0026-895X/03/6303-565–573$7.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 63:565–573, 2003

Vol. 63, No. 3
1788/1049871
Printed in U.S.A.

N-(4-Hydroxyphenyl)retinamide Inhibits Retinoblastoma Growth through Reactive Oxygen Species-Mediated Cell Death

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Received April 10, 2002; accepted November 27, 2002

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Retinoblastoma arises from a subset of developing retinal cells lacking the RB-1 gene product pRB, which have lost the ability to respond to apoptotic signals. A better understanding of retinoblastoma biological response to therapeutic agents with low toxicity could improve the development of novel approaches for treatment and prevention of the disease. Naturally occurring retinoids inhibit growth and induce differentiation of Y79 human retinoblastoma cells in vitro. The synthetic retinoid *N*-(4-hydroxyphenyl)retinamide (4HPR) has been shown to induce apoptosis and/or necrosis of tumor cells of neuroectodermal origin. We examined the sensitivity of Y79 retinoblastoma cells to 4HPR in vitro, and in a xenograft model of tumor growth in nude mice in vivo. 4HPR treatment in the range 2.5 to 10 μM induced a loss of Y79 cell viability, as determined by crystal violet, trypan blue exclusion, and long-term clonogenic assays, and impairment of mitochondrial function detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Reactive oxygen species were elevated in 4HPR-treated cells and antioxidants rescued cell viability, indicating that 4HPR-induced cell death was mediated by oxidative stress. 4HPR inhibited growth of Y79 xenografts in vivo in both chemoprevention and intervention settings. Tumor growth inhibition by 4HPR was also associated with significant inhibition of angiogenesis in vivo. These findings could have an important translational value for chemoprevention or early intervention in the treatment of retinoblastoma.

Retinoblastoma is a locally invasive tumor of the eye derived from a multipotential stem cell of the developing neural retina. It occurs only in the first years of childhood as a result of the loss of function of both alleles of the RB-1 retinoblastoma tumor-suppressor gene (DiCiommo et al, 2000). In the absence of a functional pRB-1, a subset of human retinal cells undergoes defective differentiation and uncontrolled proliferation and is induced to develop tumors (Gallie et al., 1999). Loss of apoptotic signals regulating cell death during tissue remodeling in the developing retina has been indicated as the cause of tumor development from retinal cells lacking pRB-1 (Gallie et al., 1999; DiCiommo et al, 2000).

Although generally confined to the ocular tissue at the time of diagnosis, retinoblastoma in humans is rarely detected at early stages. Current aggressive therapy includes enucleation of the eye with subsequent loss of vision and facial deformity. In hereditary cases where both eyes may be affected, and in more advanced stages of the disease, aggressive radiotherapy and chemotherapy increase the risk for development of second tumors, including osteosarcoma and melanoma. Consequently, novel therapeutic interventions aimed at preventing or limiting tumor development and spreading to extraocular tissues are urgently needed to preserve the visual function and survival in children affected by retinoblastoma.

Although tissue-specific RB-1 inactivation in mice provided a valuable tool to study cancer etiology, phenotypes derived from germline mutations of RB-1 in mouse show profound differences with the human disease, RB-1 loss being insufficient for retinoblastoma development in the mouse retina (Mills et al., 1999).

Because of its high differentiation potential, the human retinoblastoma Y79 cell line represents a suitable model system to study cancer-related alterations in gene expression and control of apoptosis, as well as early events of neuroretinal development.

This study was supported by grants from the Istituto Superiore di Sanità-Progetto Italia-USA sulla Terapia dei Tumori, the Associazione Italiana per la Ricerca sul Cancro, the Ministero della Sanità, Progetto Finalizzato, the Ministero dell'Università e della Ricerca Scientifica e Tecnologica, and the Compagnia di San Paolo. R.V. is recipient of a Fondazione Italiana per la Ricerca sul Cancro fellowship.

ABBREVIATIONS: RB, retinoblastoma; RA, retinoic acid; 4HPR, *N*-(4-hydroxyphenyl)retinamide; ROS, reactive oxygen species; NAC, *N*-acetyl-L-cysteine; PDTC, pyrrolidinedithiocarbamate; PBS, phosphate-buffered saline; H_2DCFDA, dichlorofluorescin diacetate; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; DPI, diphenyleneiodonium chloride; CM, conditioned medium; ANOVA, analysis of variance; HBSS, Hanks' balanced salt solution.

Retinoids profoundly regulate the development of the vertebrate eye. In the mouse embryo the neural retina is the tissue richest in retinoic acid (RA) (Li et al., 2000), and congenital vitamin A deficiency syndrome is characterized by severe eye malformations (Morriss-Kay and Ward, 1999). In the adult eye, retinoids are indispensable regulators of signaling pathways intrinsic to the photoreceptor cell function and gene expression. RA, by mimicking the effects of light, induces the expression of arrestin (Wagner et al., 1997), which in photoreceptors terminates the visual signal transduction cascade.

The biological activity of retinoids is mediated by specific cytoplasmic and nuclear receptors. Data from gene knockout mouse models indicate an essential role for nuclear RA receptors (retinoic acid receptors and retinoic acid X receptors) and cytoplasmic retinoid binding proteins (cellular retinol binding protein-I, cellular retinoic acid-binding protein-I and -II) during retinal morphogenesis (Dolle et al., 1990). Alterations in retinoid binding proteins and RA receptor expression have also been shown to affect target gene response and the phenotype of cancer cells (Picard et al., 1999; Sun et al., 2000). A potential tumor suppressor function has been postulated for retinoic acid receptors (Sun et al., 2000).

Y79 cells can be induced to differentiate to the main cell types of the mature retina (neurons, glia, and photoreceptors) by several agents, including sodium butyrate and cAMP analogs (Kyritsis et al., 1987) and are sensitive to the antiproliferative and differentiating action of retinoids (Conway et al., 1997; Tsukamoto et al., 1998).

The synthetic retinoid *N*-(4-hydroxyphenyl)retinamide (4HPR), a cancer chemopreventive and therapeutic agent (Decensi and Costa, 2000), showed enhanced activity and reduced toxicity compared with the natural compounds. 4HPR induces cell death in most of the cancer cell lines so far analyzed, including cells of neuroectodermal origin such as neuroblastoma (Reynolds, 2000) and melanoma cells (Montaldo et al., 1999). Elevation of reactive oxygen species (ROS) and mitochondrial damage have been shown to be involved in 4HPR-induced cell death in some cancer cell types (Sun et al., 1999b; Hail and Lotan, 2001).

Given the effects of 4HPR on tumor cells of neuroectodermal origin, we examined the effects of 4HPR on Y79 retinoblastoma cells in vitro and on tumors generated by Y79 cells in vivo. We found that 4HPR induces extensive retinoblastoma cell death associated with ROS elevation. Survival of 4HPR-treated Y79 cells was rescued by the antioxidants *N*-acetyl-L-cysteine (NAC) and catalase and partially by pyrrolidinedithiocarbamate (PDTC). 4HPR inhibited growth of tumors formed by retinoblastoma cells in nude mice when used both in chemoprevention and intervention protocols, which was also associated with a significant reduction of angiogenesis in vivo.

