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Inhibition of angiogenesis in vivo and growth of Kaposi's sarcoma xenograft tumors by the anti-malarial artesunate

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Abstract

Artesunate (ART) is a semi-synthetic derivative of the sesquiterpene artemisinin used for the second line therapy of malaria infections with *Plasmodium falciparum*. ART also inhibits growth of many transformed cell lines. In the present investigation, we show that ART inhibited the growth of normal human umbilical endothelial cells and of KS-IMM cells that we have established from a Kaposi's sarcoma lesion obtained from a renal transplant patient. The growth inhibitory activity correlated with the induction of apoptosis in KS-IMM cells. Apoptosis was not observed in normal endothelial cells, which, however, showed drastically increased cell doubling times upon ART treatment. ART strongly reduced angiogenesis in vivo in terms of vascularization of Matrigel plugs injected subcutaneously into syngenic mice. We conclude that ART represents a promising candidate drug for the treatment of the highly angiogenic Kaposi's sarcoma. As a low-cost drug, it might be of particular interest for areas of Kaposi's sarcoma endemics. ART could be useful for the prevention of tumor angiogenesis.

Keywords: Angiogenesis; Apoptosis; Drug resistance; Kaposi's sarcoma

1. Introduction

Kaposi's sarcoma (KS) is a highly angiogenic multifocal tumor and produces several angiogenic cytokines [1–3]. It derives from endothelial cells of the skin vasculature. Aggressive forms of KS can also involve the mucosa and viscera. Human herpesvirus 8 (HHV8), also called Kaposi's sarcoma associated herpesvirus, is strictly correlated with the occurrence of KS and most probably causally involved in its etiology [4,5]. Classic KS is a rare tumor that occurs in elder HHV8-positive subjects. Iatrogenic KS occurs in transplant patients under immunosuppressive treatment who either received HHV8 with the transplant or reactivated a latent infection. Epidemic KS is also associated with HIV infections, since the lost immunosurveillance caused by the lentiviral infection increases the susceptibility of patients for the development of KS [6,7]. The development of drug resistance frequently hampers the success of chemotherapy, and the response duration is limited [8]. Despite progresses in treatment, KS can be fatal. There is, therefore, an urgent need for novel drugs to improve the treatment outcome of KS patients. We have recently developed the concept of angio-prevention, a strategy that aims at suppressing or retarding the development of aggressively growing primary cancers or metastases through the inhibition of angiogenesis [9,10]. We have shown that chemopreventive drugs such as the thiolic anti-oxidant *N*-acetyl cysteine [11], the green tea polyphenol (−)-epigallocatechin gallate [12,13], the synthetic retinoid 4-HPR [14], the neuroprotective anti-oxidant α-lipoic acid (unpublished observations) and the hormones chorionic gonadotropin [15] and somatostatin [16] act at least in part by inhibiting tumor vascularization.

Abbreviations: ARAB, Arteanuine B; ARE, Arteether; ARM, Artemether; ARS, Aartemisinin; ART, Artesunate; ARTEMIS, Artemisitene; ARTEST1/2, Dihydroartemisinyl Ester Stereoisomers 1/2; HAART, Highly Active Antiviral Therapy; HHV8, Human Herpesvirus 8; HUVEC, Human Umbilical Endothelial Cells; IC_{50}, Inhibition Concentration 50%; KS, Kaposis Sarcoma; N.C.I, National Cancer Institute

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Artesunate (ART) is a semi-synthetic derivative of the sesquipertene artemisinin extracted from the leaves of *Artemisia annua* that has been used in traditional Chinese medicine for the treatment of fever for more than 2000 years [17]. Today, ART and the related compound, artemether, are used as second line treatment for malarial infections with *Plasmodium falciparum*. These compounds are well-tolerated, and no major side effects are observed in patients [18,19]. The molecular mechanisms of action are not fully explored yet. Formation of carbon-centered free radicals has been suggested [17,20–24]. A recent meta-analysis revealed that ART-containing regimens substantially reduce treatment failure compared to standard anti-malarial treatments [25]. ART can significantly prolong the time span of anti-malarial treatment efficacy [26]. This may at least in part be due to ART's effects on otherwise drug-resistant strains of the parasite [27].

