

Inhibition of Kaposi's Sarcoma *in Vivo* by Fenretinide

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ABSTRACT

Purpose: We examined the effects of fenretinide [*N*-(4-hydroxyphenyl)retinamide; (4HPR)] on highly angiogenic Kaposi's sarcoma tumors *in vivo* and investigated the mechanisms involved for potential clinical applications.

Experimental Design: (CD-1)BR nude mice bearing KS-Imm cell tumors were randomized to receive 4HPR or vehicle until sacrifice. *In vitro*, KS-Imm and endothelial cells were treated with 4HPR to study the effects on proliferation, apoptosis, migration, and invasion; *in vivo* angiogenesis was evaluated in the Matrigel model. Angiogenesis-related and retinoid receptor molecules were examined at the mRNA and protein expression levels.

Results: *In vivo*, 4HPR significantly ($P < 0.001$) reduced growth of detectable Kaposi's sarcoma (KS) xenografts and inhibited angiogenesis in the Matrigel plug assay ($P < 0.04$). *In vitro*, 4HPR affected KS-Imm and endothelial cell growth and KS-Imm migration and invasion. 4HPR invasion inhibition was associated with decreased release of matrix metalloproteinase-2 and rapid reduction of vascular endothelial growth factor (VEGF) expression by KS cells and of vascular endothelial growth factor receptor 2 (VEGFR2) by KS and endothelial cells. Finally, 4HPR repression of angiogenesis was associated with a 4HPR-induced increase in retinoic acid receptor β expression.

Conclusions: These data indicate that 4HPR inhibits KS tumor growth *in vivo* through a mechanism involving the modulation of angiogenesis-associated growth factors and their receptors on both tumor and endothelial cells. In addition, 4HPR inhibited invasion by decreasing of matrix metalloproteinase-2 activity. Our results justify further studies to evaluate the utility of 4HPR as a chemopreventive or

therapeutic agent in KS, a malignancy associated with immune suppression that has a high risk of recurrence with highly active antiretroviral therapy failure.

INTRODUCTION

KS,⁴ a highly vascularized tumor associated with infection by the KS-associated herpesvirus/HHV8 (1), is the most common tumor associated with human immunodeficiency virus infection, which develops in 15–30% of cases during the course of AIDS (2). KS contributes to morbidity and mortality through invasion of the lungs, the gastrointestinal tract, and other visceral organs. Although the use of highly active antiretroviral therapy (HAART) has led to decreased KS tumor incidence and burden in AIDS-KS patients, the risk for recurrence of KS, once HAART is discontinued, is very high. KS is also associated with therapeutic immune suppression in HHV8-infected patients. In geographical areas where a high endemic HHV8 infection rate overlaps with widespread, inadequately treated HIV infection, KS has become the leading tumor type (3).

The main histological features of KS are spindle cells, an inflammatory infiltrate, and the formation of a dense, poorly organized capillary network recruited from the host (2). KS spindle cells release a mixture of potent stimulators of endothelial cell migration and invasion (4) that induce angiogenesis *in vivo* (5). The process of angiogenesis is tightly regulated, depending on a dynamic balance between stimulators and inhibitors. In the early 1970s, inhibition of angiogenesis was proposed as a possible strategy for the treatment of solid tumors (6), and the search for mediators and inhibitors of tumor angiogenesis began (7). Recently, we have noted that numerous drugs used as cancer chemoprevention agents appear to target tumor angiogenesis, suggesting that this may be a main mechanism for this class of molecules (8).

Retinoids have been shown to exert chemopreventive and antitumor activities in a variety of normal and malignant cells (9–11). RA and 9-*cis*-RA have produced tumor responses in KS both *in vitro* and *in vivo* (12–14). RA does not control recurrences of the original tumor but can block the development of new primary tumors (15). The vitamin A analogue fenretinide (4HPR) has been shown to inhibit breast carcinogenesis in preclinical studies (16), and premenopausal women treated with 4HPR have shown a significantly lower incidence of new breast cancers than the corresponding control group in a clinical trial

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Note: Drs. Ferrari and Morini contributed equally to this study.

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⁴ The abbreviations used are: KS, Kaposi's sarcoma; HHV, human herpesvirus; HAART, highly active antiretroviral therapy; RA, retinoic acid; fenretinide, [*N*-(4-hydroxyphenyl)retinamide (4HPR)]; CAM, chorio-allantoic membrane; RAR, retinoic acid receptor; RXR, retinoid X receptor; AP, activator protein; VEGF, vascular endothelial growth factor; MMP, matrix metalloproteinase; VEGFR, vascular endothelial growth factor receptor; FGF, fibroblast growth factor; SFM, serum-free medium; CM, conditioned medium; TNF, tumor necrosis factor; VTH, 100 ng/ml VEGF, 2 ng/ml TNF α , and heparin; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

(17). These patterns are typical of anti-angiogenic agents that prevent further development of hidden metastases, even if they are unable to control growth of the original primary tumor (18).

Several studies have indicated that retinoid treatment is associated with angiogenesis inhibition and a decreased vascular response *in vitro* and *in vivo* (19–23). In particular, it has been shown that 4HPR is capable of inhibiting angiogenesis in the chick CAM assay (24, 25), an activity associated with several different potential mechanisms; it has also been shown that 4HPR represses tumor growth *in vivo* in prevention protocols (24).

Retinoids exert their effects through two families of nuclear receptors, RARs and RXRs, which are ligand-dependent transcription factors belonging to the superfamily of steroid-thyroid-vitamin D₃ hormone receptors. Retinoid receptors activate gene expression by binding to response elements present in the promoter regions of RA-responsive genes (26). Retinoid receptors have also been shown to negatively regulate the expression of certain genes by antagonism of the activity of transcription factor AP-1 (27).

Here we demonstrate that administration of 4HPR potently inhibited growth of established KS tumors *in vivo* in early intervention protocols. This activity was associated with decreased tumor vessel density, repression of the proangiogenic release of VEGF and MMP-2 by the tumor cells, as well as reduced endothelial cell proliferation and *VEGFR2* expression. The 4HPR-mediated down-regulation of angiogenesis may be *RARβ*-dependent, because *RARβ* expression was strongly induced by fenretinide, whereas other retinoids that did not effect cell growth *in vitro* or angiogenesis *in vivo* were unable to induce *RARβ* expression. Our studies indicate that 4HPR may be useful in the prevention and therapy of KS.