Materials and Methods

Cell Culture and Retinoid Treatments. Human retinoblastoma Y79 cells (ATCC HTB-18) were grown in suspension in RPMI 1640 medium supplemented with 15% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 mM penicillin/streptomycin. For adherent cultures, cells were seeded on poly-D-lysine-coated dishes (5 μg/cm^2) and treated on day 2 for 24, 48, or 72 h until 7 days with 4HPR (kindly provided by Dr. James A. Crowell, Division of Cancer Prevention, National Cancer Institute, Bethesda, MD, and Dr. Gregg Bullard, McKessonBio, Rockville, MD) dissolved in ethanol (1, 2.5, 5, and 10 μM). Medium was changed every 2 days.

Cell Viability Assays. To assess 4HPR cytotoxicity, three assays were used: a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay; a crystal violet assay; and a trypan blue exclusion assay. A suspension of Y79 cells was seeded at 7×10^3 cells/well in 96-wells microtiter dishes in 100 μl of complete medium per well containing 4HPR dissolved in ethanol (0.1% final ethanol concentration) at the concentrations indicated, and incubated for 24, 48, or 72 h at 37°C. For the MTT assay, 2 h before the end of each incubation time 10 μl of MTT (5 mg/ml stock in PBS) was added per well and the incubation continued for 2 h. Finally, 100 μl of a solution of 50% dimethylformamide in 20% SDS, pH 4.8, was added and the next day the absorbance at 570 nm was determined with an automatic microtiter plate reader (Molecular Devices Corp., Sunnyvale, CA). For the crystal violet assay, the cells were fixed and stained in a solution of 0.75% crystal violet, 0.35% sodium chloride, 32% ethanol, and 3.2% formaldehyde. The stain was then dissolved in 50% ethanol, 0.1% acetic acid and read with a microtiter plate reader as described above at 595 nm. The trypan blue exclusion was performed on 8×10^4 cells seeded in quadruplicates in 24-well plates. The cells were suspended a 0.4% trypan blue solution and counted with a hemocytometer.

Clonogenic Assay. Y79 cells were plated in complete medium at 5×10^3 density in 24-well plates coated with poly-D-lysine as described and allowed to attach to the substrate overnight. The cells were treated with 1, 2.5, 5, and 10 μM 4HPR and the medium was changed every 3 days. After 10 days, the cultures were either fixed and stained with crystal violet or with the vital dye dichlorofluorescin diacetate (H_2DCFDA) (Molecular Probes, Europe BV, Leiden, The Netherlands) at 50 μM for 15 min in Hanks' balanced salt solution (HBSS), or were examined with phase contrast optics. The cells were photographed with a charge-coupled device camera at 20 or 100× magnification on a DM-IRB inverted microscope (Leitz, Wetzlar, Germany) equipped with phase contrast and fluorescence optics.

Detection of Apoptosis and Necrosis by Flow Cytometry and Histone-Associated DNA Fragments. Suspension and adherent cultures of Y79 cells in a logarithmic phase of growth were treated with 4HPR dissolved in ethanol in complete medium as described above for 24, 48, and 72 h. At the end of the incubation times, 5×10^5 cells were collected and prepared for fluorescence-activated cell sorting (FACS) analysis using an Annexin V-FITC apoptosis detection kit (Oncogene Research, Darmstadt, Germany) following the manufacturer's instructions. This method allows discrimination between viable cells (FITC negative, propidium iodide negative), early apoptotic cells with intact cell membranes (FITC positive), late apoptotic and secondary necrotic cells (FITC positive, propidium iodide positive), and primary necrotic cells (FITC negative, propidium iodide positive). Analysis was performed on 10,000 gated cells to exclude cell debris using a Coulter Epics XL FACS with excitation set at 488 nm and emission at 518 nm (FITC detector) or 620 nm (phycoerythrin fluorescence detector). Apoptotic cells were also determined by an enzyme immunoassay kit (Cell Death Detection ELISA^{PLUS}; Roche Diagnostics, Milano, Italy) to detect fragmented DNA and histones (mono- and oligonucleosomes) in the cytoplasm of cell lysates.

Detection of ROS. Y79 cells (1×10^6) suspended in 5 ml of complete medium were treated with 2.5, 5, or 10 μM 4HPR for 2 h. The antioxidant *N*-acetylcysteine was used at 10 and 25 mM (1 M stock solution dissolved in culture medium); RA was used at 5 μM. Twenty minutes before the end of the treatment, the cells were incubated with 50 μM H_2DCFDA (Molecular Probes), washed twice with phenol-free HBSS at the end of the incubation, and resuspended in the same medium. Cell suspensions were then analyzed with a flow cytometer (Coulter Epics XL) with excitation set at 488 nm and emission at 530 nm. The analysis was performed on 10,000 gated cells.

Treatment with Antioxidants. Cell viability was determined by the crystal violet assay, trypan blue exclusion, and the MTT assay in adherent Y79 cells grown in serum-supplemented medium in the presence of 4HPR as described above and with different antioxidants. The crystal violet assay was performed on 4HPR-treated cells in the presence of NAC at 10 mM (stock solution 1 M in culture medium). Because catalase interfered with the crystal violet assay, trypan blue exclusion was performed on 4HPR-treated cells in the presence of catalase 4 mg/ml (80 mg/ml stock solution in PBS), NAC, and PDTC used at 25 μM (10 mM stock solution in dimethyl sulfoxide). For the MTT assay, the following antioxidants were used: catalase, 4 mg/ml (80 mg/ml stock solution in PBS); allopurinol, 300 μM (3.5 mM stock solution in PBS); and diphenyleneiodonium chloride (DPI), 2 μM (0.3 mM stock solution in PBS). All reagents were purchased from Sigma (Milano, Italy).

Tumor Cell Growth in Vivo. CD1 nu/nu female mice (age 6–7 weeks) were obtained from Charles River Italica (Calco, Italy) and housed in pathogen-free conditions. Animals were treated with 4HPR dissolved in drinking water following a published protocol. In this study, two protocols were followed: a chemoprevention protocol and an early intervention protocol. In the chemoprevention protocol, 4HPR was dissolved in 100% ethanol and diluted in water to the concentrations of 7.2 μg/ml (low-dose group, approximately, 1.2 mg/kg/day) or 72 μg/ml (high-dose group, approximately, 12 mg/kg/day). Treatment with 4HPR in the water was started 3 days before tumor cell inoculation and continued throughout the course of the experiment, the ethanol vehicle alone was used for the controls. Fresh drinking water was prepared every 2 days, the treatments did not influence the average amount of water consumed. On day 0, 10^6 Y79 cells mixed with liquid Matrigel to a final volume of 250 μl were s.c. injected in the flanks of the nude mice. These experiments were repeated twice with essentially identical results for a total number of 12 animals per treated group and 14 controls. Tumor dimensions were measured every 2 days with calipers and the tumor volumes were calculated from the length $\times$ width^2/2.