The observation that ART inhibits the growth of many transformed cell lines has led to the hypothesis that the drug can also be useful for the treatment of human neoplasia [28–37]. A salient feature is that ART is active against multidrug-resistant cell lines expressing either the *MDR1*, *MRP1*, or *BCRP* genes [32]. In the present investigation, we have tested ART for its effects on KS for the following reasons: (i) it has direct effects against transformed cells, it is well tolerated and it is already widely used as an antimalarial drug; (ii) KS has a high incidence in regions of Africa that are endemic for the human herpesvirus 8 and in part also for malaria; (iii) KS is the most frequent neoplasia diagnosed in some of these areas [38]; (iv) there is an urgent need of low-cost therapies in these regions and (v) ART has been shown to also exert anti-viral activities [39]. We show here that ART has a strong anti-angiogenic activity in vivo and negatively affects growth of normal endothelial cells and KS cells and induces apoptosis in the latter. These activities translate into inhibition of KS cell growth in nude mice. We have addressed the question, whether ART reveals anti-tumor activity in vivo and whether this activity is related to anti-angiogenic effects.

2. Materials and methods

2.1. Animals and materials

Six to seven weeks-old C57BL/6 mice and male (CD-1) BR nude mice were obtained from Charles river (Calco) and housed in specific pathogen-free conditions in conformity with European Community legislation. Matrigel, an extract of the murine Engelbreth-Holm-Swarm tumor grown in C57BL/6 mice, was produced as previously described [40]. Briefly, Engelbreth-Holm-Swarm tumor material was homogenized with a polytron homogenizer in a high-salt buffer (3.4 M NaCl; 50 mm Tris–Cl, pH 7.4; 4 mm EDTA with 2 mm *N*-ethyl-maleimide). The homogenate was separated from the supernatant by centrifugation and the procedure was repeated three times. The remaining pellet was solubilized in 2 M urea, 50 mm Tris–Cl (pH 7.4), 150 mm NaCl overnight. The supernatant was cleared by centrifugation and extensively dialyzed against Tris–saline and finally against DMEM. The resulting material, Matrigel, is rich in basement membrane components (laminin, collagen IV, nidogen, and perlecan) with limited quantities of growth factors. Heparin was obtained from Clarisco (Schwarz Pharma). ART was obtained from Saokim Co. Ltd.

2.2. Cell culture

Kaposi's sarcoma-IMM is a spontaneously immortalized iatrogenic KS cell line derived from a renal transplant patient. The KS line was grown in Roswell Park Memorial Institute 1640 medium containing glutamine (300 μg/ml) and 10% heat-inactivated FCS. Human umbilical vascular endothelium (HUVE) cells were grown in M199 medium containing 10% FCS, 10 ng/ml of acidic fibroblast growth factor and basic fibroblast growth factor, 20 ng/ml epidermal growth factor, and 160 μg/ml heparin.

2.3. In vitro response to cytostatic drugs

For growth analysis of KS-IMM and HUVE cells, 1000 and 2500 cells/well, respectively, were seeded in 96-well plates and grown in complete medium or treated with ART at the concentrations between 0.1 and 15 μM. The wells were coated with gelatine for the growth assay of HUVE cells. Media were changed every 48 h. The number of viable cells was measured over-time using the crystal violet assay. Briefly, after fixation and staining in a solution of 0.75% crystal violet, 0.35% sodium chloride, 32% ethanol and 3.2% formaldehyde, the cells were dissolved in 50% ethanol, 0.1% acetic acid and read in a microtiter spectrophotometer at 540 nm. Eight data points were collected for each drug concentration and time interval. Data were statistically evaluated using one-way ANOVA test followed by Bonferroni's multiple comparison test.

2.4. Apoptosis assay

Apoptosis was measured using a photometric enzyme immunoassay (Cell death detection ELISA kit, Roche Biochemicals). This assay is based on the quantitative sandwich-enzyme-immunoassay principle using mouse monoclonal antibodies directed against DNA and histones. These antigens become available upon nucleosomal fragmentation during apoptosis. Degradation of chromatin into (poly-) nucleosome particles and their release into the culture medium is diagnostic for advanced phases of apoptosis. HUVE cells and KS-IMM cells were grown

for 24 or 48 h as described and treated with ART at different concentrations. Vincristine (10 μM) was used as positive control for inducing apoptotis. The cells were harvested, resuspended at a density of 10^5 cells/ml, centrifugated at 500 × *g* for 5 min and lysed for qualitative and quantitative determination of cytoplasmic histone associated DNA fragments, according to the protocol of the manufacturer.