MATERIALS AND METHODS

Cell Culture and Chemicals. The previously described KS-Imm cell line, isolated in our laboratory from a kidney transplanted immuno-suppressed patient (28), was grown in RPMI containing 10% FCS and 1% glutamine. Endothelial HUVE cells were obtained from the ATCC (Rockville, MD) and cultivated on gelatin coated plates (1% in PBS) in M199 containing 10% heat-inactivated FCS, 100 μg/ml heparin, 10 ng/ml acidic FGF, 10 ng/ml basic FGF, 10 ng/ml epidermal growth factor, 10 μg/ml hydrocortisone. 4HPR (Fenretinide; Calbiochem, La Jolla, CA) was dissolved in absolute ethanol at a stock concentration of 10 mM and stored in aliquots at –70°.

Tumor Growth *in Vivo*. The effect of 4HPR on the KS growth *in vivo* was tested by using 18 seven-week old male (CD-1)BR nude mice (Charles River, Lecco, Italy) having an average weight of ~25 g, housed in pathogen-free conditions. KS-Imm cells at 80% confluence were harvested by trypsinization, counted and resuspended in SFM. The cells (5×10^6) were then mixed with liquid Matrigel (10 mg/ml) to a final volume of 250 μl at 4°C and injected s.c. into the flanks of nude mice. Ten days after tumor cell injection, when a distinct tumor mass was detectable in all animals, the animals were randomized into three groups with the same average tumor size in an early intervention protocol. Each group was given daily an intragastric administration of 100 μl sesame seed oil either alone (con-

trol group) or containing 1.2 mg/kg or 12 mg/kg body weight of 4HPR, based on previous studies (24). The animals were weighed and the tumor diameters measured every 2–3 days. Animals were sacrificed after 14 days of treatment, and the tumor was removed and processed for histological evaluation. Vessel densities were estimated by counting the vessels in stained sections. Random fields were selected for section of several different tumors derived from control and treated animals. Housing and treatments of animals were in accordance with the Italian National and European Community guidelines (D.L. 2711/92 No. 116; 86/609/EEC Directive).

Cell Proliferation and Apoptosis Assays. To study cell growth, 750 KS-Imm or 2,000 HUVE cells per well were seeded in 96-well plates and grown in complete medium or treated with various concentrations of 4HPR. Media were changed every 48 h and the number of viable cells was measured with 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 at 595 nm. To measure any enrichment of cytoplasmic histone-associated DNA fragments after 4HPR-induced cell death, a commercially available kit was used (Cell Death Detection ELISA, Roche, Mannheim, Germany) using 24-well plates seeded with 10,000 (KS-Imm) or 30,000 (HUVE) cells per well and grown in complete medium with various concentrations of 4HPR.

Chemotaxis and Invasion Assays. Chemotaxis and chemo-invasion assays on KS-Imm cells were carried out in Boyden chambers as described previously (29). KS-Imm cells were exposed to different concentrations of 4HPR for 15 h in SFM with 0.1% BSA. Trypan blue exclusion under these conditions showed no decreased cell viability as compared with controls. After treatment, 5×10^4 cells in SFM with 0.1% BSA were placed in the upper compartment with or without 4HPR as above. The two compartments of the Boyden chamber were separated by a 8-μm pore-size polycarbonate filters coated with 0.3 mg/ml gelatin for the chemotaxis assay or with Matrigel (15 μg/ml), a reconstituted basement membrane, for the invasion assay. Supernatants from NIH3T3 cells (NIH3T3-CM) were used as chemo-attractants in the lower chamber. After 6 h of incubation at 37° in 5% CO₂, the filters were recovered, the cells on the upper surface were mechanically removed and those on the lower surface were fixed and stained. The migrated cells were counted in 5–10 fields for each filter under a microscope. The experiments were performed in triplicate and repeated three times.

***In Vivo* Angiogenesis.** We used the Matrigel sponge model of angiogenesis described previously (5). KS-CM and heparin or VTH (100 ng/ml VEGF, 2 ng/ml TNFα, and heparin) either alone or in combination with 4HPR were added to unpolymerized liquid Matrigel at 4°C and the mixture brought to a final volume of 600 μl. The Matrigel suspension was then injected slowly s.c. into the flanks of C57/bl6 male mice (Charles River, Lecco, Italy) with a cold syringe. At body temperature *in vivo*, the Matrigel quickly polymerizes to form a solid gel. Different groups of animals (four mice in each group) were used for the different treatments. The control group was treated with vehicle alone and provided plain drinking water *ad libitum*. One group received 4HPR (5 μM final concentration)

only in the Matrigel sponge, whereas another group was given 4HPR in the drinking water (12 mg/kg/day) three days before Matrigel injection and continued 4 days after. A third group received both oral and Matrigel incorporated 4HPR treatments. After 4 days, gels from all groups were collected and weighed. Samples were either minced and diluted in water to measure the hemoglobin content with a Drabkin reagent kit (Sigma, Milano, Italy) or processed for histology.

Gelatin Zymography. Supernatants of cells incubated for 20 h in regular SFM with 0.1% BSA or in the presence of different concentrations of 4HPR were removed and pelleted by centrifugation. The protein content was measured by the Bradford method (Bio-Rad, Hercules, CA) and gelatin zymography performed as described previously (30). Briefly, SDS-PAGE gels were prepared containing copolymerized gelatin at a final concentration of 1.6 mg/ml. After electrophoresis, the gels were washed in 2.5% Triton X-100 for 30 min to remove SDS and incubated for 18 h at 37°C in collagenase buffer [40 mM Tris, 200 mM NaCl, and 10 mM CaCl₂ (pH 7.5)]. Gels were then stained in 0.1% Coomassie brilliant blue followed by destaining. The enzyme-digested regions were observed as white bands against a blue background.