In the intervention protocol, Y79 cells suspended in Matrigel were injected in untreated mice as described above (13 animals) and tumor growth was allowed to proceed until day 15, when clear tumor masses were detectable. The animals were then randomized into two groups with similar mean tumor volumes (0.09 $\pm$ 0.03 cm^3). To one group (six animals) 4HPR was administered in the drinking water at 12 mg/kg/day as described above; the other group (seven animals)

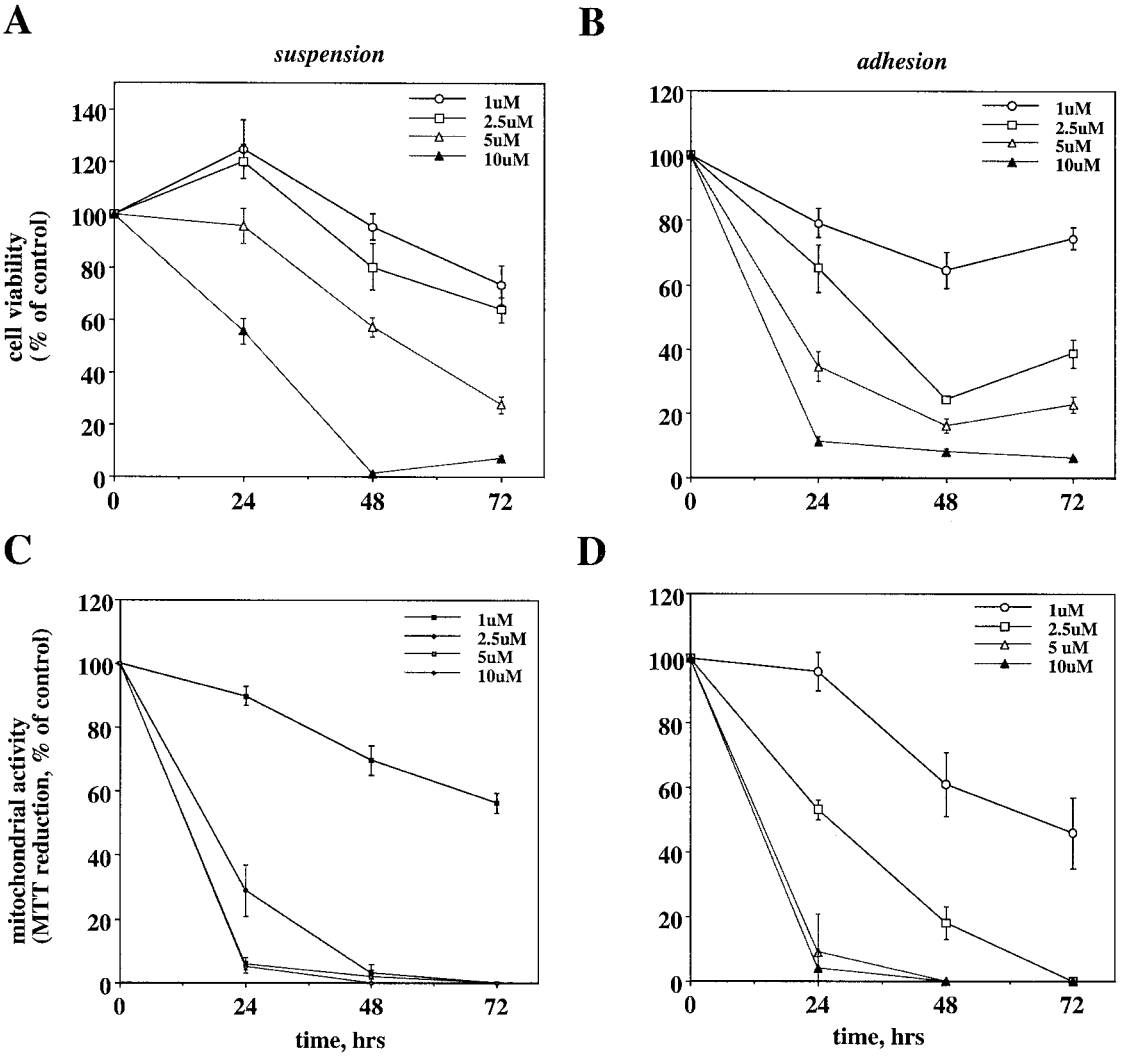

Fig. 1. Concentration- and time-dependent effects of 4HPR on cell survival and MTT reduction as an indicator of mitochondrial function in Y79 cells. To assess cell viability, a trypan blue exclusion assay was used for suspension cultures (A), the crystal violet assay for adherent cultures (B), and an MTT assay for both conditions (C and D). Crystal violet and MTT assays were performed on 7 $\times$ 10^3 Y79 cells seeded on 96-well plates, whereas for trypan blue exclusion cells were seeded at the density of 8 $\times$ 10^4 cells/well in 24-well plates. The results are the mean $\pm$ S.D. of data obtained from at least two independent experiments run in sextuplicate. Results are expressed as percentage of relative to untreated control cells. Treatment with 4HPR dose dependently reduced mitochondrial function.

was given ethanol alone. Tumor growth was followed as described above.

Mice were sacrificed 32 days after tumor cell injection, the tumors were removed, photographed, and fixed in 10% buffered formalin for histological examination and immunohistochemistry. The fixed tumors were embedded in paraffin and 4-μm sections were stained with hematoxylin and eosin, or processed for immunohistochemistry by standard procedures. For immunohistochemistry, rehydrated sections were blocked with irrelevant serum, followed by incubation with anti-Van Willebrand factor VIII (DAKO, Carpinteria, CA) monoclonal antibodies to highlight vessels, alkaline phosphatase-linked secondary antibody, and visualized with the UltraVision Detection System AP (LABVISION Corp., Fremont, CA). Vessel density was estimated by counting the vessels in random fields from anti-factor VIII antibody-stained sections of several different tumors from control and 4HPR-treated mice, counts were done "blindly" as to the origin of the tissue sections.

In Vivo Angiogenesis. To confirm the antiangiogenic effect of 4HPR, we used a modified Matrigel sponge model of angiogenesis in vivo (Albini et al., 1994). Conditioned medium (CM) from Y79 cells grown in RPMI 1640 medium supplemented with 2 mM glutamine for 24 h was concentrated 10-fold by centrifugation with Centricon 3000 devices (Millipore, Vimodrone, Italy). Sixty microliters of the concentrated CM and 20 U/ml heparin were then added to unpolymerized liquid Matrigel at 4°C in a final volume of 600 μl and slowly injected s.c. into the flanks of C57/bl6 mice using a cold syringe. One group of animals had 5 μM 4HPR added to the Matrigel pellets along with the CM from untreated Y79 cells before injection. Another group of animals received 4HPR in the drinking water to 12 mg/kg/day as described above. Finally, another group of animals received conditioned medium from Y79 cells that had 5 μM 4HPR added to the RPMI 1640 medium supplemented with 2 mM glutamine and processed as described above. Matrigel with heparin alone was used for the negative controls. After 4 days the Matrigel pellets were removed, weighed, and either the hemoglobin content determined with a Drabkin reagent kit (Sigma) using a mouse blood standard curve and normalization to 100 mg of recovered gel, or the samples were formalin fixed and processed for histology as described above.