2.5. Matrigel tubulogenesis assay

A 24-microwell plate was kept at −20 °C and carefully filled with 300 μl/well of Matrigel (10 mg/ml) with a cold pipette avoiding bubbles. The Matrigel was polymerized for 1 h at 37 °C. 70,000 HUVE cells per well were suspended in regular medium in the absence or presence of 0, 1, or 10 μg ART and carefully layered on top of the polymerized Matrigel. The effects on the formation of vessel-like structures were recorded after 6 and 24 h with an inverted microscope (Leitz DM-IRB).

2.6. Zymography of metalloproteinases

Zymographic analysis of the enzymatic activity of matrix metallproteinases was performed as described [41]. Briefly, proteins contained in serum free KS-IMM and HUVE cell culture supernatants were precipitated and analyzed by SDS–PAGE using gelatin containing gels. After the gel run, SDS was removed and gel slices were incubated in the presence (15 μM) or absence of ART. After digestion, gels were stained with 0.1% Coomassie brilliant blue. Enzyme-digested areas were identified as white bands against a blue background.

2.7. Angiogenesis in vivo

Matrigel (12 mg/ml) is liquid at 4 °C, but solidifies rapidly to form a gel upon subcutaneous inoculation. Addition of VEGF (100 ng/ml), TNFα (2 ng/ml) and heparin (24–26 U/ml) as angiogenic stimuli (VTH) to a final volume of 600 μl results in a strong angiogenic response with formation of new vessels that grow into the Matrigel, whereas no reaction is induced by Matrigel with buffer alone [42].

Four days after injection of the Matrigel pellets, the animals were killed and the gels were recovered and weighed. The haemoglobin content was measured as an indicator of angiogenesis. The recovered gels were minced and dispersed in water, and haemoglobin released was measured using a Drabkin reagent kit 525 (Sigma), the concentration was calculated from a standard curve and normalized to 100 mg of recovered gel as previously described [42]. In order to assess the effect of ART on angiogenesis the drug was added either directly to the Matrigel (7.2 μM) containing VTH or given orally in the drinking water of the animals (100 mg/kg/day).

2.8. Kaposi's sarcoma (KS) xenografts

Kaposi's sarcoma-IMM cells are tumorigenic in immune-deficient mice when injected together with Matrigel [11,43]. Ten animals for each treatment were injected with 5.0 × 10^6 KS-IMM cells suspended in 250 μl of 10 mg/ml Matrigel. Matrigel is liquid at 4 °C and rapidly solidifies at 37 °C. ART was solved in ethanol at a concentration of 300 mg/ml and 100 μl were added to 30 ml drinking water per day (final concentration: 1 mg/ml). This equals to a dosage of 167 mg/kg/day. Control animals received the same amount of drinking water containing ethanol alone. Drinking water was limited to 5 ml per mouse in both, treated and control groups, according to the daily need of the animals under the conditions in the animal house. Both groups consumed all the water supplied so that there were no differences in hydration between the two groups and the drug-treated animals had a controlled dose (two factors that could not be guaranteed, if unlimited access to drinking water were allowed). Tumor growth was monitored by measuring the maximal and the minimal tumor diameters. The experiment was stopped when the animals of the control group has reached tumor volumes of over 1.0 cm^3. At the end of the treatment, mice were killed and tumors were recovered and weighted.

3. Results

3.1. Growth inhibition

Artesunate (ART) has been described to inhibit growth of many, but not all transformed cell lines. Since nothing is known on ART's activity against KS cells, we analyzed, whether the immortalized KS-IMM cell line is susceptible to the drug. As KS is of endothelial origin, we also investigated growth inhibitory effects on normal human umbilical endothelial cells (HUVE). Both cell types were treated with 0.1 to 10 μM ART and compared to vehicle-treated control cells. At concentrations above 5.0 μM, a dose-dependent inhibition of cell growth was observed in both cell types (Fig. 1). For clarity, data for concentrations below 1.0 μM are not shown. Growth inhibition was significantly different from untreated controls for 7.5 and 10.0 μM ART ($p < 0.05$ and $p < 0.01$), respectively, according to one-way ANOVA group comparison and Bonferroni's multiple comparison test). Thereby, an increase in cell doubling times from 98 to 236 h (7.5 μM) and 387 h (10.0 μM) for HUVE cells and from 47 to 79 h (7.5 μM) and 92 h (10 μM) for KS-IMM cells was measured by means of non-linear regression analysis.

