# ARTICLES

# Involvement of Reactive Oxygen Species in N-(4-Hydroxyphenyl)retinamide-Induced Apoptosis in Cervical Carcinoma Cells

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*Background:* The inhibitory effects of *N*-(4-hydroxyphenyl)-retinamide (4HPR) on tumorigenesis and tumor growth may result from its ability to induce apoptosis (programmed cell death). Since antioxidants inhibit 4HPR-induced apoptosis, experiments were planned to determine whether the levels of reactive oxygen species increase in cells undergoing apoptosis after exposure to 4HPR. *Methods:* Cells of the human cervical carcinoma cell line C33A and normal human cervical epithelial cells were treated with 4HPR and analyzed for survival, induction of apoptosis, generation of reactive oxygen species, and expression of the apoptosis-related proteins Bcl-2 and Bax. *Results:* Treatment with 4HPR decreased C33A cell number by inducing apoptosis in a time- and dose-dependent fashion. DNA fragmentation typical of apoptosis was observed in cells exposed to 4HPR at concentrations of 3 $\mu M$ or higher for 6–24 hours. The generation of reactive oxygen species was enhanced by 1.85-fold to 4.5-fold after a 1.5-hour treatment with 0.4–10 $\mu M$ 4HPR. Pyrrolidine dithiocarbamate, an oxygen radical scavenger, suppressed the rate of generation of reactive oxygen species and inhibited 4HPR-induced apoptosis. 4HPR failed to modulate cellular levels of the Bcl-2 and Bax proteins. *N*-(4-Methoxyphenyl)-retinamide, the major 4HPR metabolite, and several other retinoids that bind to nuclear retinoic acid receptors or retinoid X receptors failed to enhance the generation of reactive oxygen species and to induce apoptosis. 4HPR was much less effective in generating reactive oxygen species and in inducing apoptosis in normal human cervical epithelial cells than in C33A cervical carcinoma cells. *Conclusions:* Enhancement of the generation of reactive oxygen species may be involved in apoptotic pathway induction by 4HPR. [J Natl Cancer Inst 1997;89:1191–8]

The synthetic retinoid *N*-(4-hydroxyphenyl)retinamide (4HPR) has been shown to inhibit carcinogenesis in animal models for cancers of breast, bladder, lung, ovary, and prostate tissues *(1–3)*. Some activity of 4HPR in humans has been reported in preneoplastic oral leukoplakia patients and in patients at risk of developing breast or ovarian cancer *(2)*. Various clinical chemoprevention trials targeting cancers of the breast, prostate, cervix, skin, and lung are ongoing *(2,3)*. In addition, 4HPR

has exhibited therapeutic effects by causing tumor regression in animals bearing carcinogen-induced or xenotransplanted human tumors [reviewed in *(2)*].

Reports that 4HPR can induce apoptosis in a variety of tumor cell types [reviewed in *(4)*] suggest that this activity may be important for the chemopreventive and therapeutic effects of this retinoid. The mechanism by which 4HPR induces apoptosis is not well understood. Many retinoids act by activating gene transcription following their binding to nuclear receptors, which include the retinoic acid receptors (RARs) α, β, and γ and retinoid X receptors (RXRs) α, β, and γ *(5)*. Although several studies have shown that 4HPR can increase the expression of RAR β *(6)* and activate transcription of retinoic acid response elements by nuclear RARs, primarily RAR γ *(7,8)*, the ability of 4HPR to induce apoptosis in cells that are resistant to all-*trans*-retinoic acid (ATRA) *(4,9–12)*, a retinoid with high affinity for nuclear RARs *(5)*, has led to the suggestion that 4HPR induces apoptosis by a mechanism that appears to be independent of nuclear retinoid receptors *(4,9,10)*.

Some clues related to the mechanism of 4HPR action were provided by the finding of Delia et al. *(13)* that the antioxidants vitamin E and *N*-acetyl-L-cysteine can inhibit 4HPR-induced apoptosis in human HL-60 leukemia cells. Using the antioxidants pyrrolidine dithiocarbamate (PDTC) and nordihydroguaiaretic acid, we confirmed this finding in the human cervical carcinoma cell line C33A *(14)*. In addition, expression of transfected Bcl-2, an apoptosis inhibitory protein that can function in an antioxidant pathway *(15)*, delayed 4HPR-induced apoptosis in HL-60 cells *(13)*. These results suggested that 4HPR can act as a prooxidant. Such an activity may be important

because oxidants such as hydrogen peroxide and nitric oxide have been found to induce apoptosis directly, and reactive oxygen species (e.g., hydrogen peroxide, hydroxyl radicals, and superoxide anions) have been found to mediate apoptosis induced by a variety of agents, including tumor necrosis factor-$\alpha$, transforming growth factor-$\beta$1, anti-Fas antibodies, and several chemotherapeutic agents, as well as radiation (16–20).

We previously reported that 4HPR is a potent inducer of apoptosis in head and neck and cervical cancer cell lines (11,12) and that certain antioxidants can inhibit 4HPR-induced apoptosis in C33A cervical carcinoma cells (14). We designed the present study to determine more directly whether 4HPR increases the generation of reactive oxygen species in cells that undergo apoptosis after exposure to 4HPR.

## Materials and Methods

### Retinoids

4HPR and N-(4-methoxyphenyl)retinamide (4MPR), the major 4HPR metabolite (2), were obtained from Drs. Ronald Lubet and Vernon Steele, respectively (both from the Division of Cancer Prevention and Control, National Cancer Institute, Bethesda, MD). ATRA was obtained from Dr. Werner Bollag (F. Hoffmann-La Roche, Basel, Switzerland). CD2665, i.e., 4-[7-(1-adamantyl)-6-(2-methoxyethoxy)methoxy-2-naphthalenyl]benzoic acid (an antagonist of nuclear RARs $\beta$ and $\gamma$), and CD437, i.e., 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (an RAR $\gamma$-specific agonist) (21,22), were obtained from Dr. Braham Shroot, CIRD/Galderma, Valbonne, France. The RXR agonist SR11236, i.e., 2-(4-carboxyphenyl)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)1,3-dioxane (23), was obtained from Dr. Marcia Dawson (SRI International, Menlo Park, CA).

