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 (IC50) 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. IC50 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 angiogenesis 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.

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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, melanomas, 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...
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), Dulbecco’s modified eagle’s medium (DMEM) were supplied by Gibco (BRL, Mereibcke, 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 μg ml⁻¹ streptomycin), at 37 °C, 5% CO₂ in air. HUVECs were grown in DMEM medium with 1% penicillin and 10 ng ml⁻¹ VEGF and antibiotics. HUVECs were used within 10 passages.

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 × 10⁴ cells per well. After 24h 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 4h. 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 IC₅₀ values (50% inhibitory concentration).

2.4. Growth inhibition assay of HUVECs

HUVECs were seeded at a density of 1 × 10⁴ cells per well into 24-well plates. After 24 h incubation at 37 °C in a 5% CO₂ 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 × 10⁴ HUVECs per well were seeded into 24-well plates and grown to confluence. The ‘scratch wound’ in the confluent 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% CO₂ in air for 24 h. The number of cells that had migrated from the edge of the wound in each 250 μm × 500 μm 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 Na₂CO₃ 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 × 10⁴ 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 microscope 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
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 IC₅₀ 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 IC₅₀ 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 IC₅₀ 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 24h. Fig. 4 shows the branching vessel-like structures formed by HUVECs. When ART or dihydroartemisinin was added to the culture, there was a...

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**Table 1**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Cell line</th>
<th>IC₅₀ (μM)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Hela</td>
<td>JAR</td>
</tr>
<tr>
<td>ART</td>
<td>38.6 ± 4.3</td>
<td>40.4 ± 5.8</td>
</tr>
<tr>
<td>Dihydroartemisinin</td>
<td>13.7 ± 3.7</td>
<td>24.3 ± 5.3</td>
</tr>
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Various cells were cultured with ART or dihydroartemisinin for 48h and cell growth was assessed by the MTT colorimetric method. Results of experiments in triplicate are expressed as the IC₅₀ values that suggest the inhibitory activity of ART and dihydroartemisinin against four cancer cell lines.

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**Fig. 1.** Quantification of inhibitory effects of ART and dihydroartemisinin on HUVECs. HUVECs were plated in 24-well plates, allowed to attach for 24h 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 the wound within each 125 μm × 500 μm area were counted. 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 24h. 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.
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 IC₅₀ values of these four cell lines were different according to their different sensitivities towards ART and dihydroartemisinin. Ovarian cancer line showed...