3.2. Apoptosis

Next, we analyzed the activation of programmed cell death (apoptosis) by ART. For this reason, we performed

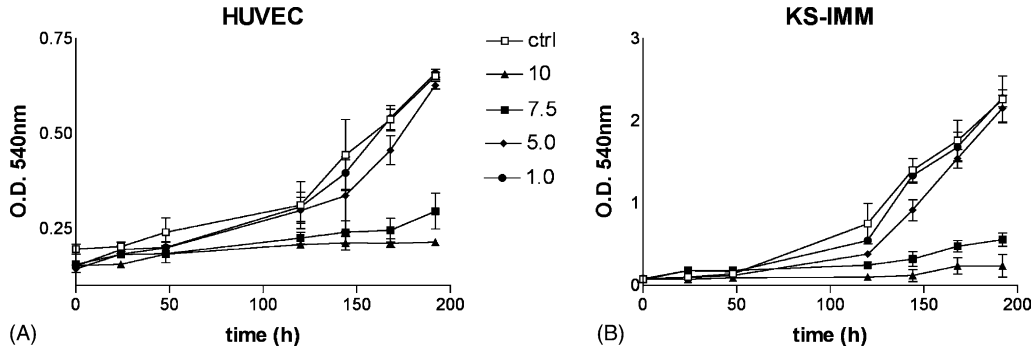

Fig. 1. Growth analysis of HUVE cells (Panel A) and KS-IMM cells (Panel B). HUVE and KS-IMM cells were grown in the presence of ART at concentrations between 1 and 10 μM in comparison to untreated controls. The number of viable cells was measured over-time using the crystal violet assay. Concentrations of 7.5 and 10 μM strongly inhibit cell growth.

ELISA assays that specifically measure the release of histone–DNA complexes. Treatment of HUVE cells with 1–15 μM ART for 24 h did not induce apoptosis (Fig. 2). On the contrary, apoptosis was induced in KS-IMM cells even at the lowest concentration tested (1 μM, $p < 0.01$) and increased in a dose-dependent manner (2.5–15 μM, $p < 0.001$ according to one-way ANOVA with Bonferroni's post-test; Fig. 2).

Induction of the cellular suicide program explains KS-IMM cell growth inhibition at least in part, but growth retardation of HUVE cells cannot be attributed to apoptotic cell death.

3.3. Matrigel tubulogenesis assay

Human umbilical endothelial cells (HUVE) cells, when seeded onto Matrigel, form capillary like structures and anti-angiogenic effects can be mediated by the interference with this morphogenetic capacity. ART did not consistently interfere with tubologenesis at doses up to 15 μg/ml (data not shown).

3.4. Zymography of metalloproteinases

We also wished to know whether ART directly inhibits matrix metalloproteinases (MMPs). For this purpose we performed zymographic analyses of MMP-2 and MMP-9 secreted by KS-IMM and HUVE cells. The treatment of gel slices containing gelatine as a substrate and the electrophoretically separated proteins of cell culture supernatants with 15 μg/ml ART did not reveal any inhibition of the activity of the MMPs that appear as unstained bands in the substrate gels (data not shown).

3.5. Angiogenesis in vivo

Activation and growth of endothelial cells is a prerequisite for vessel formation during angiogenesis. Therefore, the question arises, whether the cytotoxic effect of ART in vitro is correlated to an anti-angiogenic effect in vivo. Using the Matrigel plug assay, we injected gel containing the strong angiogenic stimuli, VEGF, TNFα and heparin subcutaneously into nude mice. After 4 days, the gel was heavily vascularized and the pellet contained