Results

Cytotoxic Effects of 4HPR in Y79 Retinoblastoma Cells. 4HPR effects on tumor cell lines has mainly been focused on its cell death-inducing activity. However, the chemopreventive activity of 4HPR when administered at low doses (e.g., in breast cancer patients) could be related to other less defined properties, including induction of differentiation. To investigate 4HPR activity on Y79 cell growth and differentiation, we first examined its effects on Y79 cell viability. Y79 cells can either be propagated in suspension or monolayer cultures on poly-D-lysine-coated substratum. The manipulation of the culture conditions can modify the response of Y79 cells to differentiating or cell death-inducing agents, including retinoids (Kyritsis et al., 1987; Campbell and Chader, 1988a; Fassina et al., 1997). We therefore tested 4HPR activity on Y79 cells grown in suspension and adherent cultures. Three assays were used: the crystal violet assay, a trypan blue exclusion assay, and a modified MTT assay that measures the reduction of MTT by mitochondrial dehydrogenases to a colored formazan dye, an indicator of mitochondrial function. For technical reasons, not all the assays can be used in all conditions, the crystal violet assay was used for monolayer cultures, trypan blue exclusion for suspension cultures, and the MTT assay for both conditions.

In both suspension and adherent Y79 cultures (Fig. 1), 4HPR at the lowest concentrations tested (1 and 2.5 μM) had a slight inhibitory effect on cell survival at early times, as determined by all the viability assays. At 24 h 4HPR at 1 and 2.5 μM had a modest stimulatory effect on Y79 cell growth in suspension cultures (Fig. 1A). 4HPR at 5 to 10 μM significantly lowered viable cell number and mitochondrial activity within 24 h of treatment in adherent cultures (Fig. 1, B and D). Loss of mitochondrial function as indicated by MTT reduction (Fig. 1, C and D) was more rapid than the decrease in cell viability in suspension and adherent cultures. 4HPR cytotoxicity in monolayer cultures seemed to be enhanced at early times (24 h) compared with suspension cultures, and was in general more effective on adherent Y79 cells, which seem to be more prone to differentiation (Kyritsis et al., 1987; Campbell and Chader, 1988b; Fassina et al., 1997) and death (Kyritsis et al., 1987).

4HPR activity was also studied in long-term cultures of Y79 cell seeded at low density (25 cells/mm^2) on poly-D-lysine-coated dishes (Fig. 2). Under these conditions, Y79 cells form circular "rosette" colonies of cells (Kyritsis et al., 1987). 4HPR at 1 μM did not inhibit rosette formation in long-term cultures (Fig. 2). However, 4HPR starting at the dose of 2.5 μM inhibited rosette formation and cell viability in a dose- and time-dependent manner (Fig. 2).

To investigate the cause of cell death of 4HPR-treated Y79 cells, we examined both suspension and adherent cultures and assessed apoptosis and/or necrosis by FACS upon double-staining of cells with Annexin V-FITC and propidium

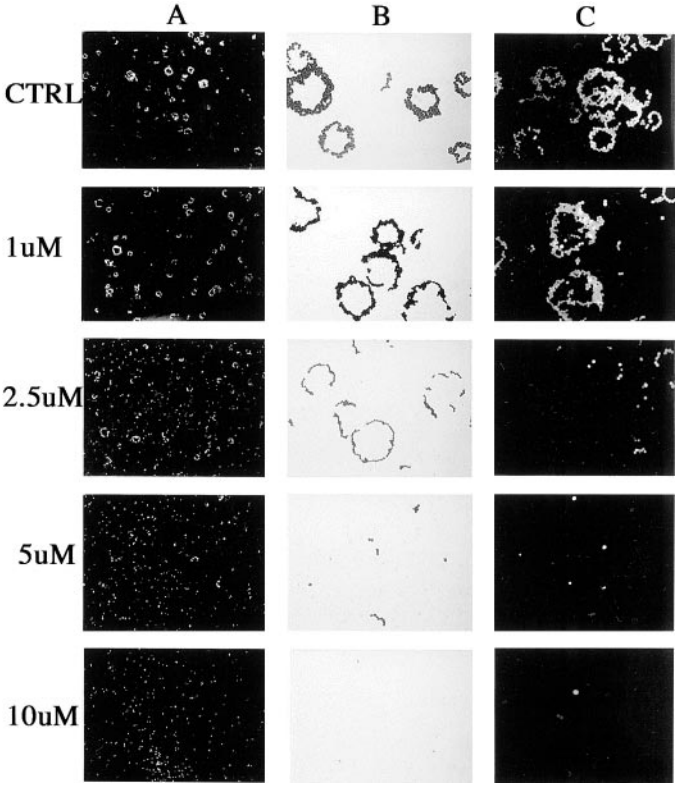

Fig. 2. Rosette colony formation is inhibited by 4HPR. Y79 cells were seeded at 5×10^3 density in poly-D-lysine–coated 24-well plates. After 10 days, the cells were either directly photographed with phase contrast (A), stained with crystal violet (B), or stained with the vital dye H_2DCFDA as described under *Materials and Methods* and photographed. Magnification is 20× in A and 100× in B and C. 4HPR treatment dose dependently inhibited rosette colony formation.

TABLE 1

Effect of 4HPR on ROS generation in Y79 cells

Intracellular ROS elevation was analyzed in 4HPR-treated Y79 cells. 4HPR or *all-trans*-RA was administered to Y79 cell suspensions at the concentrations indicated for 2 h. Cells treated with 4HPR and with NAC were preincubated with the antioxidant for 80 min before the end of the incubation, 50 μM H_2DCFDA was added to the culture medium, and the cells were washed and analyzed by flow cytometry. Elevation of ROS was analyzed by H_2DCFDA green fluorescence emission detected with a Coulter Epics XL flow cytometer (excitation, 488 nm; emission, 530 nm). Treatment with 4HPR clearly elevated ROS production by Y79 cells that was blocked by NAC.

	Mean Fluorescence Intensity	Relative Increase
Control	0.35 ± 0.08	1
2.5 μM 4HPR	1.30 ± 0.10	3.7
5 μM 4HPR	2.12 ± 0.20	6.1
10 μM 4HPR	2.55 ± 0.25	7.3
5 μM 4HPR + 10mM NAC	0.74 ± 0.10	2.1
5 μM 4HPR + 25mM NAC	0.27 ± 0.03	0.8
25 mM NAC	0.20 ± 0.10	0.7
10 μM RA	0.90 ± 0.10	2.6

iodide. Annexin V-FITC binds to externalized phosphatidyl serine on the surface of intact membranes of early apoptotic cells, whereas propidium iodide is taken up by damaged cells in late apoptotic phases and by primary necrotic cells. A dose- and time-dependent increase in the number of early (Annexin V-FITC positive) and late apoptotic cells (Annexin V-FITC/propidium iodide positive), along with primary necrotic cells (propidium iodide positive), was detectable in 5 and 10 μM 4HPR-treated cells at 24, 48, and 72 h in suspension and adherent cultures (data not shown). The increase of early apoptosis in 4HPR-treated cells was confirmed by immunoenzymetric detection of mono- and oligonucleosomes in cell lysates, indicative of DNA fragmentation, giving analogous results (data not shown).