RNA Extraction, RT-PCR, and Real-time RT-PCR. Total RNAs were isolated from control and treated cells that were grown in complete medium using the RNeasy Mini Kit (Qiagen, Milano, Italy). RT was performed with oligodeoxythymidylic acid primers and PCR for each *RAR* and *RXR* subtype was performed as described previously (31). *VEGF*, *VEGF-B*, and *RAR-β* mRNA expressions were analyzed by quantitative real-time RT-PCR by using the following specific primers: *VEGF* sense, 5'-CAGACGTGTAATGTTCCCTGC; *VEGF* antisense, 5'-GCAGCGTGGTTTCTGTATC; *VEGF-B* sense, 5'-TGTCTCCCAGCCTGATG; *VEGF-B* antisense, 5'-AGTCAA-GGGCACCACCA; *RAR-β* sense, 5'-TTCTACTGCGAG-TCCGT; *RAR-β* antisense, 5'-GCTGGTGCTCTGTGTTTCAA. Relative expression of each gene was assessed in comparison to the house keeping gene *GAPDH* amplified with the primers: sense 5'-GAAGGTGAAGGTCGGAGT and antisense 5'-CAT-GGGTGAATCATATTGGAA. cDNAs were amplified for 50 cycles using iQ Supermix (Bio-Rad, Hercules, CA) containing the intercalating agent SYBR Green in a two-step amplification scheme (95°C, 15 s; 60°C, 30 s). Fluorescence was measured during the annealing step on a Bio-Rad iCycler iQ instrument (Bio-Rad, Hercules, CA). Blank controls that did not contain cDNA were run in parallel. All samples were run in triplicate. After amplification, melting curves with 80 steps of 15 s and a 0.5°C temperature increase were performed to control for amplicon identity. Relative expression values with standard errors and statistical comparisons (unpaired two-tailed *t* test) were obtained using Qgene software (32).

Protein Extraction, Immunoprecipitation, and Western Blotting. KS-Imm and HUVE cells were grown in 100-mm culture dishes in regular media in the presence or absence of 5 μM 4HPR for the indicated times. Cellular proteins were isolated in lysis buffer containing 6 M urea, 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, and 10% glycerol. Samples containing 50 μg of proteins as determined by Bradford analysis were subjected to electrophoresis and transferred to Hybond ECL membranes (Amersham, Milano, Italy). For West-

ern blotting, membranes were blocked in 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20 for 1 h at room temperature and then incubated for 3 h with an anti-VEGFR2 antibody (Santa Cruz Biotechnology, Santa Cruz Biotechnology, CA) at room temperature. The membranes were subsequently incubated for 1 h with peroxidase-labeled secondary antibody (Amersham, Milano, Italy) and specific complexes were revealed by enhanced chemiluminescence solution (Amersham, Milano, Italy). For VEGF detection, KS-Imm cells were grown as above and during the last 8 h were labeled in 1.5 ml of methionine and cysteine-free MEM containing 2 mM glutamine, 10% dialyzed FCS, and 100 μCi ³⁵S-*trans* label (ICN, Irvine, CA). Culture media were collected, centrifuged at maximum speed, and precleared with a 1:1 suspension of Protein A-Sepharose in PBS for 2 h at 4°C. Immunoprecipitation on the same amounts of radiolabeled material was then performed at 4°C overnight with a mouse monoclonal antihuman VEGF antibody (Sigma, Milano, Italy) and 50 μl of Protein A-Sepharose in PBS. The immunoprecipitated protein was washed three times in radioimmunoprecipitation assay buffer, once in PBS, and eluted from the resin with 20 μl of SDS sample buffer at 100°C for 2 min. The eluted protein was loaded on 12.5% SDS-polyacrylamide gels and electrophoresed. Gels were then dried under vacuum and exposed to Hyperfilm MP (Amersham, Milano, Italy).

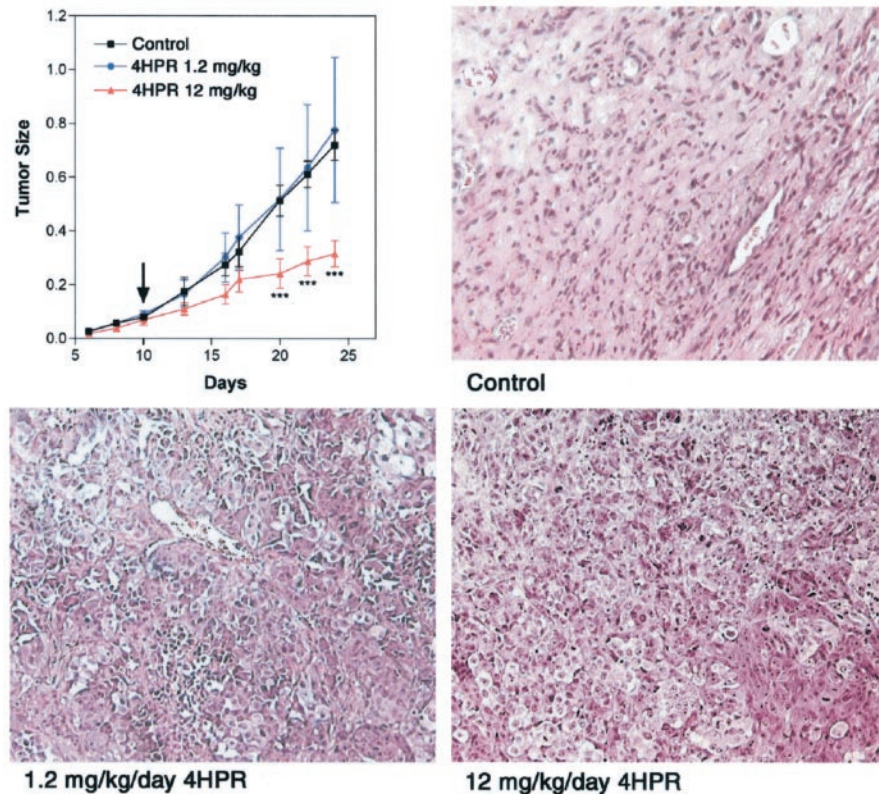
VEGF Quantification by ELISA. VEGF protein released into the media by KS-Imm cells was measured using a commercial ELISA kit (LISTARFISH; CYTImmune Sciences, College Park, MD) following the manufacturer's instructions. The supernatants from cells grown in complete medium either without or with 0.1, 1, and 5 μM 4HPR were collected after 24 or 48 h. The ELISA assay was performed on 100 μl, and the values obtained were compared with a calibration curve prepared by serial dilution of standard VEGF in complete medium. The assay was run in triplicate and repeated with similar results.