### Antioxidant

PDTC (18) was purchased from Sigma Chemical Co., St. Louis, MO.

### Cell Culture, Retinoid Treatment, and Cell Survival Assay

The human cervical carcinoma cell line C33A was purchased from the American Type Culture Collection, Rockville, MD. This cell line is negative for human papillomavirus and contains a mutated p53 gene that is transcribed to give a high level of messenger RNA and protein (24). The cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle medium:Ham's F12 (DMEM:F12) medium supplemented with 5% fetal bovine serum (FBS); however, for the cell survival assay, cells were cultured in medium with 1% FBS only. A primary culture of normal human cervical epithelial cells (lot #17074; negative for human immunodeficiency virus, *Mycoplasma,* and hepatitis B virus) was purchased from Clonetics Corp., San Diego, CA, and analyzed after one subculture in Clonetics proprietary serum-free medium supplemented with human epidermal growth factor (0.5 ng/mL), bovine pituitary extract (30 $\mu$g/mL), hydrocortisone (0.5 $\mu$g/mL), fatty acid-free bovine serum albumin (0.5 mg/mL), epinephrine (0.5 $\mu$g/mL), triiodothyronine (6.5 ng/mL), transferrin (10 $\mu$g/mL), retinoic acid (0.1 ng/mL), insulin (5 $\mu$g/mL), gentamicin sulfate GA-1000 (50 $\mu$g/mL), and amphotericin B (50 $\mu$g/mL).

The retinoids were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 m$M$ and diluted in growth medium immediately before addition to cell cultures. Control cultures received the same amount of DMSO as the treated cultures. Cell survival was estimated by the colorimetric sulforhodamine B (SRB) assay with the use of 96-well plates (25). Briefly, after the designated treatment times, the medium was discarded and the adherent cells were fixed *in situ* by adding 100 $\mu$L of cold trichloroacetic acid (10% wt/vol) to each well and incubating the plates for 60 minutes at 4 °C. The plates were then washed five times with deionized water and air dried. Each well then received 50 $\mu$L of SRB (Sigma Chemical Co.) solution (0.4% wt/vol in 1% acetic acid), and the plates were incubated for 10 minutes at room temperature. Unbound SRB was removed by washing the plates five times with 1% acetic acid. The plates were then air dried, the bound stain was solubilized with 100 $\mu$L of 10 m$M$ unbuffered Tris base (pH 10.5), and the optical densities were read with the use of a microtiter plate reader (Dynatech Laboratories, Inc., Chantilly, VA) at 492 nm. Six replicate wells were used for each experimental condition.

### Extraction of Soluble DNA and Gel Electrophoretic Analysis of DNA Fragmentation

Soluble DNA was extracted as described previously (12). Briefly, after treatment, floating and adherent cells were detached, collected, and centrifuged at 4 °C for 10 minutes at 2000$g$ into a pellet. They were then resuspended in Tris–EDTA (Sigma Chemical Co.) buffer (pH 8.0). The cells were lysed as described above, and the lysate was centrifuged at 12 000$g$ for 15 minutes at 4 °C to separate soluble DNA from the insoluble fraction. Soluble DNA was treated with ribonuclease A (50 $\mu$g/mL; Sigma Chemical Co.) at 37 °C for 1 hour and then treated with proteinase K (100 $\mu$g/mL; Boehringer Mannheim, Indianapolis, IN) in 0.5% sodium dodecyl sulfate at 50 °C for 2 hours. The residual material was extracted with phenol–chloroform, precipitated in ethanol, subjected to electrophoresis in a 2% agarose gel, stained with ethidium bromide, and photographed under UV illumination.

### Quantitative Assay for DNA Fragmentation

DNA fragmentation was analyzed by quantitation of DNA solubilization induced by 4HPR by use of a modification of the method described by Higuchi et al. (26). Briefly, C33A cells were prelabeled with tritiated thymidine by the incubation of semiconfluent cultures in a 100-mm plate in a 1:1 mixture of DMEM:F12 medium supplemented with 10% FBS with 0.5 $\mu$Ci/mL of [*methyl*-$^3$H]thymidine (20 Ci/mmol; ICN Radiochemicals, Irvine, CA) at 37 °C for 16 hours. The cell monolayers were washed twice with complete medium, trypsinized, suspended by repeated pipetting, and washed once with complete medium. The cells were then resuspended in a 1:1 mixture of DMEM:F12 medium supplemented with 10% FBS and incubated in 96-well plates ($2.5 \times 10^4$/well) at 37 °C for 24 hours. The cultures were washed once with complete medium and then treated with different concentrations of 4HPR (0.06–10 $\mu M$) in a total volume of 200 $\mu$L at 37 °C for 24 hours. After incubation, the cells were solubilized by the addition of 50 $\mu$L of lysis buffer (10 m$M$ Tris–HCl [pH 8.0] containing 5 m$M$ EDTA and 2.5% Triton X-100) and incubation for 1 hour at 4 °C. The mixture was suspended, transferred to microcentrifuge tubes, and centrifuged in an Eppendorf microcentrifuge at 12 000$g$ for 1 minute at 4 °C. The radioactivity released in the supernatant was determined by a liquid beta scintillation counter (Packard Instrument Co., Downers Grove, IL), and this radioactivity represents DNA solubilized as a result of DNA fragmentation. For the total count of labeled DNA, the cells were lysed by the addition of 50 $\mu$L of 0.5% sodium dodecyl sulfate. The percentage of DNA release was calculated as follows: percent DNA fragmentation = (cpm in test sample supernatant/total cpm) × 100. Each assay was done in triplicate, and results are shown as the means ± standard error.