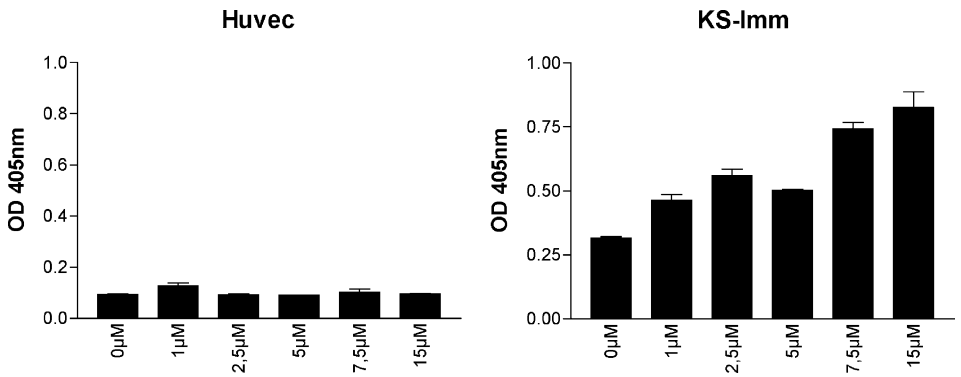

Fig. 2. Artesunate (ART) induces apoptosis in KS-IMM but not in HUVE cells. Apoptosis was measured using a sandwich enzyme immunoassay that detects nucleosomes released into culture supernatants during apoptosis. HUVE cells (left hand panel) and KS-IMM cells (right hand panel) were grown for 24 h as described and treated with ART at the concentrations indicated. No apoptosis could be detected for HUVE cells, whereas KS-IMM cells showed a dose-dependent induction of nucleosome fragmentation.

