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# Inhibition of Proliferation and Induction of Apoptosis in Cervical Carcinoma Cells by Retinoids: Implications for Chemoprevention

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**Abstract** The effects of retinoids including all-*trans*-retinoic acid (ATRA), 13-*cis*-retinoic acid (13CRA), and *N*-(4-hydroxyphenyl)retinamide (4-HPR) on several cervical carcinoma cell lines in culture were investigated as a prelude to investigating the mechanisms underlying the chemopreventive potential of retinoids in cervical cancer. We found that when used at a concentration of 1 μM, 13CRA and ATRA inhibited the proliferation of three cell lines (ME-180 [HPV 68], SiHa [HPV 18], and HT-3 [HPV$^-$]) by about 80% after a seven-day treatment. Three other cell lines (MS-751 [HPV 18], HeLa [HPV 18], C-33A [HPV$^-$]) were moderately inhibited (30–48%), and two (C-4 II [HPV 18], CaSki [HPV 16]) responded poorly (< 25% inhibition). 4-HPR failed to inhibit the growth of any of these cell lines when used at 1 μM; however, when used at 5 or 10 μM, it induced apoptosis as evidenced by DNA fragmentation in several of the cell lines and was more potent in this effect than 10 μM ATRA. Retinoids that induce apoptosis in malignant cells may be able to exert similar effects on premalignant cells. Such retinoids would be expected to exhibit greater potency as chemopreventive agents than retinoids that exert only cytostatic effects. © 1995 Wiley-Liss, Inc.

**Key words:** Apoptosis, cervical cancer, chemoprevention, *N*-(4-hydroxyphenyl)retinamide, retinoids

Cervical carcinoma is the second most prevalent cancer in women worldwide and a leading cause of mortality among relatively young women in developing countries. Worldwide in 1989, there were an estimated 500,000 cases, with an overall 5-year survival of 40% [1]. Yet the problem is not unique to developing countries. Despite advances in diagnosis and therapy, the morbidity and mortality from this malignancy are still high. In the U.S. alone, 1995 will see an estimated 65,000 new cases of carcinoma *in situ* (CIS), 15,800 new cervical cancer cases, and 4,800 deaths [2].

Cervical carcinoma is thought to develop through a multistep process in which premalignant dysplastic lesions (cervical intraepithelial neoplasia, or CIN) of increasing severity (*i.e.*, CIN I, CIN II, and CIN III) progress to CIS and eventually to invasive cancer [1,3]. Because CIN can often be detected several years (> 10) before invasive cancer, cervical cancer is eminently suited for prevention [1].

Chemoprevention with retinoids is one approach to preventing cervical cancer. Reti-

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noids are essential for the proper differentiation of most epithelial tissues [4]. Deficiency in vitamin A can lead to squamous metaplasia [4], and epidemiologic studies have shown an inverse relationship between dietary intake or serum levels of vitamin A and the development of certain malignancies including cervical dysplasia and/or cervical cancer [5]. Retinoids have already been used in several clinical trials to prevent cervical cancer. A Phase II trial of all-*trans*-retinoic acid (ATRA) delivered locally to the cervix resulted in a complete clinical response rate of 50% [6]. A randomized Phase III trial with a short course of topically applied ATRA demonstrated an increase in the complete histologic regression rate of CIN II from 27% in the placebo group to 43% in the treatment group; however, no treatment difference between the two arms was evident in the severe dysplasia group [7]. In yet another trial, although severe dysplasias failed to respond to short-term treatment with ATRA, advanced stage cancers regressed markedly after treatment with 13-*cis*-retinoic acid (13CRA) in combination with interferon-α [8]. These studies all demonstrated the potential of retinoids in prevention of cervical cancer.