4HPR Generates ROS. Generation of ROS has been indicated as an intracellular mechanism mediating the cytotoxic response in several tumor cell lines by 4HPR, which seems to activate at least one pathway that results in free radical damage (Sun et al., 1999b; Suzuki et al., 1999). The generation of ROS by 4HPR-treated Y79 cells in suspension cultures was measured with a fluorometric method that allows detection of intracellular peroxides by reaction with the membrane-permeant H_2DCFDA, which, once inside the cell, is converted to the fluorescent 2,7-dichlorofluorescein after oxidation. A dose-dependent increase in the intracellular mean fluorescence intensity was observed in cells treated with 2.5, 5, and 10 μM 4HPR (about 4-, 6-, and 7-fold, respectively) relative to untreated control cells (Table 1; Fig. 3). Retinoic acid has been shown to act as an activator of oxidative stress in embryonic stem cells (Castro-Obregon and Covarrubias, 1996). In Y79 cells, 5 μM retinoic acid increased ROS levels to a lesser extent than that observed with 4HPR (Table 1). The antioxidant NAC at 10 mM reduced the generation of ROS induced by 5 μM 4HPR by about 65%, whereas 25 mM brought the ROS levels to those of the controls treated with NAC alone (Fig. 3; Table 1).

ROS Are Involved in 4HPR-Induced Cell Death. We then assessed cell viability of 4HPR-treated cell in the presence of NAC and other antioxidants. Cell viability of adherent cultures was rescued by NAC treatment at 10 mM (Fig. 4A). In adherent cultures cell viability at 72 h was 22.8 ± 2.5% of control with 5 μM 4HPR and 104.1 ± 12.0% in

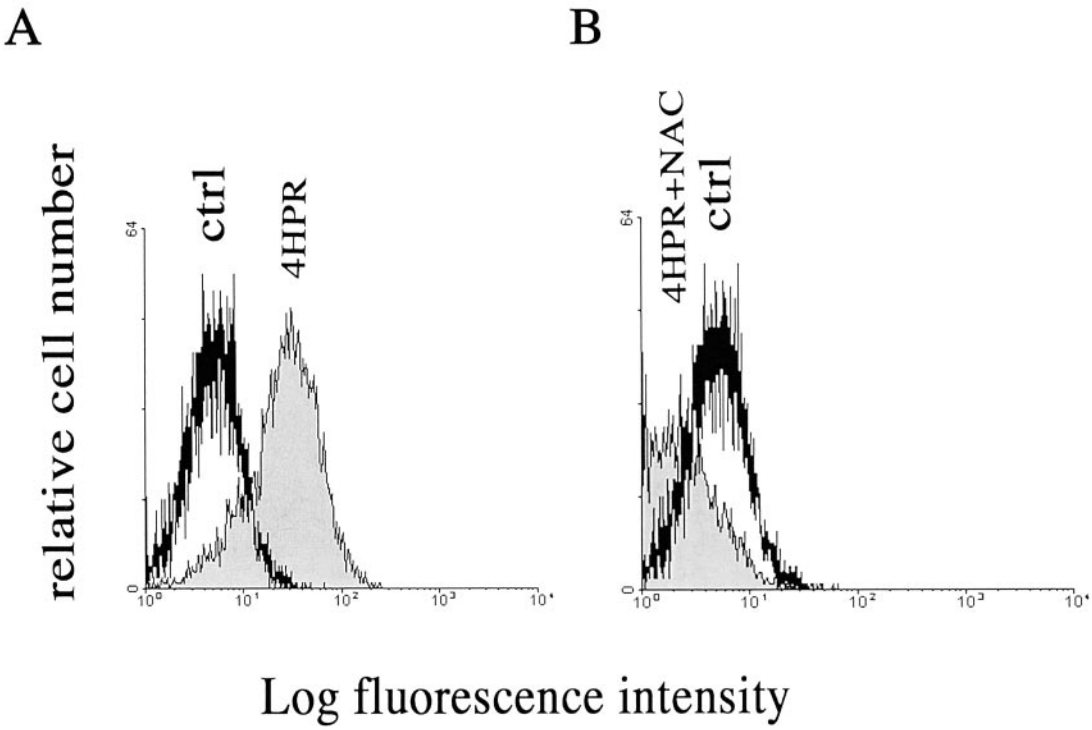

Fig. 3. ROS increase in 4HPR-treated cells is inhibited by the antioxidant NAC. Y79 cell suspensions at 5 × 10^5/ml were treated with 5 μM 4HPR alone for 2 h (A) or preincubated with NAC at 25 mM for 1 h (B). H_2DCFDA at 50 μM was then added 20 min before the end of the treatment, cells were washed in HBSS, resuspended in phenol red-free Dulbecco's modified Eagle's medium, and analyzed by flow cytometry as detailed under *Materials and Methods*. Flow cytometric fluorescence histograms are shown. The mean fluorescence intensity of 4HPR-treated cells in the presence of NAC returns to values detected in control cells treated with NAC alone. The histogram relative to control NAC-treated cells is not shown in B for clarity of the figure.

NAC-treated cells, and 6.2 ± 0.4% with 10 μM 4HPR versus 84.9 ± 9.0% in cells treated with 4HPR plus NAC (compare Fig. 4A with Fig. 1B). To confirm the protective effect on cell viability of antioxidants, we tested the effects of the general antioxidant PDTC and of the H_2O_2-degrading enzyme catalase, which can counteract the effects of ROS production, on viability of 4HPR-treated cells. Reagents that lower H_2O_2 concentration in the extracellular space, which constitutes the majority of the culture volume, should reduce H_2O_2 concentration in cells and therefore reduce 4HPR toxicity. Because catalase gave a high background in the crystal violet assay, we used trypan blue exclusion assays in these experiments. The addition of 4 mM catalase completely prevented 4HPR-induced loss of cell viability in adherent Y79 cells after 24 h, bringing the cell death rates down to control levels in 2.5, 5, and 10 μM 4HPR-treated cells (Fig. 4B). The general antioxidant PDTC (25 μM) decreased in part 4HPR induced-cytotoxicity (about 75% of control cell viability versus 25% in cells treated with 4HPR alone). These data clearly indicate that 4HPR toxicity is mediated by H_2O_2 production in Y79 cells. Because catalase cannot penetrate the plasma membrane, to further investigate the intracellular sources of superoxide or H_2O_2, more specific inhibitors of different oxygen radical-generating pathways were tested for their ability to