### Assay of Generation of Reactive Oxygen Species

The net intracellular generation of reactive oxygen species was measured by use of the oxidation-sensitive fluorescent dye 5,6-carboxy-2′,7′-dichlorofluorescin diacetate (DCFH-DA) (Molecular Probes, Inc., Eugene, OR). After uptake by cells, this nonpolar compound is converted into the nonfluorescent polar derivative 2′,7′-dichlorofluorescin (DCFH) by cellular esterases. Because DCFH is membrane impermeable, it is trapped in the cells and can be oxidized to the highly fluorescent compound 2′,7′-dichlorofluorescein by intracellular reactive oxygen species, primarily hydrogen peroxide and hydroxyl radicals (27). Cells were seeded in 96-well cluster tissue culture plates and grown for 3 days. They were then washed twice in buffer A (Krebs–Ringer buffer containing 20 m$M$ HEPES, 10 m$M$ D-glucose, 127 m$M$ NaCl, 5.5 m$M$ KCl, 1 m$M$ CaCl$_2$, and 2 m$M$ MgSO$_4$ [pH 7.4]) and covered with 200 $\mu$L of buffer A to which were added 4HPR (diluted from a 10 m$M$ stock solution in DMSO) at final concentrations of 0.08, 0.4, 2, or 10 $\mu$M and DCFH-DA (diluted from a 10 mg/mL stock solution in DMSO) at a final concentration of 10 $\mu$g/mL. Control cells received DCFH-DA but no 4HPR. After a 20-minute incubation at 37 °C for temperature equilibration in the plate reader, the fluorescence was measured at 538 nm after excitation at 485 nm at 2-minute intervals for up to 6 hours in a Fluoroskan II ELISA (i.e., enzyme-linked immunosorbent assay) plate reader equipped with temperature control (Labsystems, Helsinki, Finland). Generation of reactive oxygen species increased in a linear fashion for a period of 4 hours. The rate of generation of reactive oxygen species was measured in the linear range. Six wells were used for each control or treatment analysis, and the means ± standard deviation were determined.

## Western Immunoblotting for Detection of Bcl-2 and Bax Proteins

Cells were solubilized by suspension in a 10 m$M$ Tris–HCl (pH 7.5) buffer containing 0.5% Nonidet P-40, 10% glycerol, 1 m$M$ EDTA, 10 m$M$ KCl, 1 m$M$ phenylmethylsulfonylfluoride, 0.5 μg/mL leupeptin, and 0.5 μg/mL aprotinin and incubation on ice for 15 minutes. The cytosolic extracts were cleared by centrifugation at 12 000$g$ for 15 minutes at 4 °C, and the protein concentration in the supernatant was determined by a kit from Bio-Rad Laboratories, Hercules, CA. Protein samples (80 μg) were subjected to electrophoresis in polyacrylamide slab gels in the presence of sodium dodecyl sulfate and transferred to nitrocellulose membranes for western blotting. Polyclonal rabbit anti-human Bcl-2 antibodies and mouse monoclonal anti-human Bax antibodies were purchased from Santa Cruz Laboratories (Santa Cruz, CA). The binding of these antibodies to the membranes was probed by the chemiluminescence ECL method according to the manufacturer's instructions (Amersham Life Science Inc., Arlington Heights, IL).

## Results

### Differential Effects of 4HPR, 4MPR, and Other Retinoids on Survival of C33A Cells

4HPR induced a time- and dose-dependent decrease in the rate of survival of C33A cells (Fig. 1, A). These effects were observed as early as 6 hours after treatment with 10 μ$M$ 4HPR, and more than 90% of the cells were eliminated by 24 hours. The lower dose of 4HPR (3 μ$M$) was also effective in decreasing survival of the C33A cells, albeit at a slower rate. In contrast, 4MPR, the major 4HPR metabolite *(2,28)*, failed to exert a sig-
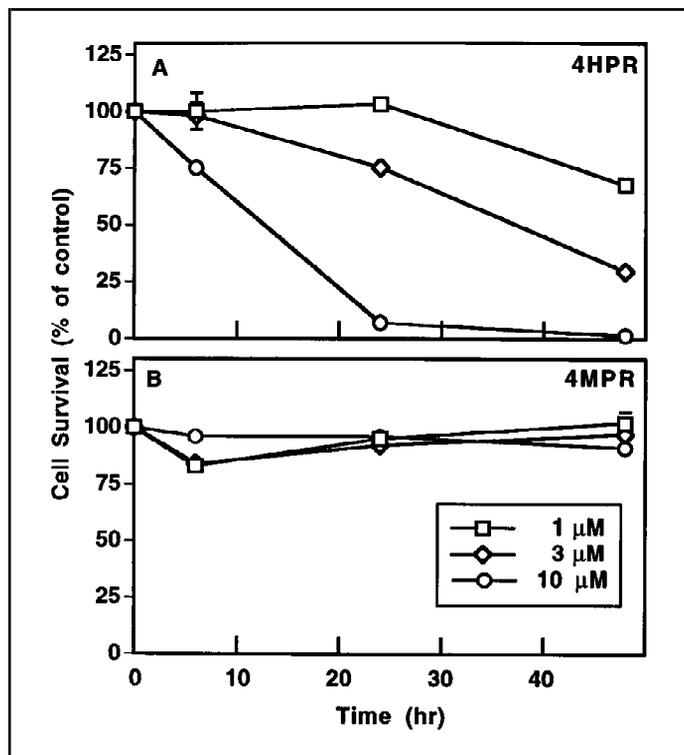


**Fig. 1.** Effects of *N*-(4-hydroxyphenyl)retinamide (4HPR) and *N*-(4-methoxyphenyl)retinamide (4MPR) on the survival of human cervical carcinoma C33A cells. Cells were seeded in 96-well plates (10 000 cells/well); after 24 hours, they were treated with medium containing 0, 1, 3, or 10 μ$M$ 4HPR (**A**) or the same concentrations of 4MPR (**B**) for 6, 24, or 48 hours. Cell numbers were estimated by the sulforhodamine B assay as described in the ''Materials and Methods'' section, and the values represent the mean ± standard deviation of six replicate wells.

nificant effect on cell survival even when used at 10 μ$M$ for 48 hours (Fig. 1, B). Likewise, ATRA (10 μ$M$) failed to inhibit cell growth or decrease survival of the C33A cells, although CD437 (0.1 or 1 μ$M$) did induce extensive apoptosis under the same treatment conditions (data not shown).