Unfortunately, prolonged use of ATRA or 13CRA has significant side effects, but there are synthetic retinoids that exhibit fewer side effects. One of these retinoids is *N*-(4-hydroxyphenyl)-retinamide (4-HPR). In animal models, 4-HPR was found to be effective alone or in combination with tamoxifen against breast, bladder, lung, and prostate cancer [9,10]. This retinoid also exhibited antiproliferative effects against fresh human tumors in an *in vitro* colony formation assay [11], and therapeutic effects against breast, prostate, and ovarian cancer in rodents [12–14]. Various clinical chemoprevention trials are in progress to test the effects of 4-HPR in breast [15] and preneoplastic oral leukoplakia [16]. Furthermore, it was recently reported that 4-HPR may prevent ovarian cancer [17] and suppress the growth of ovarian cancer xenografts in nude mice [14]. These preclinical and clinical activities of 4-HPR have raised our interest in using this retinoid for chemoprevention of cervical carcinogenesis. As a prelude to our Phase II clinical trial of 4-HPR in patients with CIN, we examined the effects of ATRA, 13CRA, and 4-HPR on several established human cervical carcinoma cell lines, and found that 4-HPR was more potent than the natural retinoic acids in inducing apoptosis in some of the cell lines.

## MATERIALS AND METHODS

### Cell Culture and Retinoic Acid Treatment

The human cervical carcinoma cell lines MS-751, C-4 II, HeLa, C-33A, CaSki, SiHa, and HT-3 were purchased from the American Type Culture Collection (Rockville, MD). The ME-180 cells were obtained from Dr. Nicholas Donato (M.D. Anderson Cancer Center, Houston, TX). All cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium:Ham's F12 medium (DMEM/F12) supplemented with 10% fetal bovine serum. 4-HPR was obtained from Dr. Ronald Lubet (Division of Cancer Prevention and Control, National Institutes of Health, Bethesda, MD) via Ogden Bioservices, Inc. (Rockville, MD). ATRA and 13CRA were obtained from Dr. Werner Bollag (F. Hoffmann-La Roche, Basel, Switzerland). The retinoids were dissolved in dimethylsulfoxide (DMSO) at a concentration of 10 mM and diluted in growth medium immediately before addition to cell cultures. Control cultures received the same amount of the solvent as did the treated cultures.

### Assay of Growth Inhibition

Cells were plated in 48-well tissue culture plates in DMEM/F12 with 10% fetal bovine serum and the appropriate supplements (0.01% DMSO as control solvent; 1 μM retinoid) on day 1 and day 3, and the experiments were terminated on day 6. Growth inhibition was determined using calcein-AM, a polyanionic molecule that is fluorescent within viable cells (Molecular Probes, Eugene, OR). The fluorescence of cells in control cultures (Fc) and in treated cultures (Ft) were measured using a fluorescent plate scanner (Cytofluor Corp., Bedford, MA) connected to an NEC Power Mate 386/33i computer and an NEC Pinwriter printer. The data were gathered, then used to calculate growth inhibition according to the equation, $\% \text{ Inhibition} = (1 - Ft/Fc) \times 100$. The optimal concentration of calcein-AM that gives a linear fluorescence with increase in cell number in the range of 50 to 100,000 cells was determined for each of the cell lines.

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

Soluble DNA was extracted as described previously [18]. In brief, after a 7-day incubation with solvent only, 10 µM ATRA, or 10 µM 4-HPR, the cell cultures were processed as follows. Cells floating in media were collected by centrifugation; the cells that remained attached to the dish were resuspended in Tris-EDTA buffer (TE), pH 8.0. The plasma membranes of the cells were lysed in 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% Triton X-100 on ice for 15 min. The lysate was centrifuged at 12,000 x g for 15 min to separate soluble (fragmented) from pellet (intact genomic) DNA. Soluble DNA was treated with RNase A (50 µg/ml) at 37°C for 1 hr, followed by treatment with proteinase K (100 µg/ml) in 0.5% SDS at 50°C for 2 hr. The residual material was extracted with phenol/chloroform, precipitated in ethanol, and electrophoresed on a 2% agarose gel.

