemisinin; (c) 50 μM dihydroartemisinin.

decrease in both the number and length of tube formation in a dose-dependent manner (Fig. 5). There was approximately 70 or 90% reduction in the total tube length per field following 50 μM ART or dihydroartemisinin treatment for 48 h, respectively. The inhibitory activity of dihydroartemisinin is also greater than that of ART ($P < 0.01$).

4. Discussion

Although some studies have shown that the anti-malarial ART and dihydroartemisinin were active against many cancer cell lines in vitro [5,6,15], their effects on these four cancer cell lines Hela, JAR, RD and HO-8910 were not reported. In this investigation, we examined ART and dihydroartemisinin's anti-tumor activity on the above four cancer cell lines to extend the anti-tumor spectrum of the two drugs. The IC_{50} values of these four cell lines were different according to their different sensitivities towards ART and dihydroartemisinin. Ovarian cancer line showed

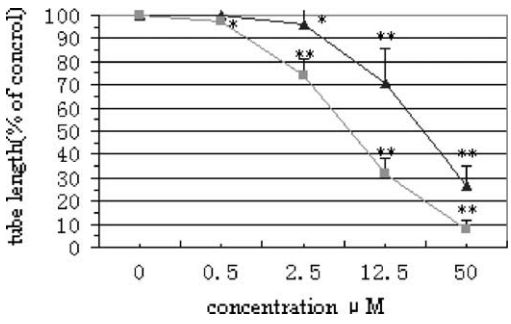

Fig. 5. Dose-dependent inhibition of HUVEC tube formation by ART and dihydroartemisinin. HUVECs were plated in a three-dimensional culture system on collagen gels and then treated by ART or dihydroartemisinin at different concentrations for 2 days. Total length of tube formation per field was measured and results were expressed as percent of control (average ± S.D.) ($n = 3$). Symbols indicate ART (■) and dihydroartemisinin (▲). (*) $P < 0.05$; (**) $P < 0.01$, compared to control.

the highest IC_{50} values indicating the lowest sensitivity to both ART and dihydroartemisinin in this test panel. While, either ART or dihydroartemisinin was most active against embryo transversal cancer cell line RD. Compared to ART, dihydroartemisinin had greater anti-tumor activity in vitro.

Angiogenesis plays a vital role in tumor growth, intrasavation, metastatic spread [10,11]. Inhibition of angiogenesis provides a good chance of preventing cancer from becoming malignant [16,17]. Angiogenesis is composed of several process dissociations of pericytes from preexisting vessel, digestion of extracellular matrix with proteases growth, migration and invasion of ECs, tube formation, then finally remodeling occurs. Among these processes, growth, migration and tube formation of ECs are essential for angiogenesis. This motivates us to determine anti-angiogenic activities of ART and dihydroartemisinin by inhibiting HUVECs growth, migration and tube formation. Our data showed that the inhibition of HUVECs growth of dihydroartemisinin occurred at higher concentration than the concentrations needed to inhibit cell migration and tube formation. It was expected that the suppression of angiogenesis by dihydroartemisinin might not be induced only by inhibiting ECs proliferation. The mechanism of such effect should be studied further. In the present investigation of anti-angiogenic activity, results suggested that dihydroartemisinin and ART were two potent inhibitors. It was known that dihydroartemisinin was the main product of artemisinin and its derivatives including ART by metabolization of human bodies. This information together with our results indicated ART and other artemisinin drugs might continue to be active or even more against cancer after metabolization in human bodies.

The mechanism of inhibition of ART and dihydroartemisinin on tumor growth is not studied exhaustively. It is well known that the artemisinin and its derivative molecules contain an endoperoxide bridge that reacts with a ferrous iron atom to form free radicals which contributes to their anti-malarial activity [18,19]. However, whether the formation of radical molecules and/or reactive oxygen species of artemisinin drugs contributes to their anti-tumor activity is not completely proved. Moreover, it is not known whether genetic pathways are involved in cancer cells and to which extent they vary in different derivatives [20,21]. Singh and Lai have shown that dihydroartemisinin and ART are selectively toxic to human cancer cells and with relatively low toxicity on normal human cells [22,23]. It is also reported that artemisinin derivatives are active against many drug-resistant cancer cell lines, such as small-cell lung cancer (SCLC) [24]. Compared to normal cells, cancer cells contain higher rates of iron intake correlated with their high transferrin receptor concentration. So, cancer cells including drug-resistant cancer cells are more susceptible to artemisinin drugs under conditions of high iron availability [25,26].

Although we suggested the inhibitory effects of ART and dihydroartemisinin on angiogenesis in vitro, the mechanism of inhibition is still not clear at the present time and further studies are needed to gain a full understanding of the anti-angiogenic activity in vivo.

Since many identified tumor and angiogenesis inhibitors have problems concerning their therapeutic applications because of their excessive toxicity and limited efficacy. The anti-tumor and anti-angiogenic efficacy together with the known low and selective toxicity make it possible that ART and dihydroartemisinin may be promising novel candidates for cancer chemotherapy.