Stable Transfections. The *RARβ* cDNA was cloned into the *pZeoSV* expression vector as described previously (33). Empty vector and the *RARβ*-expressing vectors were transfected into KS-Imm cells with Lipofectamine 2000 (Invitrogen, Irvine, CA). Stable transfectants were isolated by selection with 400 μg/ml of Zeocin (Invitrogen, Irvine, CA), and the total pool of transfectants was used.

RESULTS

4HPR Inhibition of KS Growth in Nude Mice. KS-Imm cells formed highly vascular tumors when injected s.c. into nude mice, with tumor masses becoming detectable and measurable ~10 days after injection. At this time, animals were randomized into groups with the same average tumor volume and 4HPR treatment was started. Animals that were treated with a daily intragastric treatment of 12 mg/kg body weight 4HPR in sesame seed oil showed reduced tumor growth as compared with controls, which formed large tumors (Fig. 1). No alteration of tumor growth was observed for animals treated with 1.2 mg/kg 4HPR. Comparison of tumor growth curves by two-way ANOVA showed that the differences between controls and 12 mg/kg 4HPR-treated animals were statistically significant from day 20 on (*P* < 0.001). No differences were noted in animal

Fig. 1 Effect of intragastrically administered 4HPR on the growth of KS tumors *in vivo*. **Top left panel**, (CD-1)BR nude mice received, at time 0 of the experiment, a s.c. injection of KS-Imm cells (5×10^6 cells/mouse) in the flank region. Starting on day 10, when the tumor mass was measurable, the mice were treated daily with either 4HPR [1.2 (bottom left panel) or 12 mg/kg (bottom right panel) body weight in sesame oil] or the same amount of vehicle alone (top right panel). Treatment continued until the end of the experiment (day 24). The average tumor size \pm SE is shown; differences in tumor sizes of the control and 12 mg/kg treated groups were statistically significant from day 20 on (ANOVA: $P < 0.001$). H&E-stained sections of control and 1.2 mg/kg 4HPR treated tumors showed large areas of vascularization that were absent in the 12 mg/kg 4HPR treated samples. Original magnification $\times 100$.



body weights, indicating little or no toxicity of the 4HPR treatment. Histological examination showed extensive vascularization of the tumors in the control (Fig. 1) and 1.2 mg/kg 4HPR-treated animals (Fig. 1), whereas tumors in the 12 mg/kg 4HPR-treated animals showed very little vascularization (Fig. 1). Vessel counts showed a significantly (ANOVA: $P < 0.001$) reduced microvessel density in the tumors that formed in the 12 mg/kg 4HPR-treated animals (2 ± 1.07 vessels/unit field) as compared with controls (7.9 ± 2.3 vessels/unit field). These data indicated that repression of angiogenesis had an important role in the inhibition of tumor growth observed.

In Vitro Effects of 4HPR on KS-Imm and Endothelial Cells. To determine the action of 4HPR on cell proliferation, KS-Imm cells were treated with a range of concentrations of 4HPR (from 10 nM to 5 μ M) and the cell number assessed. Cell growth inhibition was apparent after 4 days of exposure to 5 μ M 4HPR, which became more evident at day 7 (two-way ANOVA: $P < 0.001$; Fig. 2A). 4HPR was also tested for its potential to induce apoptosis by determination of cytoplasmic histone-associated DNA fragments. No significant differences between controls and treated cells were observed even at the highest concentrations used (Fig. 2B).

Because angiogenesis is required for solid tumor growth and tumors from the 12 mg/kg 4HPR-treated animals showed reduced vessel densities, we also examined the effects of 4HPR on endothelial cells *in vitro*. A concentration of 5 μ M 4HPR potently inhibited endothelial cell growth with significant differences with respect to controls (two-way ANOVA: $P < 0.001$)

from 24 h to the end of the experiment (Fig. 2C). A concentration of 1 μ M 4HPR also strongly and significantly inhibited HUVE cell growth ($P < 0.001$) at later time points (5 and 7 days). Only a small percentage of cells underwent apoptosis after 24 h of exposure to 4HPR, whereas longer treatment times with 5 μ M 4HPR significantly (ANOVA: $P < 0.001$) reduced cellular apoptosis (Fig. 2D). Prolonged exposure to 5 μ M 4HPR (up to 2 weeks) protected cells from apoptosis; these remained viable, yet grew at a lower rate (data not shown).

The effects of 4HPR on KS-Imm cell migration and invasion were then tested in a broad dose/response experiment. Untreated KS-Imm cells migrated and invaded through Matrigel in response to fibroblast CM (NIH3T3; Fig. 3), whereas few cells migrated in the absence of a chemo-attractant (SFM). Pretreatment (15 h) of the cells with 5 μ M 4HPR, which was also maintained during the 6-h assay, significantly inhibited KS-Imm migration and invasion (two tailed t test: $P = 0.02$ and $P = 0.008$, respectively), whereas treatment with lower 4HPR concentrations did not show significant effects (Fig. 3). Identical results were obtained with cells exposed to 4HPR only during the assay (without pretreatment; data not shown).

Effect of 4HPR on Gelatinolytic Activity Released by KS-Imm Cells. Because invasion of basement membranes requires metalloproteases, which are essential to the invasive process, we tested whether 4HPR could inhibit the gelatinase activity associated with these enzymes. Zymographic evaluation was performed on supernatants of cells incubated for 20 h in the absence or presence of increasing concentrations of 4HPR.

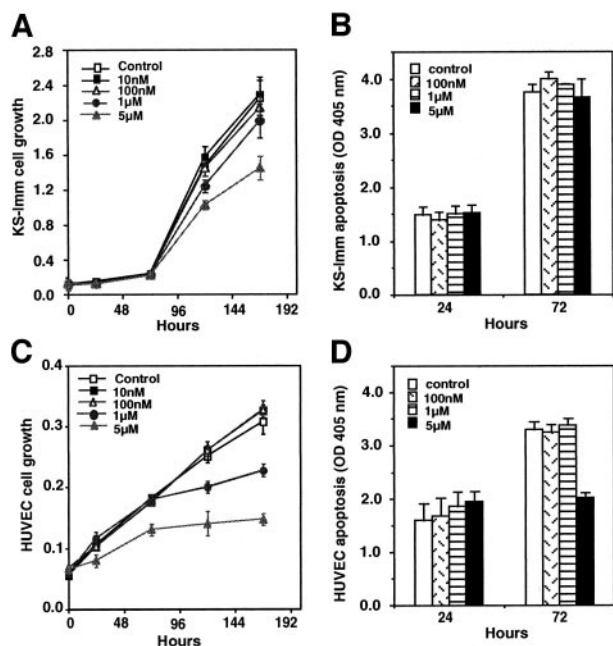


Fig. 2 A, KS-Imm cell growth *in vitro* in the presence of increasing doses of 4HPR, as assessed by the crystal violet assay; at 5 μM , 4HPR significantly inhibited cell growth. B, KS-Imm apoptosis in the presence of 4HPR, as assessed by the Cell Death Detection ELISA assay. No significant differences were observed. C, HUVEC cell growth *in vitro*; at 1 and 5 μM , 4HPR significantly inhibited cell growth. D, HUVEC cell apoptosis in the presence of 4HPR; after 72 h of treatment, 5 μM 4HPR significantly reduced apoptosis as compared with controls. Means \pm SD are shown.