### Quantitative DNA Fragmentation Assay

The percentage of apoptotic cells was evaluated by a modification of the quantitative assay described by Wright *et al.* [19]. Cells (1 x $10^5$/well) were labeled with [$^3$H]thymidine (64 Ci/mmol; 1 µCi/$10^5$ cells) for 20 hr in 24-well tissue culture plates. The cells were washed three times with 2 ml of complete medium and then incubated for 4–6 additional hours, followed by treatment with 10 µM ATRA or 10 µM 4-HPR for 24 hr. At the end of the incubation, medium containing the floating cells was separated into supernatant (cpm in medium) and pellet (cpm in floating cells) after centrifugation at 5,000 x g for 5 min. Both fractions were analyzed by scintillation counting. Attached cells were harvested by trypsinization, resuspended, and lysed in 500 µl of lysis buffer (10 mM EDTA, 0.5% Triton X-100 [TX-100] in 10 mM Tris-HCl, pH 8.0) on ice for 15 min. Intact chromatin in the insoluble fraction was separated from fragmented DNA in the TX-100 fraction by centrifugation at 12,000 x g for 15 min. The pellet was dissolved in 500 µl of 1% SDS. Both TX-100 lysate (cpm in TX-100 fraction) and the SDS-solubilized pellet (cpm in SDS fraction) were then counted in a scintillation counter. Percent DNA release in total cells and adherent cells, respectively, was calculated as follows:

The amount of unincorporated [$^3$H]thymidine in the soluble fraction of attached cells was negligible, judging from the measurement made after the soluble DNA in this fraction was precipitated in ethanol.

## RESULTS

### Differential Growth Inhibitory Effects of Retinoids on Cervical Carcinoma Cell Lines

The peak plasma levels of ATRA, 13CRA, and 4-HPR are about 1 µM in patients receiving oral retinoids. When established cervical carcinoma cell lines were treated with these retinoids at 1µM, their growth was suppressed to varying degrees (Table I). ATRA and 13CRA exhibited comparable activity and inhibited the growth of ME-180, SiHa, and HT-3 cells by about 80%, the growth of MS-751, HeLa, and C-33A cells by 30–48%, and the growth of C-4 II and CaSki cells by less than 24%. In contrast, 4-HPR was mostly inactive. There appeared to be no correlation between the presence or type of HPV in the cells and their response to retinoids.

### Differential Induction of Apoptosis by Retinoids in Cervical Carcinoma Cell Lines

Tissue levels of retinoids may be higher than peak plasma levels due to the tendency of some retinoids to accumulate in cells, especially in fatty tissues. For example, the level of 4-HPR in the nipple discharge of women treated with 200 mg/day was about 30 µM, or 30 times higher than the peak plasma level [20]. Recently, it was reported that 4-HPR (at doses > 3 µM) induced apoptosis in hematopoietic malignancies [21] and in neuroblastoma cells [22]. This prompted us to examine whether cervical carcinoma cells exposed to 10 µM of either ATRA or 4-HPR undergo apoptosis measured by DNA fragmentation. Table I shows that some of the cell lines (*e.g.*, C-4 II, C-33A, and CaSki) undergo spontaneous apoptosis. 4-HPR induced or increased apoptosis in five of the eight cell lines tested, had no effect on one cell line, and decreased apoptosis in two cell lines. In contrast, ATRA increased apoptosis in only one cell line

TABLE I. Characteristics of Human Cervical Carcinoma Cell Lines and Their Responses to the Growth Inhibitory Effects of Retinoids

| Cell Line | Age (Yr) Race[a] | Papilloma Virus[b] | % Growth Inhibition[c] 13CRA | ATRA | 4-HPR | Apoptosis[d] C | ATRA | 4-HPR |
|---|---|---|---|---|---|---|---|---|
| ME-180[e] | 66/C | HPV 68 | 84 | 83 | 16 | – | – | + |
| MS-751 | 47/C | HPV 18 | 47 | 42 | 8 | ± | ± | + |
| C-4 II | 41/C | HPV 18 | 18 | 21 | 11 | ++ | ++ | ++ |
| HeLa | 31/B | HPV 18 | 48 | 45 | 17 | + | + | ± |
| C-33A | 66/C | ND | 36 | 30 | 9 | +++ | +++ | NA |
| CaSki[a] | 40/C | HPV 16 | 24 | 16 | 6 | ++ | ++ | – |
| SiHa | 55/M | HPV 16 | 82 | 77 | 16 | – | – | ++ |
| HT-3 | 58/C | ND | 86 | 74 | 21 | – | + | NA |

[a]C, Caucasian; B, Black; M, Mongoloid; [b]HPV, human papilloma virus; ND, not detected; [c]All retinoids were used at a concentration of 1 μM; growth inhibition was determined after 6 days; [d]The retinoids were used at a concentration of 10 μ; floating and adherent cells were analyzed for DNA ladder formation after a 7-day incubation. C, control solvent alone; -, no visible DNA ladder; ±, barely discernible DNA ladder; +,++, and +++, increasingly more visible DNA ladder. For example, in Figure 1, the last two lanes were scored + and ++, respectively. NA, apoptosis so intensive that all of the cells died and were unavailable for analysis; [e]Derived from metastasis

(HT-3) and failed to alter apoptosis in the other seven cell lines (Table I).