Treatment of the cells for 24 hours with different ATRA concentrations (0.1–10 μ$M$) in combination with 4HPR (0.1–3 μ$M$) did not affect cell survival any differently than the same concentrations of 4HPR alone (data not shown). Like ATRA, the RXR agonist SR11236 was ineffective as a single agent (0.1–10 μ$M$) and also failed to augment 4HPR action when the two were used in combination. CD2665 (up to 10 μ$M$), an antagonist of nuclear RAR β and RAR γ, failed to alter the effect of 4HPR (1–3 μ$M$) on cell survival (Table 1).

### Increased Rate of Generation of Reactive Oxygen Species in C33A Cells Exposed to 4HPR

Measurements of cellular fluorescence revealed that C33A cells generate intracellular reactive oxygen species at a constitutive rate of 0.079 fluorescence unit/minute (Fig. 2, A, control). After 20 minutes of exposure to 4HPR, a linear and dose-dependent increase in the rate of generation of reactive oxygen species was observed (Fig. 2, A, 4HPR). This increase was dependent linearly on 4HPR concentration in the range between 0.08 and 10 μ$M$, with about 50% of the maximal 4.5-fold increase observed at 0.6 μ$M$ 4HPR (Fig. 2, B).

4MPR, ATRA, and CD437 all failed to increase the generation rate of reactive oxygen species in C33A cells (Table 1).

### 4HPR Dose-Dependent DNA Fragmentation

A 24-hour exposure of the cells to 4HPR resulted in a dose-dependent DNA fragmentation, which appeared to be linear at the concentration range between 1.25 and 10 μ$M$ (Fig. 2, C). An increase in DNA fragmentation relative to untreated cells was measured in cells treated with as low as 3 μ$M$ 4HPR.

### Inhibitory Effects of Antioxidant on 4HPR-Induced C33A Cell Death and Generation of Reactive Oxygen Species

PDTC, which is an effective oxygen radical scavenger (antioxidant compound) and has been reported to inhibit the cytotoxic effects of tumor necrosis factor α *(18),* inhibited in a dose-dependent fashion the decrease in cell number in C33A cell cultures treated with 6 μ$M$ 4HPR for 12 hours (Fig. 3, A). PDTC also inhibited 4HPR-induced apoptosis, as determined by solubilization of [$^3$H]thymidine-labeled DNA (Fig. 3, B) and DNA ladder formation (Fig. 3, C). That these effects of PDTC were related to its antioxidant activity was indicated by the ability of PDTC to suppress the enhancement of the generation of reactive oxygen species in 4HPR-treated C33A cells (Fig. 3, D). All of these assays were done after a short (4–6 hours) exposure of the cells to 4HPR and PDTC because prolonged treatment (>12 hours) of the C33A cells with PDTC alone was somewhat cytotoxic to the cells (data not shown).

### Expression of Bcl-2 and Bax Proteins During 4HPR Treatment

Immunoblotting analysis revealed that both Bcl-2 and Bax proteins are expressed constitutively in C33A cells and that the level of Bax appears to be much lower than that of Bcl-2 (Fig.

**Table 1.** Ability of different retinoids to induce apoptosis and to enhance generation of reactive oxygen species (ROS) in C33A cells*

| Treatment | Retinoid | Binding to retinoid receptors | Transactivation of RARE | Induction of apoptosis | Enhancement of ROS generation |
|-----------|----------|-------------------------------|-------------------------|------------------------|-------------------------------|
| A | 4HPR | No | Yes | Yes | Yes |
| B | 4MPR | ND* | ND | No | No |
| C | ATRA | Yes (RARs) | Yes | No | No |
| D | CD437 | Yes (RAR $\gamma$) | Yes | Yes | No |
| E | CD2665 | Yes (RARs) | No | No | No |
| F | SR11236 | Yes (RXRs) | No | No | No |
| A + C | | | | Yes | Yes |
| A + E | | | | Yes | Yes |
| A + F | | | | Yes | Yes |

*4HPR = N-(4-hydroxyphenyl)retinamide; 4MPR = N-(4-methoxyphenyl)retinamide; ATRA = all-*trans*-retinoic acid; CD437 = 6-[3-(l-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid; CD2665 = 4-[7-(l-adamantyl)-6-(2-methoxyethoxy)methoxy-2-naphthalenyl]benzoic acid; SR11236 = 2-(4-carboxyphenyl)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1,3-dioxane; RAR = retinoic acid receptor; ND = not determined; RARE = retinoic acid response element.

4). The Bcl-2 level remained unchanged after 2, 5, and 8 hours of treatment with 4HPR (Fig. 4, upper panel), and Bax showed no consistent changes during the same 4HPR treatment (Fig. 4, lower panel).

## Effects of 4HPR on Survival and Generation of Reactive Oxygen Species in Normal Human Cervical Epithelial Cells

The survival of normal human cervical epithelial cells after a 12-hour exposure to 1–10 $\mu M$ 4HPR was greater than 80% (Fig. 5, A). Extending the treatment to 48 hours further decreased the survival of the cells exposed to 3 or 10 $\mu M$ to about 75% and 30%, respectively (Fig. 5, A).

Analyses of cultures exposed to different concentrations of 4HPR failed to reveal an increase in the rate of generation of reactive oxygen species (Fig. 5, B).