KS-Imm cells produced a characteristic gelatinase activity corresponding to the 62-kDa activated form of gelatinase A (MMP-2). Treatment with 4HPR decreased MMP-2 activity produced by KS-Imm cells in a dose-dependent manner (Fig. 3).

Inhibition of Angiogenesis *in Vivo*. The effects of 4HPR on angiogenesis-associated endothelial cell functions observed *in vitro* were then confirmed *in vivo* in the Matrigel angiogenesis assay. Matrigel suspensions containing either KS cell conditioned media (KS-CM) or a cocktail (VTH) of VEGF (100 ng/ml) and TNF α (2 ng/ml) as angiogenic stimuli were injected s.c. into mice. The presence of KS-CM or VTH in the Matrigel sponges promoted a hemorrhagic vascularization of the gels within 4 days. Quantification of the extent of angiogenesis by hemoglobin content measurement showed that 4HPR treatment significantly (Mann-Whitney: $P < 0.04$) reduced the angiogenic response with respect to the positive control. This occurred consistently when 4HPR was (a) added directly to the Matrigel (5 μM) containing KS-CM or VEGF/TNF α ; (b) given orally in the drinking water of the animals (360 $\mu\text{g}/\text{animal}/\text{day}$, corresponding to ~ 12 mg/kg/day); or (c) administered with a combination of both the direct and oral treatment approaches (Fig. 4A). Histological examination (Fig. 4B) confirmed the absence of vascularization in the samples from 4HPR-treated irrespective of the angiogenic stimulus used (KS-CM or VTH).

Modulation of VEGF and VEGFR2 Receptor by 4HPR.

Because VEGF is a key angiogenic factor released by tumor cells in response to hypoxia, we examined the effects of 4HPR

on the expression of VEGF in KS-Imm cells. Quantitative analysis by real-time RT-PCR revealed that exposure of KS-Imm cells to 5 μM 4HPR for 4 h significantly decreased the mRNA levels for VEGF (unpaired two-tailed t test: $P < 0.05$; Fig. 5A). A significant decrease in VEGF-B (unpaired two-tailed t test: $P < 0.001$; Fig. 5A) required 24 h of exposure to 5 μM 4HPR. The effects of 5 μM 4HPR on VEGF after 4 h with quantitative RT-PCR was consistent with observations in Northern blot analyses (data not shown).

VEGF protein can be detected in culture supernatants of KS-Imm cells and, under reducing conditions, the major VEGF proteins secreted have a molecular mass of 23 kDa, corresponding to glycosylated VEGF₁₆₅, and 18 kDa, corresponding to glycosylated VEGF₁₂₁. Immunoprecipitation of VEGF from supernatants of ³⁵S-labeled KS-Imm cells showed that 24 h of treatment with 5 μM 4HPR reduced VEGF secretion (Fig. 5B). Lower 4HPR concentrations had little effect (data not shown). Using a sensitive ELISA system, we observed that KS-Imm cells released a mean of 252 ± 6.5 pg of VEGF/ 10^6 cells into the culture medium after incubation for 24 h. Treatment of KS-Imm cells with 5 μM 4HPR significantly (Student's t test: $P < 0.025$) reduced VEGF levels to 50% of the control values (130 ± 37.2 pg/ 10^6 cells). Forty-eight hours of 4HPR treatment also induced a striking decrease in VEGF levels (from 312 ± 8.1 to 199 ± 7.7 pg/ 10^6 cells; Student's t test: $P < 0.005$).

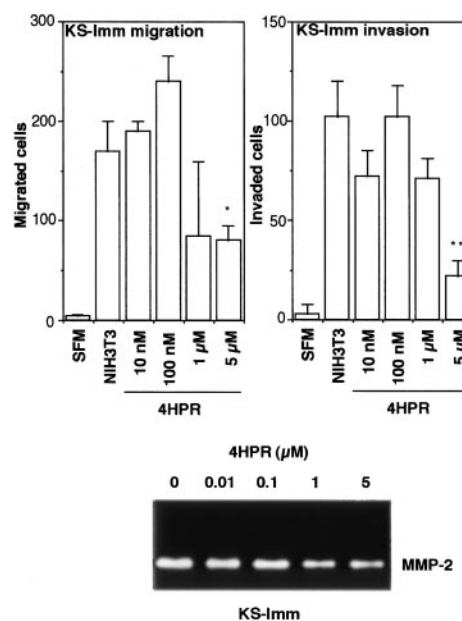


Fig. 3 Inhibition of KS-Imm cell migration and invasion (top panels) by increasing doses of 4HPR. SFM and supernatants of NIH3T3 fibroblasts were used as negative and positive controls, respectively. Experiments were performed in triplicate and repeated three times, means \pm SD are shown. 4HPR inhibited both migration and invasion. Bottom, zymographic detection of secreted gelatinase activity. Supernatants of cells treated for 20 h with various concentrations of 4HPR were analyzed by gelatin zymography at equivalent protein concentration. Gelatinolytic activity is observed as white bands on the blue-stained background of gelatin in the gel. 4HPR dose-dependently inhibited the release of enzymatic activity into the supernatants, indicating inhibition of MMP release.