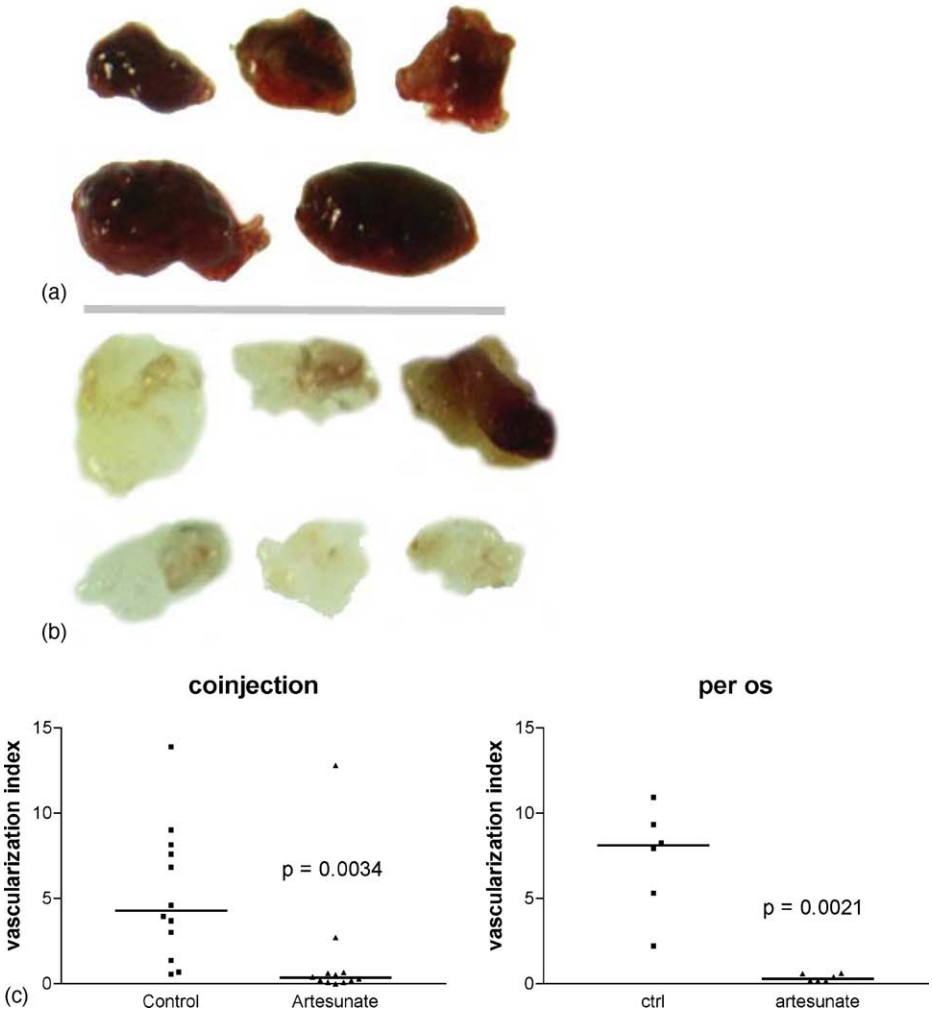

Fig. 3. Artesunate (ART) strongly inhibits angiogenesis in vivo Matrigel sponges impregnated with VEGF, heparin and TNFα as described in the methods section were injected under the skin of mice. After 4 days recovered plugs show a strong vascularization (Panel a). When the sponges also contain ART vascularization is strongly reduced (Panel b). The vascularization index (normalized haemoglobin content) shows that vascularization is significantly reduced when ART is co-injected with the plug (Panel c, left) or administered with the drinking water (Panel c, right).

functional blood filled vessels (Fig. 3, Panel a). If ART was added to the pellets or applied with the drinking water for 4 days, a drastic reduction in the vascularization of the pellet was observed (Fig. 3, Panel b). As a measure to quantify the angiogenic response, we determined the haemoglobin content. The vascularization indices are shown in Fig. 3 (Panel c). The haemoglobin content of the Matrigel plugs was normalized with the weight of those obtained from mice where ART was administered to the pellets (left hand panel) or applied with the drinking water (right hand panel). The vascularization indices showed a nearly complete suppression of angiogenesis in both cases ($p = 0.0034$ and $p = 0.0021$, respectively; t test).

3.6. Xenografts

As tumor growth depends on both the growth characteristics of transformed cells and on angiogenesis in the tumor's microenvironment, we investigated, whether ART suppresses growth of KS-IMM cells in vivo. We inoculated nude mice with 5.0×10^6 KS-IMM cells subcutaneoulsy and treated the mice with ART at the same day. Again, the drug was administered with the drinking water.

Artesunate (ART) suppressed growth of KS-IMM xenograft tumors (Fig. 4). Statistical significant differences, if compared with the untreated control tumors were reached from day 21 on. At the end of the experiment, tumors were resected and weighted. The diagram inserted in Fig. 4 shows whiskers with the entire range of tumor weights measured and boxes with the 25th to the 75th percentiles. The horizontal line indicates the 50th percentile. The weight differences between ART-treated an untreated xenograft tumors was statistically significant ($p = 0.0144$; unpaired t test) providing evidence that ART strongly retarded Kaposi's sarcoma xenograft growth in nude mice.

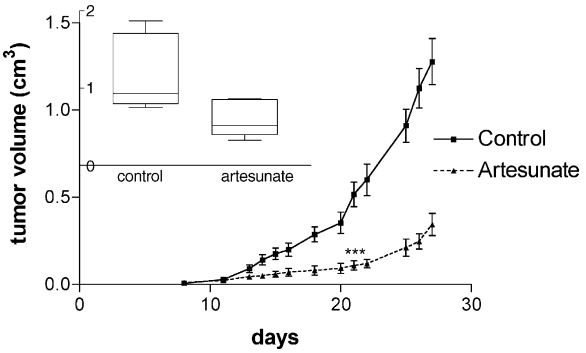

Fig. 4. Artesunate (ART) retards KS-IMM xenograft growth in nude mice 5.0×10^6 KS-IMM cells were injected together with Matrigel into the flanks of immunodeficient mice. After about 10 days palpable tumors began to form. Animals received the drug (ART) or vehicle alone (control) in the drinking water from the day of injection on. Growth differences were significant from day 21 on (***$p < 0.001$, one-way ANOVA with Bonferroni's post-test). The insert shows the weights of tumors recovered at the end of the experiment. Whiskers indicate the whole range observed, boxes indicate the 25th and 75th percentiles, the horizontal bar indicates the 50th percentile. Weight differences were statistically significant ($p = 0.0014$, t test).