## Discussion

Other investigators *(13)* have reported that vitamin E and N-acetyl-L-cysteine inhibit 4HPR-induced apoptosis of HL-60 leukemia cells, and we found that PDTC and nordihydroguaiaretic acid inhibit induction of apoptosis by 4HPR in C33A cervical carcinoma cells [*(14)*; Fig. 3]. Through distinct mechanisms, these antioxidants control the cellular redox state by quenching intracellular free radicals such as reactive oxygen species. These findings raised the possibility that reactive oxygen species are involved in 4HPR-induced apoptosis. Because of their high reactivity, reactive oxygen species affect various cellular molecules, such as fatty acids, carbohydrates, proteins, and nucleic acids. An excess of reactive oxygen species may lead to cell death when their level overwhelms the cellular antioxidant capacity, which is linked to the antioxidant level, the growth rate, and the age of the cells *(29–32)*.

Considering that reactive oxygen species can induce apoptosis *(17,32),* our study was designed to determine whether 4HPR can modulate the generation of reactive oxygen species in C33A cells. To our knowledge, we have demonstrated for the first time that 4HPR can increase the generation of reactive oxygen species in cervical carcinoma cells. We propose that this effect may be important for the induction of apoptosis after treatment with 4HPR. Several lines of evidence support this conclusion: 1) Antioxidant compound PDTC inhibited 4HPR-induced apopto-

sis as well as 4HPR-enhanced generation of reactive oxygen species; 2) 4HPR increased the generation of reactive oxygen species and DNA fragmentation in a dose-dependent fashion; and 3) 4MPR, ATRA, and SR11236 failed to induce apoptosis and were also incapable of increasing production of reactive oxygen species (Table 1).

The dose–response relationship observed for cell killing (Fig. 1) showed a greater difference between the efficacy of 3 and 10 $\mu M$ 4HPR than the dose dependence observed for the generation of reactive oxygen species at these concentrations (Fig. 2, B). The reason for this apparent discrepancy may be the assay used for analysis of cell survival. For this assay, we counted the cells that remained attached and did not include cells that were detached and floating in the medium as a result of apoptosis. Cell detachment is an event characteristic of apoptotic cells, and it is possible that the detachment of cells treated with 3 $\mu M$ 4HPR is slower than that of cells treated with 10 $\mu M$ 4HPR. The induction of DNA fragmentation required higher 4HPR concentrations than those required for the induction of reactive oxygen species. This observation suggests that a certain threshold level of reactive oxygen species is required to induce the apoptotic pathway, which culminates in DNA fragmentation. Such a threshold may depend on the antioxidant capacity of a given cell. The peak plasma level of 4HPR in women who received 200 mg/day of the retinoid was approximately 1 $\mu M;$ however, tissue levels may be much higher, as indicated by a concentration of greater than 10 $\mu M$ 4HPR in the nipple discharge of a woman who had taken 4HPR for 15 days *(28)*. Thus, it is possible that 4HPR levels required to induce reactive oxygen species and apoptosis can be achieved pharmacologically in certain tissues in patients.

4HPR has been reported to activate transcription of retinoic acid response elements most probably through RXR–RAR heterodimers *(7,8)*. However, the findings that ATRA, which is an effective activator of nuclear retinoid receptors, was ineffective in inducing apoptosis of C33A cells and that the RAR antagonist CD2665 failed to inhibit 4HPR's effects on cell survival suggested that it is unlikely that the nuclear retinoid receptors mediate these effects in the C33A cells. This conclusion is in agreement with several reports on 4HPR-induced apoptosis of various carcinoma cells *(9–12)*.