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References

[1] Klayman DL. Qinghaosu (artemisinin): an antimalarial drug from China. Science 1985;228:1049–55.
[2] Benakis A, Paris M, Loutan L, Plessas CT, Plessas ST. Pharmacokinetics of artemisinin and artesunate after oral administration in healthy volunteers. Am J Trop Med Hyg 1997;56:17–23.
[3] Hien TT, White NJ. Qinghaosu. Lancet 1993;341:603–8.
[4] Dhingra V, Rao VM, Narasu L. Current status of artemisinin and its derivatives as anti-malarial drugs. Life Sci 2000;66:279–300.
[5] Efferth T, Dunstan H, Sauerbrey A, Miyachi H, Chitambar CR. The anti-malarial artesunate is also active against cancer. Int J Oncol 2001;18:767–73.
[6] Efferth T, Davey M, Olbrich A, Rücher G, Gebbart E, Daveu R. Activity of drugs from traditional Chinese medicine towards sensitive and MDRI- or MRPI-overexpressing multidrug-resistant human CCRF-CEM leukenna cells. Blood Cells Mol Dis 2002;28:160–8.
[7] Yang X, Pan Q, Liang Y-G. Cancer 1997;16:186–7 (in Chinese).
[8] Wang Q, Wu L-M, Li A-Y, Zhao Y, Wang N. China J Chinese Materia Med 2001;26:707–8 (in Chinese).
[9] Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med 1971;285:1182–6.
[10] Folkman J, Klagsburn M. Angiogenic factors. Science 1987;235: 442–7.
[11] D'Amore PA, Thompson RN. Mechanism of angiogenesis. Annu Rev Physiol 1987;49:453–64.
[12] Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS, Ferrata N. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumor growth in vivo. Nature 1993;362:841–4.
[13] Kawasaki J, Hirano K, Hirano M, Nishimura J, Nakatsuka A, Fujishima M, Kanaide H. Dissociation between the Ca^{2+} signal and tube formation induced by vascular endothelial growth factor in bovine aortic endothelial cells. Eur J Pharm 2000;398:19–29.
[14] Soeda S, Kozako T, Iwata K, Shimèno H, Malingre TM, Feraly FS, Kampinga HH, Kenings AW. Oversulfated fucoidan inhibits the basic fibroblast growth factor-induced tube formation by human umbilical vein endothelial cells: its possible mechanism of action. Biochim Biophy Acta 2000;1497:127–34.
[15] Woerdenbag HJ, Moskal TA, Pras N, Kishimoto S, Sudo K, Kanamaru T, Brem H. Cytotoxicity of artemisinin-related endoperoxides to Enrlich ascites tumor cells. J Nat Prod 1993;56:849–59.
[16] Ingber D, Fujita T, Folkman J, et al. Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumor growth. Nature 1990;348:555–7.

[17] O'Reilly MS, Holmgren L, Shing Y, Chen CC, Rosenthal RA, Moser M, Lane WS, Cao Y, Sage EH, Folkman J. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell 1994;79:315–28.
[18] Berman PA, Adams PA. Artemisinin enhances heme-catalysed oxidation of lipid membranes. Free Radic Biol Med 1997;22:1283–8.
[19] Zhang F, Grosser DK, Meshnick SR. Hemin-catalyzed decomposition of artemisinin (qinghaosu). Biochem Pharmacol 1992;43:1805–9.
[20] Efferth T, Olbrich A, Bauer R. mRNA expression profiles for the response of human tumor lines to the anti-malarial drugs artesunate, arteether, and artemether. Biochem Pharmacol 2002;64:617–23.
[21] Kihara C, Tsunoda T, Tanaka T, Yamana H, Furukawa Y, Ono K, Kitahara O, Zembutsu H, Yanagawa R, Hirata K, Takagi T, Nakamura Y. Prediction of sensitivity of esophageal tumor to adjuvant chemotherapy by cDNA microarray analysis of gene-expression profiles. Cancer Res 2001;61:6474–9.
[22] Singh NP, Lai H. Selective toxicity of dihydroartemisinin and holotransferrin toward human breast cancer cells. Life Sci 2001;79:49–56.
[23] Lai H, Singh NP. Selective cancer cell cytotoxicity from exposure to dihydroartemisinin and holotransferrin. Cancer Lett 1995;91:41–6.
[24] Sadava D, Phillips T, Lin C, Kane SE. Tranferrin overcomes drug resistance to artemisinin in human small-cell lung carcinoma cells. Cancer Lett 2002;179:151–6.
[25] Reizenstein P. Iron, free radical and cancer. Med Oncol Tumor Pharmacother 1991;8:229–33.
[26] Shterman N, Kupfer B, Moroz C. Comparison of transferrin receptors, iron content and isoferritin profile in normal and malignant human breast cell lines. Pathobiology 1991;59:19–25.