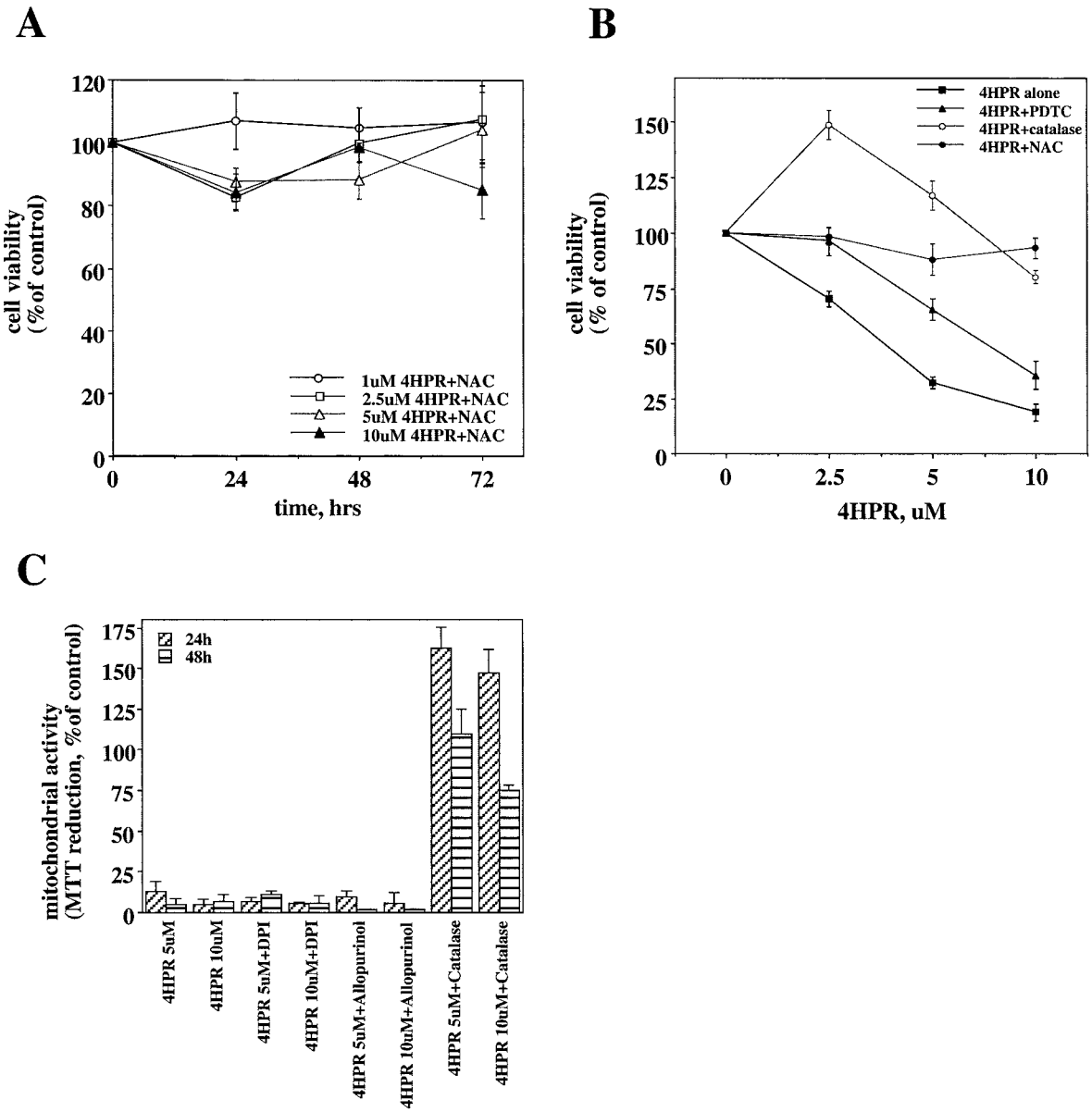

Fig. 4. Role of antioxidants in inhibition of 4HPR-induced Y79 cell death. A, NAC was added along with 4HPR to adherent Y79 cell cultures. Results from crystal violet cell viability assays are shown, the data relative to Y79 cells treated with 4HPR alone are reported in Fig. 1B. NAC protected Y79 cells from 4HPR toxicity. B, cell viability was completely rescued by the antioxidant enzyme catalase and, partially, by the general antioxidant PDTC in Y79 cells treated with 4HPR at 2.5, 5, and 10 μM for 24 h. Because catalase interferes with the crystal violet assay, trypan blue exclusion with visual counting was used in this experiment. NAC was used as a positive control. C, effect of catalase and of the antioxidants allopurinol and DPI on MTT reduction by Y79 cells was tested by MTT assays. Catalase was used as a positive control. Concentrations of the antioxidants used were NAC, 10 mM; catalase, 4 mg/ml; allopurinol, 300 μM; DPI, 2 μM; and PDTC, 25 μM. The results are presented as mean ± S.D. of data obtained with at least two independent experiments run in sextuplicate. Results are expressed as percentage of viability or reduction of MTT relative to untreated control cells, or cells treated with antioxidants alone. Antioxidants alone did not substantially affect cell viability.

inhibit the decrease of mitochondrial activity induced by 4HPR in Y79 cells. The MTT assay was used for this experiment, because MTT reduction can evaluate ROS-mediated mitochondrial dysfunction. Catalase, used as a positive control, rescued MTT reduction to control levels in Y79 cells treated with 10 μM 4HPR at 24 and 48 h (Fig. 4C), confirming its protective role on 4HPR-induced cytotoxicity. However, allopurinol, an inhibitor of the H_2O_2-generating enzyme xanthine-xanthine oxidase, and DPI, an inhibitor of flavin oxidases such as NADPH oxidase, did not exert any affect on the 4HPR-induced decrease of MTT reduction in Y79 cells (Fig. 4C). DPI and allopurinol similarly did not counteract 4HPR cytotoxicity in Y79 cells as detected by crystal violet assay (data not shown).

4HPR Inhibits the Growth of Y79 Cells in Vivo. Our data demonstrated that 4HPR can directly inhibit retinoblastoma cell growth in vitro, suggesting that it may also be able to inhibit retinoblastoma growth in vivo. Furthermore, the cancer-chemopreventive effect of 4HPR has been suggested to be linked to a mechanism involving neovascularization inhibition (Pienta et al., 1993), which may be common for all effective cancer chemopreventive agents (Tosetti et al., 2002). To investigate the effects of 4HPR on retinoblastoma tumor growth in vivo, Y79 cells suspended in liquid Matrigel were injected subcutaneously in the flanks of nu/nu nude mice in a chemoprevention protocol and an early intervention protocol. In the chemoprevention protocol, 4HPR was administered 3 days before cell injection at two doses (1.2 and 12 mg/kg/day). In the intervention protocol 4HPR was administered at the higher dose on day 15 after cell injection. Xenografts of 1×10^6 Y79 cells produced tumors in all control nude mice. In contrast, in the chemoprevention protocol both tumor incidence and mass were reduced in 4HPR-treated animals (Fig. 5, top). Statistical analysis of the tumor sizes showed a significant inhibition of tumor growth for the 4HPR-treated animals, reaching $P < 0.001$ (two-way ANOVA) by the end of the experiment for both groups. Notably, the ratio of mean tumor volumes, treated to control, at day 32 was 0.17 for the low-dose group and 0.30 for the high-dose group (inhibition of tumor growth by 83 and 70%, respectively). Tumor incidence was also reduced by 25 and 43% in the low- and high-dose groups, respectively, compared with 93% in controls. Overall, there was no significant difference between the inhibition induced by the high- and the low-4HPR treatment groups, further suggesting that this may be effective in a chemoprevention setting.

In the intervention protocol, further growth of established Y79 tumors was significantly inhibited by 4HPR treatment ($P < 0.05$, two-way ANOVA) by day 32 at termination of the experiment (Fig. 5, bottom). The ratio of mean tumor volume of the treated to control tumors was 0.50 (reduction of tumor volume by 50%) at termination. No signs of toxicity from 4HPR or the ethanol vehicle were observed in any of the experiments.