The combined treatment with ATRA and 4HPR enhanced differentiation in HL-60 cells *(33)* and enhanced inhibition of
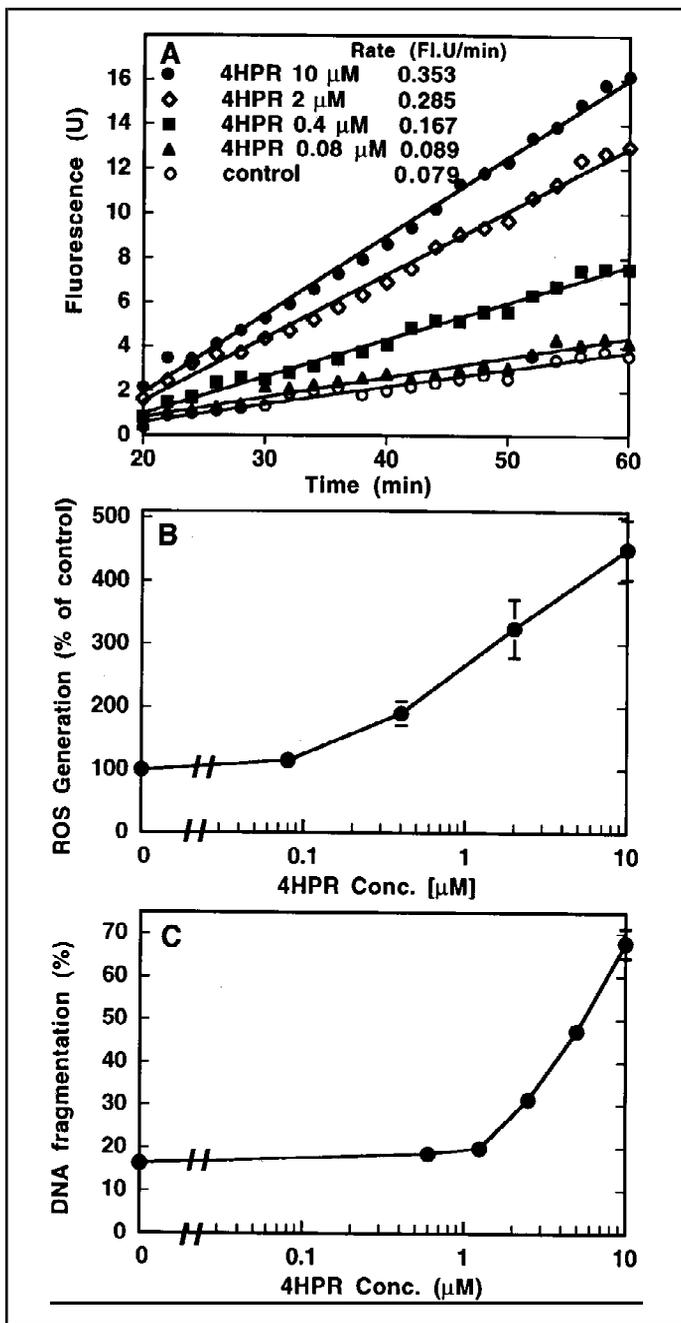
**Fig. 2.** Effects of $N$-(4-hydroxyphenyl)retinamide (4HPR) on the generation of reactive oxygen species (ROS) and on DNA fragmentation in human cervical carcinoma C33A cells. Cells were seeded in 96-well plates (10 000 cells/well); after 3 days, they were treated with 5,6-carboxy-2′,7′-dichlorofluorescin diacetate without (control) or with 0.08, 0.4, 2, or 10 $\mu M$ 4HPR. Control cultures received dimethyl sulfoxide. The cells were maintained at 37 °C, and the fluorescence emission at 538 nm was measured at 2-minute intervals after excitation at 485 nm. **A)** Rates of increase in fluorescence (representing ROS generation rate) in cells treated with different 4HPR concentrations (Conc.) between 20 and 60 minutes (data from a single well for each treatment). **B)** 4HPR dose-dependent increase in the rate of ROS generation relative to untreated cells (mean ± standard deviation of six wells per treatment). **C)** C33A cells were incubated with tritiated thymidine to label the DNA and then treated with 4HPR for 24 hours. The solubilization of DNA was used as a measure of DNA fragmentation as described in the ''Materials and Methods'' section. Fl.U = fluorescence units.
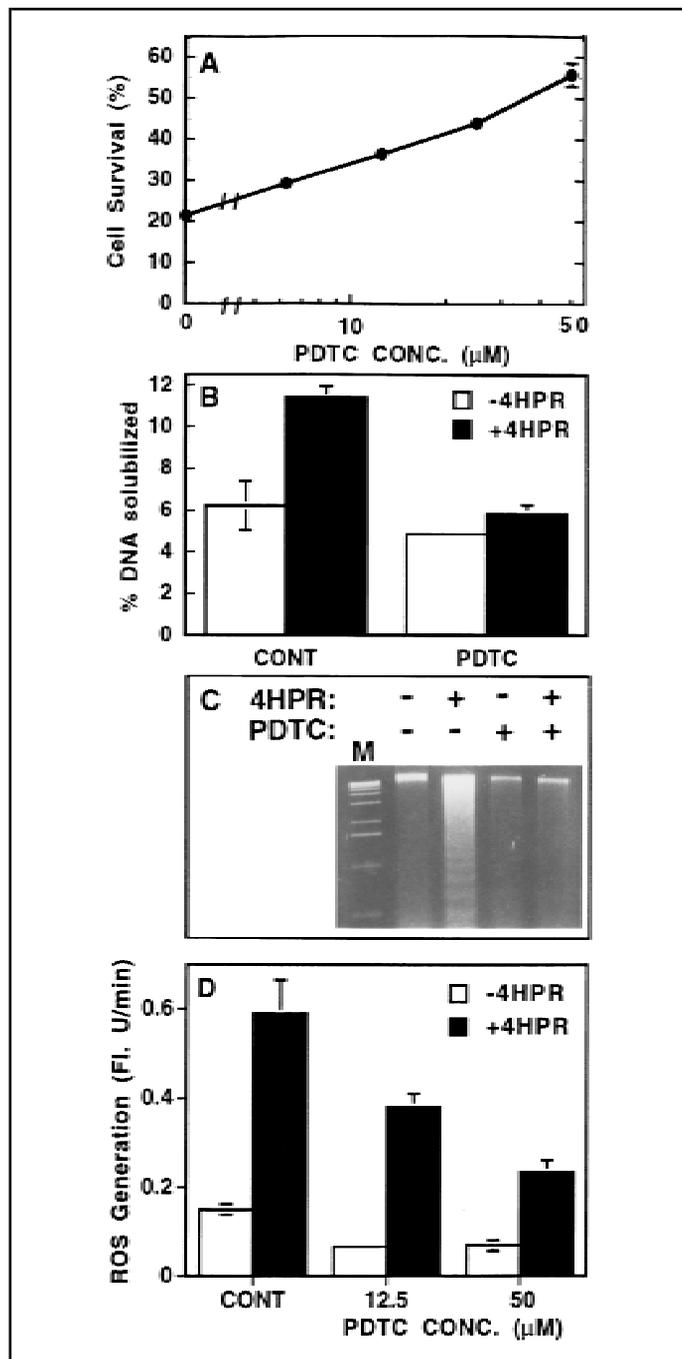