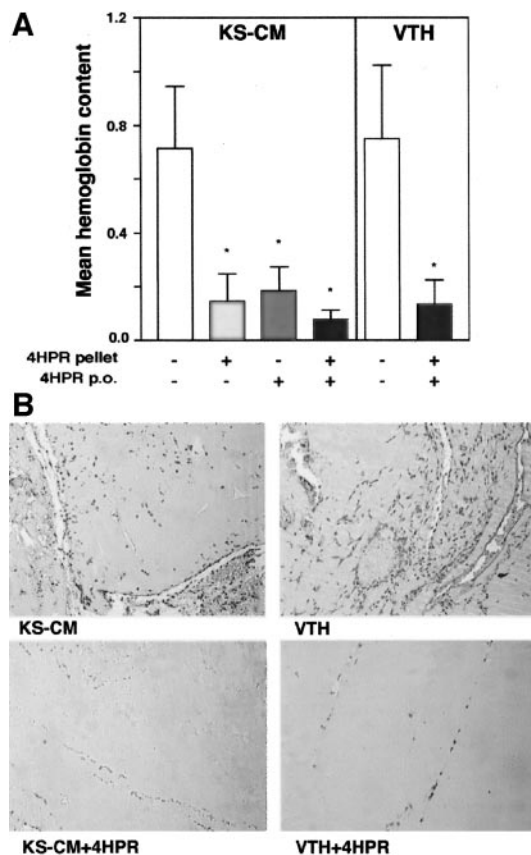


Fig. 4 Effects of 4HPR on angiogenesis *in vivo*. **A**, the angiogenic response in Matrigel sponges as measured by hemoglobin content of the gels. Matrigel sponges containing supernatants of KS cells (KS-CM) and heparin or VTH were markedly vascularized and hemorrhagic 4 days after *s.c.* implantation. Inclusion of 4HPR in the pellets ($5 \mu\text{M}$ final concentration) or continuous oral administration of 4HPR in the drinking water (12 mg/kg/day) or a combination of both significantly reduced (Mann-Whitney: $P < 0.04$) the formation of new blood vessels. **B**, hematoxylin- and eosin-stained sections of the Matrigel angiogenesis assay pellets. Vascularization is obvious in the control samples (KS-CM and VTH), whereas only a few cells penetrated the Matrigel in the samples from animals receiving 4HPR in the drinking water. Means \pm SE are shown; original magnification $\times 100$.

VEGF-C, the only form of *VEGF* mRNA we found expressed by HUVE cells, remained unaltered in both HUVE and KS-Imm cells after 4HPR treatment (data not shown).

The effect of 4HPR on VEGFR2, a VEGF key receptor, was evaluated by Western blotting of lysates from control and 4HPR treated KS-Imm and HUVE cells. Although 4HPR treatment of both cell lines had no apparent effect on *VEGFR2* mRNA levels (data not shown), treatment with $5 \mu\text{M}$ 4HPR markedly down-regulated the protein levels of VEGFR2 in HUVE cells, with a 50% decrease observed after 24 h (Fig. 6A). KS-Imm produced low amounts of VEGFR2 protein that was also down-regulated by $5 \mu\text{M}$ 4HPR, readily observed after 8 h ($>80\%$ decrease; Fig. 6B) that remained present at later time points ($\sim 50\%$; Fig. 6B). Lower 4HPR concentrations had little or no effect (data not shown).

Expression and Regulation of *RAR* β . 4HPR acts through retinoid receptor-dependent and -independent mecha-

nisms (34). To determine whether retinoid receptors mediated the effects we described, we examined the expression of RARs and RXRs by RT-PCR analysis using specific primers that allow the clear distinction among the receptor subtypes (31). KS-Imm cells showed basal expression of high levels of *RAR* α , *RAR* γ , *RXR* α , and *RXR* γ and very low or absent levels of *RXR* β (Fig. 7A). Real-time RT-PCR revealed that exposure to 4HPR dose dependently induced *RAR* β expression, reaching a maximum after 24 h (Fig. 7B). In contrast, 24 h of exposure to $10 \mu\text{M}$ all-*trans*-RA, 9- or 13-*cis*-RA failed to produce *RAR* β induction (Fig. 7B). However, the extent of *RAR* β mRNA induction in KS-Imm cells by 4HPR did not appear to be sufficient to generate protein levels detectable by Western blotting using commercially available antibodies. We, therefore, examined the effect of exogenously expressed *RAR* β in these cells by stably transfecting them with an expression vector (*pZeoSV-RAR* β). Real-time RT-PCR evaluation of *RAR* β mRNA levels indicated that the amount of the transgene was only 10% greater than that obtained after 24 h exposure to $5 \mu\text{M}$ 4HPR (data not shown). Interestingly, growth analysis showed that constitutive expression of this level of exogenous *RAR* β was sufficient to significantly (two-way ANOVA: $P < 0.001$ at 168 h) reduce cell growth as compared with parental and empty vector transfected cells (Fig. 7C).

DISCUSSION

Gains in the knowledge of endothelial cell physiology and tumor angiogenesis are providing the necessary background for

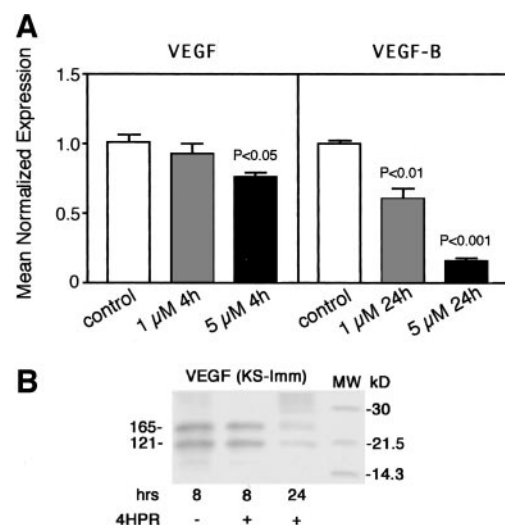


Fig. 5 Modulation of expression of VEGF by 4HPR. **A**, real-time RT-PCR analysis for VEGF and VEGF-B. KS-Imm cells were incubated with 4HPR at 1 and $5 \mu\text{M}$ for the indicated times. Total RNA was extracted and mRNA levels were determined, relative to that of *GAPDH*, by real-time RT-PCR. Data from three independent experiments were normalized and pooled. 4HPR down-regulated the mRNAs for *VEGF* (most evident at 4 h with $5 \mu\text{M}$) and *VEGF-B* (most evident at 24 h and dose dependent). **B**, detection of VEGF proteins in supernatants of ^{35}S -methionine-labeled KS-Imm cells. Equal quantities of radiolabeled supernatants from cells exposed to $5 \mu\text{M}$ 4HPR for 0–24 h were immunoprecipitated with antibodies to *VEGF*, SDS-PAGE fractionated, and autoradiographed. After 24 h, 4HPR clearly decreased the levels of VEGF protein synthesis.