Histological examination of the hematoxylin- and eosin-stained tumors from the chemoprevention group revealed a reduced invasion of the surrounding matrix by the Y79 cells in 4HPR-treated animals, and a lower cellularity in xenografts from nude mice treated with the higher dose of 4HPR (12 mg/kg/day) (Fig. 6). The retinoblastoma xenografts seemed highly vascular by macroscopic examination (data not shown) and by hematoxylin and eosin staining (Fig. 6, top). In the low-dose group, a reduced vascularization was

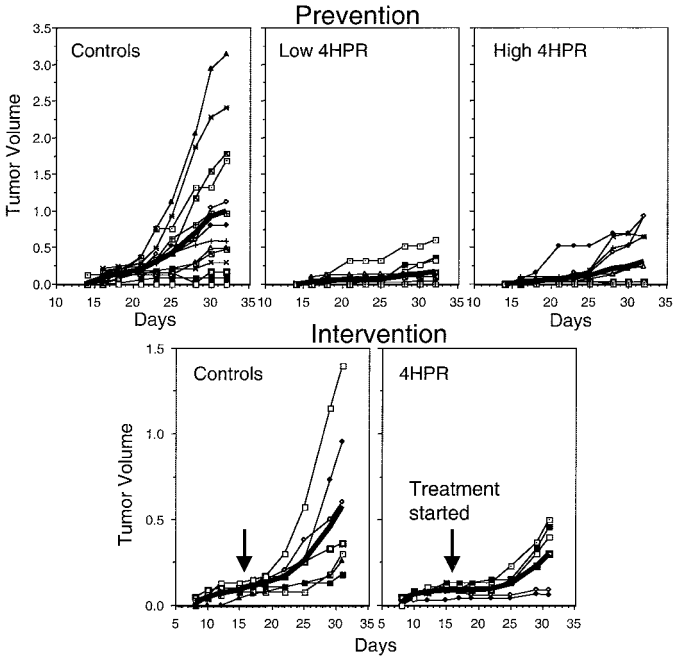

Fig. 5. In vivo growth of Y79 xenografts in nude mice is inhibited by 4HPR. Y79 cells (10^6) were mixed with liquid Matrigel to a final volume of 250 μl and injected subcutaneously in the flanks of nude mice. Tumor volumes were measured every 2 days for 32 days. 4HPR was administered in a chemoprevention protocol (top) and in an intervention protocol (bottom). The growth curves for tumors from individual mice are shown. The bold curves are the means of the values measured within each group. In the chemoprevention protocol 4HPR was administered 3 days before cell injection. The differences between tumor volumes of control and treated animals were statistically significant (two-way ANOVA; $P < 0.001$ at 32 days). The experiment was repeated twice for a total number of 12 treated animals per group and 14 controls, giving similar results. Data for all mice are shown. In the intervention protocol 4HPR at the higher dose (12 mg/kg/day) was administered on day 15 after cell injection. The differences between the treated group (six animals) and the controls (seven animals) were statistically significant (two-way ANOVA; $P < 0.05$ at 32 days).

noted; this was confirmed by vessel staining with specific anti-factor VIII antibodies (Fig. 6, bottom). Microvessel counts were statistically different in controls (23.5 ± 4.2) and in 4HPR-treated samples from the low-dose group (1.2 mg/kg/day)

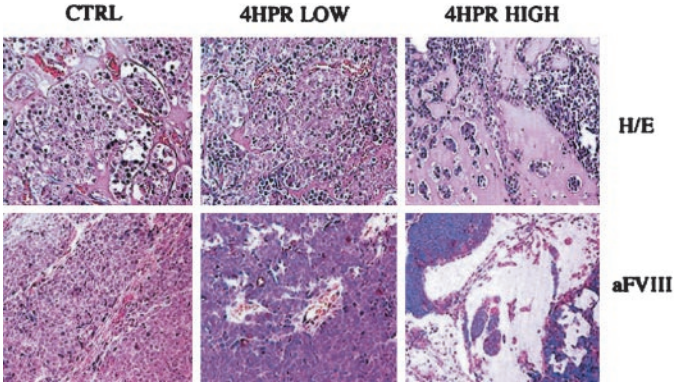

Fig. 6. Histology of hematoxylin- and eosin (H/E)-stained serial sections of Y79 tumor xenografts (top) and immunohistochemistry with monoclonal anti-factor VIII antibodies to highlight microvessels (bottom; aFVIII) from the chemoprevention protocol. Scale bar, 50 μm. Invasion of the Matrigel matrix by retinoblastoma cells is reduced by 4HPR treatment at the high dose used. The large areas of vascularization in control tumors are reduced in low dose 4HPR-treated samples.

(3.8 ± 1.7) ($P = 0.002$; t test), indicating a reduced angiogenesis in the 4HPR-treated retinoblastoma tumor xenografts.

4HPR Inhibits Angiogenesis Induced by Y79 Cell Supernatants. To confirm whether 4HPR could affect retinoblastoma tumor angiogenesis, its effects on Y79 cell product-induced angiogenesis was examined in vivo in a short-term angiogenesis assay (Albini et al, 1994). CM from Y79 cells produced a strong angiogenic response in the Matrigel sponge model in vivo. Vascularization of the Y79 CM containing Matrigel sponges was readily detectable by macroscopic (Fig. 7A) and microscopic examination of hematoxylin- and eosin-stained sections of the Matrigel pellets (Fig. 7C) and by quantification of the hemoglobin content of the sponges (Fig. 7B). The angiogenesis induced by Y79 cell products was characterized by the formation of large, leaky vessels and limited inflammatory infiltrate in the Matrigel. The addition of 4HPR to the Matrigel pellets completely blocked the angiogenic response ($P < 0.001$; ANOVA) (Fig. 7B). Administration of 4HPR in the drinking water of the mice also significantly inhibited the angiogenic response ($P < 0.05$; ANOVA). Interestingly a significant inhibition of angiogenesis ($P < 0.001$; ANOVA) was observed when the conditioned medium was prepared from cells pretreated with 5 μM 4HPR for 24 h, suggesting that 4HPR also suppresses the release of angiogenic factors by the Y79 tumor cells themselves (Fig. 7B).

Discussion

Neural cells negative for pRB expression or expressing a defective pRB protein undergo apoptosis during neuronal development (Slack et al., 1995). In contrast, retinoblastoma cells lacking pRB can replicate and escape the apoptotic fate for poorly understood reasons (DiCiommo et al., 2000). Targeting programmed cell death signaling in retinoblastoma has been suggested to be a possible tool for the development of new therapeutic and even chemopreventive strategies (Gallie et al., 1999). Preclinical studies have shown that the chemopreventive retinoid 4HPR effectively kills tumor cells of neuroectodermal origin, including neuroblastoma cells (Reynolds, 2000), and a pediatric phase I trial is in progress to determine the maximum tolerated dose of 4HPR in children with solid tumors unresponsive to standard therapy.

Here, we observed that 4HPR is able to restore the cell death response of retinoblastoma cells in vitro and to reduce retinoblastoma tumorigenicity in an ectopic model of retinoblastoma tumor growth in vivo, apparently as a consequence of cell death induction and tumor angiogenesis inhibition.