4. Discussion

In the present investigation, we describe the anti-angiogenic and anti-tumoral effects of artesunate (ART). ART suppressed growth of KS as well as normal endothelial cells. ART strongly induced apoptosis in KS-IMM cells in a dose-dependent manner and this probably explains the growth suppression. On the contrary, normal endothelial cells are not driven to the apoptosis program nor did they show signs of necrotic cell death. Interestingly, ART reduced endothelial cell activation and/or cell cycle transit time. Cell doubling time is increased four-fold in HUVE cells but only two-fold in KS-IMM cells. Increasing the cell cycle by ART may explain, why ART inhibited cell growth of HUVE cells without involvement of apoptosis. It has been shown recently, that fast growing cells are more vulnerable than slowly growing cells [32]. The prospect that ART may first accelerate cell cycle transit time and then reveal cytotoxicity to these cells is interesting and merits further investigation in the future. The finding that ART inhibited tumor xenograft growth in vivo is interesting, since it raises the possibility that ART may be suited to inhibit tumor cell growth in human beings. In this context, it is worth mentioning that the ART-treated mice did not suffer from severe toxicity (data not shown). This is an observation that is consistent with the experience with ART for human malaria treatment.

In recent investigations, we found that other "angio-preventive" drugs either induce apoptosis in both, KS-IMM and endothelial cells, as it is the case for the green tea polyphenol, epigallocatechin gallate or do not induce apoptosis at all, as observed for the anti-oxidant *N*-acetyl-cysteine [11], the synthetic retinoid 4-hydroxyphenylretinamide (4HPR); which, at high doses, induces apoptosis in HUVE but not in KS-IMM cells [14], or the neuroprotective anti-oxidant, α-lipoic acid (Pfeffer, unpublished observations). It is possible that the differential effects of ART observed for KS-IMM and HUVE cells are a consequence of the different cellular transformation state. This view is in accordance with results obtained with melanoma cells and normal fibroblast cells (Efferth and Funk, unpublished data). In order to identify potential mediators of apoptosis we are currently performing gene expression profiling studies, where we compare KS-IMM and HUVE cells. Our result is in line with the lack of severe toxicity of malaria treatment with ART. The differential killing of tumor cells without affecting normal tissues is a highly desired beneficial feature for tumor therapy.

The Matrigel plug assay mimics very well normal, physiological conditions for the quantitative assessment of neo-angiogenesis. It reflects many of the features of tumor angiogenesis. Angiogenic chemokines are locally released from the pellet (or from a growing tumor) to stimulate endothelial cell proliferation and migration and extracellular matrix degradation which is needed to allow invasion and vessel formation. ART virtually abolished angiogenesis using this assay and it is highly effective also when administered with the drinking water.

Hence, a combination of two effects was achieved by ART's induction of apoptosis in tumor cells with growth retardation in endothelial cells that probably account for the observed anti-angiogenic effects. Effects on the formation of vessel-like structures by HUVE cells grown on collagen have been described for higher doses (50 μM) of ART [28]. At the concentrations that caused maximal growth inhibition in HUVE cells (10 μM), we detected, however, only slight effects on tubulogenesis (data not shown). Activity of the main matrix metalloproteinases secreted by KS-IMM and HUVE cells, MMP-2 and MMP-9, was also not affected as analyzed by zymography (data not shown).

The experience with highly active antiretroviral therapy (HAART) in HIV/HHV8 double infected patients shows that KS regresses but there are also exceptions [44–48]. The clinical benefit of HAART for patients with advanced, symptomatic KS is not clear [49], and it is also possible that recrudescence of KS in patients treated with HAART for many years is a phenomenon yet to come. Also, HAART is still not available to all HIV patients, HHV8 is endemic in Africa where it leads to KS also in HIV negative patients [38,50–52] and classic KS in the Mediterranean area, though a rare disease, needs more efficient treatment options [53].

In the present investigation, we demonstrate anti-angiogenic and anti-KS effects of ART. This is in accord with a recently published study on the mother drug artemisinin and its metabolite dihydroartemisinine [54]. We conclude that inhibition of angiogenesis may be a general mechanism of artemisinin derivatives to inhibit tumor growth in vivo. Our concept of angio-prevention [9,10] is based on

non-toxic drugs that can be taken for extended periods, eventually for life-long treatments that are needed to control tumor or metastasis growth that would resume once the inhibition of angiogenesis is removed. It is unknown yet, whether ART belongs to this category, since its prolonged use has not been reported. However, given its efficacy against drug-resistant tumor cells [30,32] and the anti-angiogenic activities described here, it appears to be well-suited for adjuvant therapy in combination with classical chemotherapy. In addition, it might prove useful for AIDS-associated prevention of KS in HHV8 endemic areas, where HAART is not available.

Acknowledgements

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