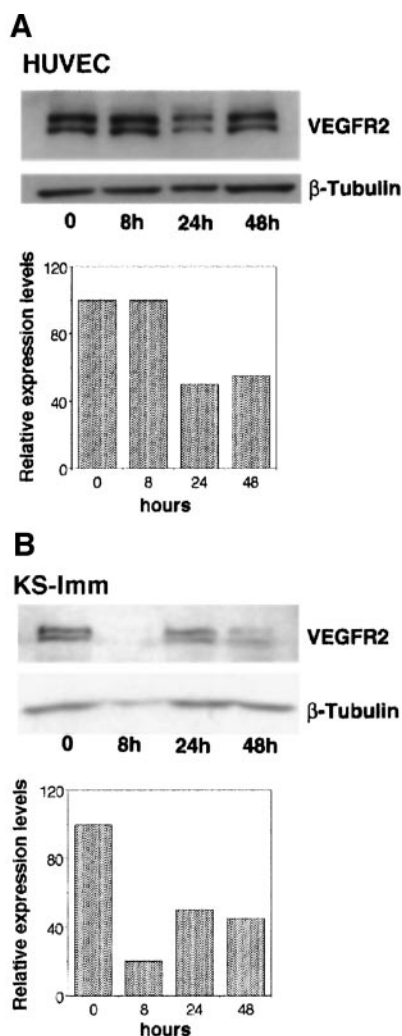


Fig. 6 Inhibition of VEGFR2 production in HUVE (A) and KS-Imm cells (B) in response to 4HPR. Western blot analyses for VEGFR2 were carried out on lysates from cells exposed to 5 μ M 4HPR for 0–48 h. As a control for loading, the same blots were reprobed with a β -tubulin antibody. Relative expression levels after densitometric analyses (*histograms*) show an evident repression of VEGFR2 protein content in samples treated with 4HPR.

developing effective anti-angiogenic strategies that may specifically target tumor angiogenesis by inhibiting endothelial-cell recruitment and proliferation. It is currently thought that pre-malignant lesions depend on angiogenesis for conversion into invasive cancer (35, 36). It is becoming more and more evident that angiogenesis is critical in the early stages of tumor progression (37, 38), and inhibition of angiogenesis before the “angiogenic switch” may be much more effective than inhibition of established angiogenic tumors. Chemopreventive agents may be particularly effective in angiogenesis inhibition; the “angioprevention” hypothesis suggests that angiogenesis inhibition is a common, if not critical, action of effective chemopreventive agents (8) that prevents the angiogenic switch and subsequent tumor growth.

In a prevention protocol, Pienta *et al.* (24) reported that

4HPR effectively inhibits the growth of prostate cancer at an early stage when given from the time of tumor cell injection. Here we show similar inhibition of detectable tumors in an early intervention protocol, where 4HPR was administered after tumor take. In addition, we show that 4HPR treatment leads to

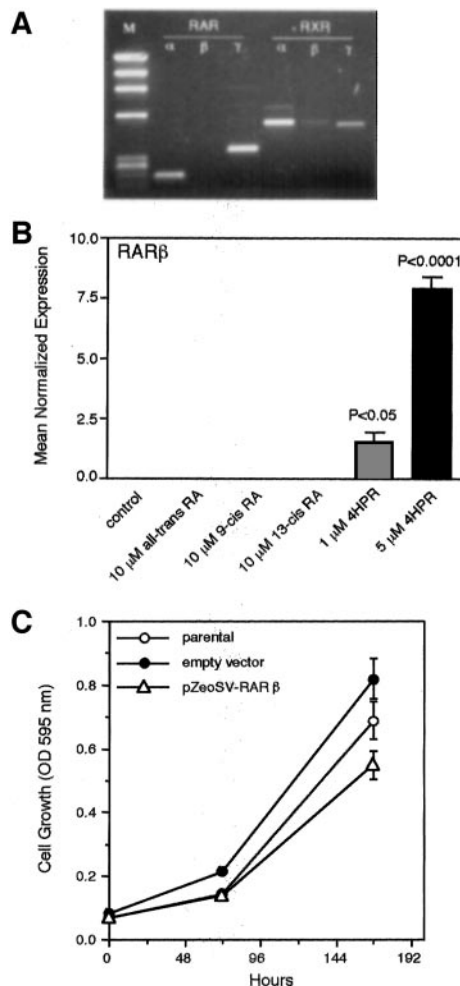


Fig. 7 mRNA expression analysis of *RARs* and *RXRs* in KS-Imm cells. **A**, total RNA from control cells was reverse-transcribed and amplified using primers specific for all of the *RARs* and *RXRs*. Under basal conditions, KS-Imm cells expressed *RAR* α and γ and all *RXRs*, but not *RAR* β . **B**, analysis of 4HPR-dependent induction of *RAR* β in KS-Imm cells by real-time RT-PCR. Total RNA were isolated from control cells and cells exposed for 24 h to 1 and 5 μ M 4HPR or 10 μ M all-trans-RA, 10 μ M 9-cis-RA, or 10 μ M 13-cis-RA. The RNAs were reverse-transcribed and amplified using primers specific for *RAR* β . Amplification products were measured during the reaction, and mean normalized expression values were calculated by comparison with the house keeping gene *GAPDH* amplified in parallel samples. Pooled data from three independent experiments are shown, significant *RAR* β expression was observed only after treatment with 4HPR (*P* values are shown in comparison with controls by unpaired two-tailed *t* test). **C**, the effect of exogenous *RAR* β over-expression in KS-Imm cells as assessed by crystal violet proliferation assays. Cells were transfected with either the *pZeoSV-RAR* β expression vector, the expression vector alone, or were not transfected. The data shown represent the means of 12 data points. *RAR* β over-expression significantly inhibited KS-Imm cell growth (*P* < 0.001 at 168 h by two way ANOVA) as compared with controls.

significantly reduced vessel density in the treated tumors as compared with controls, directly implicating inhibition of angiogenesis in concert with eventual direct effects on tumor cell growth potential. These data clearly link, for the first time, the anti-angiogenic activity of 4HPR with the observations of reduced tumor growth *in vivo*.