## Differential Induction of DNA Fragmentation in Cervical Carcinoma Cells

The induction of apoptosis in SiHa cells by 4-HPR is shown in Figure 1. DNA fragmentation, a hallmark of apoptosis [19], was detected in floating cells and adherent cells after seven days of culture in the presence of 10 μM 4-HPR, but not in cells treated with the same concentration of ATRA. A more sensitive assay that measures solubilization of DNA in cells prelabeled with [$^3$H]thymidine revealed that 4-HPR increased the fraction of soluble DNA about 5-fold relative to control cultures after 24 hr of treatment (Table II). In contrast, 13CRA was ineffective.

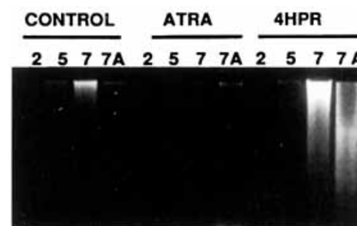


Fig. 1. Induction of apoptosis in SiHa cervical carcinoma cells by 4-HPR but not by ATRA. After reaching 80% confluence, cells were treated with DMSO (CONTROL), 10 μM ATRA, or 10 μM 4-HPR. Cells floating in the culture medium after 2, 5, and 7 days of treatment (lanes marked 2, 5, and 7, respectively) and adherent cells after 7 days (lane marked 7A) were collected, and soluble DNA from each cell fraction was extracted and electrophoresed on a 2% agarose gel. The gel was then stained with ethidium bromide and photographed under UV light.

## DISCUSSION

We found that at 1 μM, ATRA and 13CRA were potent inhibitors of proliferation of several of the cell lines examined, whereas 4-HPR was a poor growth inhibitor at the same concentration. However, at 10 μM, 4-HPR was a potent inducer of apoptosis, whereas ATRA did not exhibit this effect on most of the cell lines. This is the first report on the ability of 4-HPR to induce apoptosis in human cervical carcinoma cells in culture.

**TABLE II. DNA Fragmentation in [$^3$H]Thymidine Labeled SiHa Cells Treated with 4-HPR or 13CRA**

| | % DNA Release[a] | |
|---|---|---|
| Treatment | Total Cells | Adherent Cells |
| Control | 4.9 ± 1.0 | 2.0 ± 0.2 |
| 4-HPR | 22.0 ± 7.9 | 12.2 ± 5.1 |
| 13CRA | 3.5 ± 0.6 | 1.3 ± 0.4 |

[a] % DNA release was calculated as described in Materials and Methods.

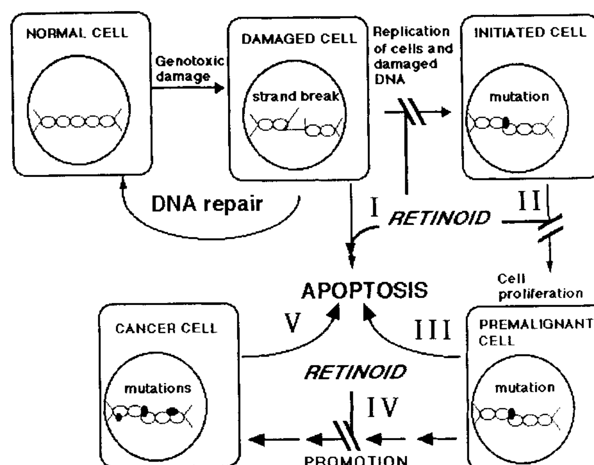


**Fig. 2.** A schematic representation of cancer development and the role of apoptosis and retinoids in cancer prevention. Cells exposed to a genotoxic agent that causes DNA damage are either restored to normalcy after DNA- repair or eliminated by apoptosis (I). However, if the DNA-damaged cells replicate before the damage is repaired, then the damaged DNA is replicated, which can result in mutations. The resulting initiated cell can proliferate and form a premalignant lesion that can undergo promotion to cancer. The ability of the retinoid 4-HPR to induce apoptosis in cancer cells (V) has been demonstrated. The hypothetical potential of 4-HPR to enhance apoptosis of DNA-damaged cells (I), initiated cells (II), or premalignant cells (III and IV) may explain the demonstrated ability of this retinoid to suppress carcinogenesis in a variety of animal models and a few human premalignancies.