Disruption of the mitochondrial chain and subsequent elevation of intracellular ROS have been indicated as central mechanisms responsible for the cytotoxic activity of 4HPR on tumor cells in vitro (Sun et al., 1999a,b; Suzuki et al., 1999). A substantial increase in intracellular ROS that was associated with retinoblastoma cell death occurred in 4HPR-treated Y79 cells. A 7-fold increase in ROS levels was detectable in suspensions of Y79 cells as early as 2 h after 10 μM 4HPR treatment, which resulted in cell death of about 40% of the total cells by 24 h. The role of ROS was confirmed by the almost complete rescue of cell viability in 4HPR-treated cells by NAC and by the antioxidant enzyme catalase and, partially, by PDTC.

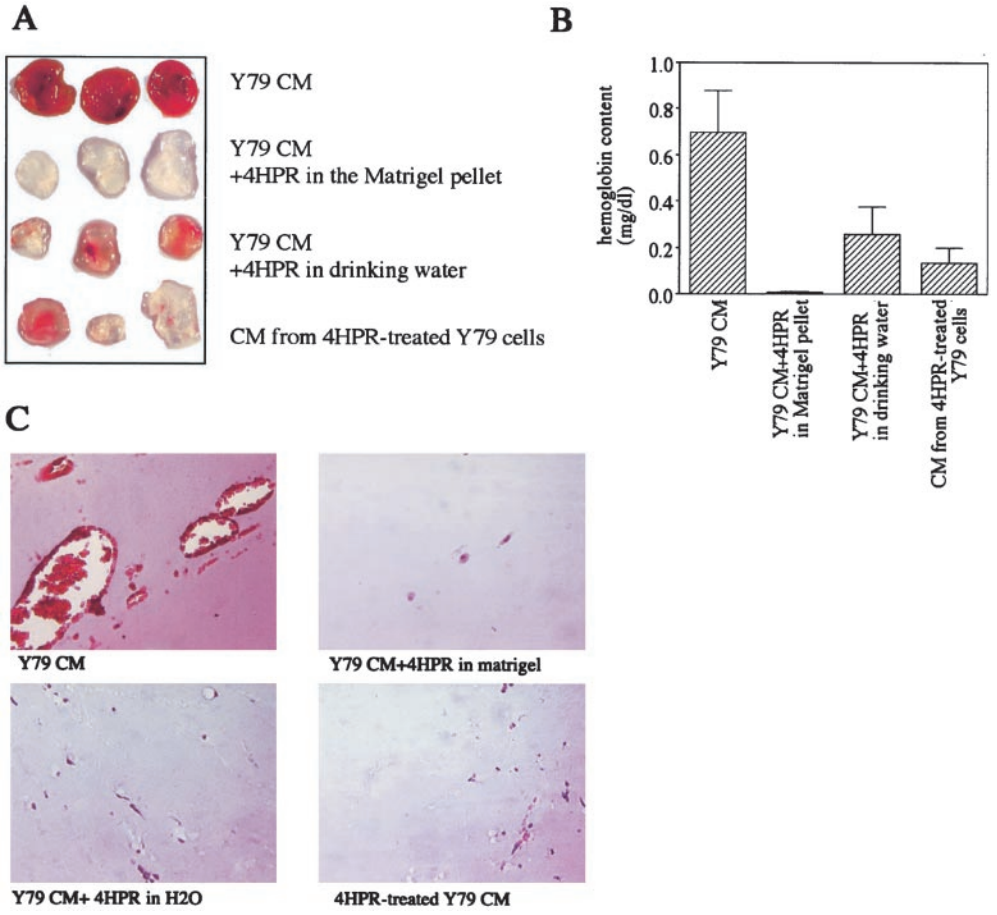

Fig. 7. Angiogenesis induced by retinoblastoma cell products in Y79-conditioned medium is inhibited by 4HPR. Matrigel was mixed with Y79 cell conditioned medium alone (Y79 CM) and injected subcutaneously. After 4 days the pellets were removed and the extent of vascularization examined. Untreated animals served as the positive control. One group of animals also had 5 μM 4HPR added to the Matrigel pellets. Another group of animals received 4HPR in the drinking water to 12 mg/kg/day as described above. Finally, a group of animals received conditioned medium from Y79 cells that contained 5 μM 4HPR. B, relative hemoglobin content of the Matrigel pellets, mean ± S.E. are shown. The differences in the hemoglobin content between pellets is statistically significant (controls versus Y79 CM plus 4HPR in pellet and versus CM from 4HPR-treated cells, $P < 0.001$; controls versus CM plus 4HPR in water, $P < 0.05$; ANOVA, 9–12 samples/group). C, hematoxylin- and eosin-stained sections of the Matrigel angiogenesis assay pellets: the reduction of vascularity is evident.

In the in vivo retinoblastoma xenograft model 4HPR, both administered in a chemoprevention and in an intervention protocol, significantly inhibited tumor growth. In the chemoprevention model, reduced tumor incidence in the 4HPR-treated groups was also observed.

We observed that the control Y79 tumors were highly vascular, whereas tumors in 4HPR-treated animals showed significantly reduced vascularization, consistent with an antiangiogenic effect of 4HPR in vivo, in addition to its cytotoxic activity on these cells. The potent antiangiogenic effect of 4HPR was confirmed using an in vivo angiogenesis assay in Matrigel pellets, where 4HPR significantly inhibited the intense vascularization induced by Y79 retinoblastoma cell conditioned medium. Previous studies have suggested that 4HPR may inhibit tumor angiogenesis (Pienta et al., 1993; Ribatti et al., 2001), observations consistent with the data reported here and the hypothesis that cancer chemopreventive agents can exert antiangiogenic effects (Tosetti et al, 2002).

4HPR was able to significantly reduce the growth of established Y79 tumors in the intervention protocol, despite the relatively small degree of inhibition of tumor cell growth in vitro at low doses. The tumor growth inhibitory effect was particularly striking when 4HPR was administered at the lower dose in the chemoprevention protocol in a setting of minimal disease state. This fact supports the hypothesis that the more efficient impairment of early tumor growth by 4HPR could be attributable to its antiangiogenic activity. 4HPR effectively inhibited tumor growth in other animal models of solid tumors, i.e., breast and melanoma tumors; however, 4HPR as a single agent was not effective in a phase II study of patients affected by advanced breast and melanoma cancer (Modiano et al., 1990). Retinoblastoma rarely is diagnosed at an early stage. Nevertheless, administration of this relatively nontoxic antiangiogenic agent could be of benefit to patients in a secondary prevention setting after surgical removal of the primary tumor, to prevent recurrence, progression or metastasis, as well as in patients with hereditary retinoblastoma at a high risk for bilateral disease. Our data, together with that of previous studies, suggest that antiangiogenesis is one of the main mechanisms underlying the antitumor activity of 4HPR in vivo. This suggests that this property of 4HPR should better be exploited at the clinical level at early stages of the disease, along with improvement of early detection methods, or could be used in combination with conventional cancer therapeutics, a future way to pursue indicated by current knowledge on the complexity of the cancer disease, to control progression to invasive cancer at more advanced stages of tumorigenesis (Hanahan and Weinberg, 2000).

Acknowledgments

We thank Claudia Semino and Giovanni Melioli for assistance with FACS analysis; Dr. Anna Rapetti for expert secretarial assistance; Monica Barabino for data searches; and Nicoletta Ferrari for helpful suggestions, criticism, and discussions.

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