We also demonstrate that 4HPR inhibited several *in vitro* parameters associated with angiogenesis. In agreement with previous observations (25), we observed that 4HPR reduced endothelial growth *in vitro*, was effective at concentrations where no toxicity was detectable, and appeared to protect endothelial cells from apoptosis. In a recent report Erdreich-Epstein *et al.* (39) described ceramide signaling in fenretinide-induced endothelial cell apoptosis. Depending on serum concentrations, different mechanisms can effect endothelial cell growth, and clinical trials have demonstrated that 4HPR does not induce vascular damage in humans (40). 4HPR also blocked angiogenesis in Matrigel pellets *in vivo*, similar to its reported anti-angiogenic activity in the chick CAM assay (24, 25). On the basis of previous studies (41), we estimate that the doses used *in vivo* are within the concentration range obtained in human clinical studies (42).

Although 4HPR did not strongly effect KS-Imm proliferation and apoptosis *in vitro*, it significantly inhibited KS-Imm cell migration and invasion toward angiogenic factors (fibroblast supernatants) similar to that reported previously for chemically transformed BALB/c 3T3 (43). MMPs appear to be involved in many stages of tumor progression, from inducing the angiogenic switch to tumor cell invasion itself (44). In contrast to the data reported for transformed BALB/c 3T3 (43), we observed that 4HPR induced a dose-dependent decrease of MMP-2 activity released by KS-Imm cells. This may be directly responsible for inhibition of invasion *in vitro* and may play a role in angiogenesis inhibition *in vivo*.

Among the network of growth factors and cytokines that regulate angiogenesis, VEGF is unique, being a potent endothelial cell-specific mitogen produced by normal and transformed cells. Augmented VEGF levels have been found in many different tumors and *VEGF* expression has been associated with metastasis (45). Several studies have demonstrated a role for VEGF and its receptors in the pathogenesis of KS (46, 47). Thus, therapeutic agents that target the *VEGF* pathway may be an effective strategy in reducing tumor growth, particularly for KS. RA and synthetic retinoids have been shown to inhibit basal expression of *VEGF* mRNA and block 12-*O*-tetradecanoylphorbol-13-acetate-induced *VEGF* expression in human keratinocytes (48). In contrast to the lack of effect reported for neuroblastoma (25), we found that 4HPR partially decreased the release of VEGF, which is also an autocrine growth factor for KS (47). This reduction of VEGF release by 4HPR could, in turn, limit vascularization of the tumor, resulting in reduced tumor growth. VEGF and VEGF-C are critical in regulating vascularization (49) *in vivo*, whereas the biological role of VEGF-B is less clear. One study (50) reported that *VEGF-B* expression in human primary breast cancer is associated with lymph node metastasis, but not angiogenesis, suggesting that VEGF-B may contribute to tumor progression by a nonangiogenic mechanism. Therefore, it is possible that 4HPR repression of VEGF release not only limits tumor vascularization but also

tumor spread, resulting in a reduction of both tumor growth and metastasis.

In the endothelium, VEGF exerts its action through the cell surface tyrosine kinase receptors VEGFR1, VEGFR2, and VEGFR3. VEGFR1 is a higher affinity receptor, whereas VEGFR2 is more highly expressed and involved in transduction of angiogenic signals (51) potentially in concert with VEGFR3 (52). Because VEGF is crucial to physiological and pathological processes dependent on angiogenesis and is an endothelial cell survival signal, it is reasonable to predict that VEGF-receptor modulation would alter vascular cell behavior. Our results indicate that 4HPR induced down-regulation of VEGFR2 protein in endothelial and KS-Imm cells. These data are in keeping with previous immunohistochemical studies that demonstrated that 4HPR treatment reduced *VEGFR2* and *FGF-2R2* expression on the endothelial cells of the chick CAM (25) and suggest that 4HPR inhibits tumor cell angiogenic potential.

The diverse and pleiotropic effects of retinoids are mediated through their binding to a family of nuclear receptors, the RARs and the RXRs (53). Ligand-bound RARs and RXRs are able to up-regulate transcription directly by binding to RA-responsive elements on the promoters of responsive genes (26). As an indirect effect, liganded RARs and RXRs can negatively affect gene expression via their ability to functionally interact with the transcription factor AP-1 (27). The mechanisms controlling the down-regulation of *VEGF* and *VEGFR2* in our cellular model remain to be elucidated; however, we favor the hypothesis of an RAR/RXR antagonism of AP-1, as previously reported for *VEGF* (48). It is tempting to speculate that RAR β plays a central role, because it was induced by 4HPR, but not by other retinoids (all-*trans*-RA, 9- and 13-*cis*-RA). This correlates with the observation that all-*trans*-RA, 9-, and 13-*cis*-RA failed to inhibit both cell growth (*in vitro* and *in vivo*) and angiogenesis in the assays used here (unpublished observations and Ref. 23). Moreover, over-expression of RAR β decreases the growth rate of KS-Imm cells, similar to that reported previously for other cell lines (33, 54). Previous studies have shown that cAMP-responsive element binding protein-binding protein (CBP) and the related p300 can act as transcriptional coactivators for both nuclear receptors (55) and AP-1 (56). On the basis of these data, we hypothesize that the gene down-regulation exerted by 4HPR depends on competition for limiting amounts of these two coactivator proteins, as already reported for *VEGFR2* (57). However, we cannot rule out involvement of receptor independent mechanisms.

In summary, we show that 4HPR represses KS tumor growth in an *in vivo* model and that angiogenesis inhibition is a principal mechanism of this inhibition. 4HPR hindered tumor cell-induced angiogenesis at multiple levels, impairing both the angiogenic potential of the tumor cells and the ability of the host endothelial cells to respond to tumor-derived angiogenic signals by modulating specific molecules involved, including MMP-2, VEGFs, and VEGFRs, acting as an "angiopreventive" drug. These activities may well combine *in vivo* to play a significant role in preventing the occurrence of solid tumors. To date, there are no reports of clinical use of 4HPR in KS; our data suggest that 4HPR should be tested for potential use to assist in clinical management of KS. These scenarios include both control of established KS, such as AIDS-associated KS, particularly in

areas where HAART is not an option because of economic factors or HAART failure, and prevention of KS in HHV8 carriers subjected to immunosuppression, particularly transplant patients.

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