**Fig. 3.** Effects of pyrrolidine dithiocarbamate (PDTC) on $N$-(4-hydroxyphenyl)retinamide (4HPR)-induced apoptosis in human cervical carcinoma C33A cells. **A)** Dose-dependent effects of PDTC on 4HPR-induced cytotoxicity. Cells were treated with 6 $\mu M$ 4HPR for 12 hours in the presence of various concentrations (CONC.) of PDTC, and the survival of cells was analyzed by the sulforhodamine B assay. Cell number in control cultures was taken as 100%. **B)** Inhibition by PDTC of 4HPR-induced solubilization of [$^3$H]thymidine-labeled DNA. Cells were labeled with [$^3$H]thymidine and then treated with control medium (CONT) or with medium supplemented with 10 $\mu M$ 4HPR, 25 $\mu M$ PDTC, or a combination of both agents for 4 hours. The solubilization of DNA was used as a measure of DNA fragmentation as described in the ''Materials and Methods'' section. **C)** Effects of 4HPR and PDTC on DNA ladder formation in C33A cells. Soluble DNA was extracted from both floating and attached cells after a 6-hour treatment with 10 $\mu M$ 4HPR, 25 $\mu M$ PDTC, or their combination and was subjected to agarose gel electrophoresis in 2% agarose gel. Lane M is the 1-kilobase DNA size marker (Life Technologies [GIBCO-BRL], Gaithersburg, MD). **D)** Cells were treated with 10 $\mu M$ 4HPR or 0.1% dimethyl sulfoxide (CONT) and with the indicated concentrations of PDTC. The generation of reactive oxygen speices was analyzed in these cells as in Fig. 2, A.
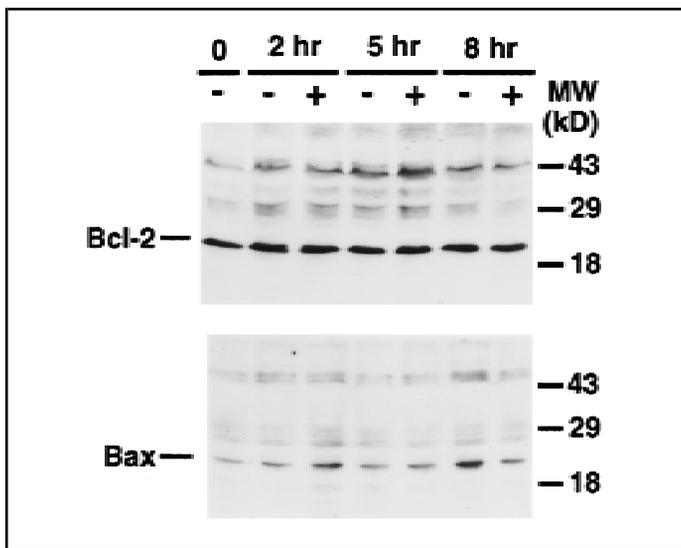
**Fig. 4.** Expression of Bcl-2 and Bax during *N*-(4-hydroxyphenyl)retinamide (4HPR) treatment of human cervical carcinoma C33A cells. Extracts from cells grown with or without 10 $\mu M$ 4HPR for the indicated periods were analyzed by immunoblotting by use of anti-human Bcl-2 (upper panel) and Bax (lower panel) antibodies. Numbers on the right indicate the kilodaltons (kD) and electrophoretic mobility of standard molecular mass markers. MW = molecular weight.

growth of an ATRA-resistant HL-60 clone *(34)*. Likewise, the combination of RAR-selective retinoids and RXR-selective retinoids enhanced growth inhibition of certain cells *(35)*. In contrast, we found that combining 4HPR with either ATRA or SR11236 did not potentiate its effect on cell survival. The inability of CD437 to increase reactive oxygen species in C33A cells, despite its ability to cause them to undergo rapid apoptosis *(36)*, suggests that 4HPR induces a specific apoptosis pathway distinct from that induced by CD437.

We examined the expression of two gene products that are relevant for reactive oxygen species-induced apoptosis, namely, Bcl-2 and Bax. Bcl-2 has been localized to intracellular sites where oxygen radicals are generated (i.e., mitochondria, endoplasmic reticulum, and nuclear membrane) and has been reported to protect cells against reactive oxygen species-induced apoptosis *(15,37)*. Heterodimerization with Bax seems to be required for Bcl-2 to repress cell death, and an increase in the ratio of Bax to Bcl-2 may enhance apoptosis *(38)*. A previous study *(13)* has implicated Bcl-2 in the mechanism of action of 4HPR on leukemia cells on the basis of the finding that expression of transfected Bcl-2 in HL-60 cells delayed 4HPR-induced apoptosis. The expression of these proteins was related to the expression of wild-type p53, which has been implicated in apoptosis induction and can suppress Bcl-2 and increase Bax expression *(39,40)*. C33A cells were reported to express a high level of mutant p53 messenger RNA and protein (codon 273; Arg to His) *(23,36)*. Thus, our finding that Bcl-2 is expressed at an apparently higher level than Bax in C33A cells may reflect the high expression of mutant p53 protein. 4HPR treatment for up to 8 hours did not alter the levels of either Bcl-2 or Bax, although apoptosis was apparent already after 6 hours in these cells. These results suggest that 4HPR-induced apoptosis in C33A cells is not mediated by suppression of Bcl-2 or by an increase in Bax levels. In some cells (e.g., neural cells depleted of glutathione), Bcl-2 prevented the generation of reactive oxygen species *(37)*;

in contrast, in other cells (e.g., pro-B lymphocytes), Bcl-2 failed to prevent the generation of reactive oxygen species *(13)*. Clearly, in C33A cells, Bcl-2 did not prevent the induction of reactive oxygen species by 4HPR and did not prevent apoptosis. Our data cannot distinguish between the possibility that the target of 4HPR action that results in enhancement of reactive oxygen species is downstream of Bcl-2 or that 4HPR acts through a pathway that is not affected by Bcl-2. In another cell type (namely, the head and neck squamous cell carcinoma cell line 1483), we have recently found that transfection of bcl-2 failed to protect the cells from apoptosis induced by either 5 or 10 $\mu M$ 4HPR (Eicher S, Lotan R: unpublished data).