Previous studies have shown that ATRA can induce apoptosis in mouse embryo limb buds [23], in HL-60 myeloid leukemia cells subsequent to their differentiation into granulocytes [24], in P39 myelomonocytic leukemia cells [25], and in neuroblastoma cells [26]. ATRA's mechanism of apoptosis induction in HL-60 myeloid leukemia and in neuroblastoma cells, and possibly other cells, has been related to induction of tissue transglutaminase [26,27] and suppression of *bcl*-2 [28]. The mechanism by which 4-HPR induces apoptosis is not clear, but it appears to be distinct from that of ATRA because the effects of 4-HPR were noticed within a few hours as opposed to a few days for ATRA [21]. Furthermore, 4-HPR was able to induce apoptosis in HL-60 cells that were resistant to ATRA [21]. Our results showed a rapid induction of apoptosis by 4-HPR within 24 hr and a lack of efficacy of ATRA in the cervical cancer cells. ATRA is thought to act by binding to nuclear retinoic acid receptors and activating gene transcription by means of retinoic acid response element [29]. The finding that ATRA is not as effective as 4-HPR in inducing apoptosis in cervical carcinoma cells suggests that 4-HPR-induced apoptosis may be mediated by another pathway.

Apoptosis is a physiological form of programmed cell death that maintains homeostasis in tissue mass and architecture during development and cellular immune responses by eliminating cells. Initiated in response to a variety of external and intracellular signals, this process is characterized by cell shrinkage, chromatin condensation, nuclear segmentation, and internucleosomal degradation of DNA [18,19,30–35]. Apoptosis can protect the organism against heritable genotypic changes in cells linked to mutagenesis and carcinogenesis [32–35]. Consequently, activation of apoptosis in cells at risk of undergoing neoplastic transformation (*e.g.*, DNA damaged cells) may constitute a physiological mechanism to protect the organism from cancer development by eliminating cells that might otherwise replicate the damaged DNA, a process that may lead to mutations and eventually to cancer. Thus, loss of the ability to undergo apoptosis is associated with cancer development and induction of apoptosis with its prevention [32–37]. Indeed, it has been shown that tumor promotion can be suppressed when apoptosis is induced by withdrawal of tumor promoters [36] or by treatment with S-adenosyl-L-methionine [37].

Eliminating tumor cells by inducing apoptosis is a promising approach to cancer therapy [38]. We have demonstrated that 4-HPR can induce apoptosis in malignant cervical carcinoma cells;

hence, it may have some effectiveness in cancer therapy. Although the mechanism of 4-HPR action is unknown, recent reports [21,22, this report] suggest that its activity in several chemoprevention trials may be the result of apoptosis induction. 4-HPR is able to induce apoptosis in fully malignant cells (Fig. 1 and Table I) that have deviated considerably from normalcy. It is tempting to speculate that this retinoid can also induce apoptosis in cells with minimal or smaller deviations, such as cells with DNA damage (Fig. 2I), initiated cells (Fig. 2II), or premalignant cells (Figs. 2III and IV). The notion that some retinoids may be effective in chemoprevention by inducing apoptosis is intriguing because, as shown in Figure 2, one could explain the inhibition of carcinogenesis by retinoids in terms of their inhibition of the proliferation of DNA-damaged cells, initiated cells, or premalignant cells. Now, induction of apoptosis can be added to the list of possible mechanisms of carcinogenesis inhibition by some retinoids. Eliminating premalignant cells by apoptosis could be more effective than suppressing proliferation because it may cause the preneoplastic lesion to disappear rather than inhibiting its growth. Furthermore, by enhancing apoptosis in slowly proliferating tissues, a cancer-preventive retinoid could be more effective than if it only inhibited cell proliferation [34]. Previous clinical trials have shown that 4-HPR is less toxic than most retinoids. This report and recent reports by others show that 4-HPR can also induce apoptosis more effectively than other retinoids; it also has the potential to be an effective agent for preventing cancer, including cervical cancer.

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