At high levels, reactive oxygen species can be deleterious to cells because of their high reactivity with various macromolecules. The interactions of reactive oxygen species with DNA can lead to genotoxic effects that can enhance carcinogenesis mostly in the promotion and progression stages *(41)*. However, various studies have demonstrated that 4HPR suppressed carcinogenesis *in vivo* *(1–3)* and exerted anticlastogenic effects in human lymphoblastoid cells exposed to DNA-damaging agents *(42)*. It is interesting that other chemopreventive agents such as oltipraz *(43)* and caffeic acid phenethyl ester *(44)* have been
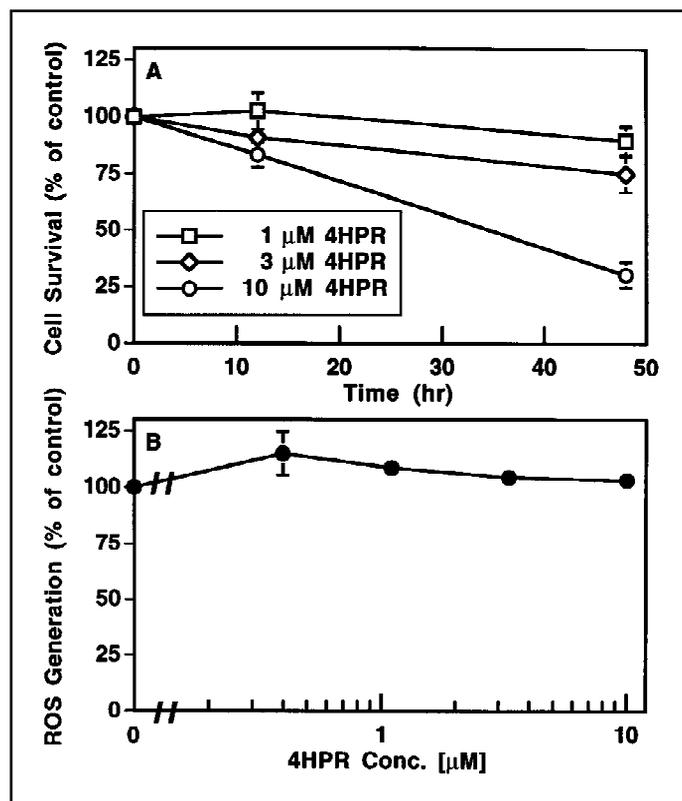


**Fig. 5.** Effects of *N*-(4-hydroxyphenyl)retinamide (4HPR) on the survival of normal human cervical epithelial cells and on the generation of reactive oxygen species (ROS). **A)** Cells were seeded in 96-well plates (2000 cells/well); after 24 hours, they were treated with medium containing 0, 1, 3, or 10 $\mu M$ 4HPR for 6 or 48 hours. Cell numbers were estimated by the sulforhodamine B assay as described in the ''Materials and Methods'' section. The values represent the mean ± standard deviation (SD) of triplicate wells. **B)** Cells were seeded in 96-well plates (2000 cells/well); after 3 days, they were treated with 5,6-carboxy-2′,7′-dichlorofluorescin diacetate without or with 0.4, 1.1, 3.3, or 10 $\mu M$ 4HPR. The generation of ROS was analyzed as described in Fig. 2, and the mean ± SD of the rate measured in six replicate wells after a 60-minute exposure to 4HPR was plotted versus 4HPR dose. Conc. = concentration.

recently reported to increase oxygen radical production. Our studies revealed that 4HPR exerts differential effects on the cervical carcinoma cells and on normal cervical epithelial cells, in that the decrease in cell survival was much more profound in the cancer cells than in the normal cells and that reactive oxygen species were generated at a higher rate in the cancer cells than in the normal cells after exposure to 4HPR. Thus, it appears that the enhancement of the generation of reactive oxygen species by 4HPR is increased preferentially in the malignant cells. Cells have an elaborate defense system for protection against free radical-induced damage that involves generation of sulfhydryls (e.g., glutathione and cysteine) and antioxidant enzymes, including glutathione peroxidase, superoxide dismutase, and catalase *(41)*. The difference between the generation of reactive oxygen species in response to 4HPR in normal and cancer cells may be due to the expression of more effective endogenous antioxidant mechanisms in the normal cells than in the malignant ones. Although 4HPR did not increase the generation of reactive oxygen species in the normal cells, it did cause a decrease in cell number; this effect may be mediated by a mechanism that is independent of the generation of reactive oxygen species. Normal cells contain presumably wild-type p53, whereas C33A cells contain a mutated p53 *(24)*. However, 4HPR-induced apoptosis appears to be independent of p53 status because, in a previous study *(11)*, we have demonstrated that 4HPR can induce apoptosis in cervical carcinoma cell lines (e.g., C4II, SiHa, MS751, and ME180), which contain wild-type p53 *(24)*.

The mechanism by which 4HPR increases the generation of reactive oxygen species is not known. A preliminary study *(45)* has demonstrated that carbonylcyanide *m*-chlorophenylhydrazone, which uncouples electron transfer and adenosine triphosphate synthesis in mitochondria and inhibits the generation of reactive oxygen species from mitochondrial respiratory chain, inhibited the 4HPR-induced generation of reactive oxygen species. Furthermore, thenoyltrifluoroacetone (an inhibitor of mitochondrial respiratory chain complex II), but not rotenone (an inhibitor of mitochondrial respiratory chain complex I) or antimycin A (an inhibitor of mitochondrial respiratory chain complex III), inhibited the 4HPR-induced generation of reactive oxygen species. These results strongly indicate that the target of the 4HPR-induced generation of reactive oxygen species is a mitochondrial respiratory chain site between complex II and complex III *(45)*.

The induction of reactive oxygen species by 4HPR could trigger a signaling pathway that is independent of nuclear retinoid receptors. Indeed, changes in the redox state of cells lead to changes in cellular signaling molecules, including protein kinases, protein phosphatases, and transcription factors; therefore, reactive oxygen species play important roles as second messengers in growth factor signaling *(16,18,19)*. Some of these pathways could lead to apoptosis.

Several chemoprevention studies *(2,3,46)* have demonstrated some efficacy of 4HPR. In the context of our study with cervical carcinoma cells, it is worthwhile to mention a randomized, double-blinded clinical trial in China in which *N*-(4-carboxyphenyl)retinamide was used as an intravaginal suppository and caused precancerous lesions to disappear in 68% of the patients *(47)*. Although the mechanism of this effect is not known, and there are no data showing that 4HPR can induce apoptosis in premalignant cells, it is tempting to speculate that apoptosis may be involved in the chemopreventive and therapeutic effects of 4HPR *in vivo*. Future studies with biopsy specimens from patients treated with 4HPR could test this hypothesis.

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# Notes