Higher Potency of N-(4-Hydroxyphenyl)retinamide than all-trans-Retinoic Acid in Induction of Apoptosis in Non-Small Cell Lung Cancer Cell Lines

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ABSTRACT

Most human non-small cell lung cancer (NSCLC) cell lines are refractory to all-trans-retinoic acid (ATRA). Recently, N-(4-hydroxyphenyl)retinamide (4HPR) was found to induce apoptosis in various tumor cells. In this study, we compared and contrasted the effects of 4HPR and ATRA on the growth and apoptosis of 10 NSCLC cell lines and normal human bronchial epithelial (NHBE) cells. All of the cancer cell lines and the NHBE cells were sensitive to 10 μM 4HPR, and their numbers decreased to <20% of the controls after a 5-day treatment, whereas ATRA decreased cell numbers to about 50% of the controls in three cell lines and was less effective in the rest of the tumor cell lines. ATRA inhibited the growth of the NHBE cells by 70–80%. 4HPR induced apoptosis in most of the cells, including the ATRA-resistant ones, as evidenced by a DNA fragmentation assay. No correlation was found between growth inhibition by 4HPR and the expression of retinoic acid receptor β (determined by Northern blotting and PCR), p53, or Bcl-2 proteins (analyzed by Western blotting). These results demonstrate that 4HPR is more potent than ATRA in inducing apoptosis in NSCLC cells and suggest that further clinical trials for prevention and therapy of NSCLC using 4HPR are warranted.

INTRODUCTION

It has been estimated that in the United States, there will be 178,100 new cases (98,300 men and 79,800 women) and 160,400 deaths (94,400 men and 66,000 women) from lung cancer in 1997 (1). These estimates place lung cancer as the leading among other cancers in both incidence (17% in men and 12% in women) and mortality (34% in men, 22% in women). Recently, the mortality from lung cancer among women has surpassed that from breast cancer. NSCLC is the predominant type (75%) of lung cancer throughout the world. The overall 5-year survival rates for lung cancer are poor (<15%) compared with most other cancers, because treatment of lung cancer is largely unsuccessful, and even early-stage NSCLCs recur in 50% of patients (2). In addition, second primary malignancies develop at an annual rate of about 4% in lung cancer patients (3).

The reasons for the refractoriness of lung cancers to treatment are not entirely clear. However, they may result, at least in part, from genetic instability and an imbalance of proliferation and apoptosis. These two characteristics are, at least in part, the result of mutations in the tumor suppressor p53 and expression of the proto-oncogene Bcl-2. p53 mutations were detected in 50–90% of lung cancers (4), whereas Bcl-2 expression was detected in about 20% of NSCLCs (5). The prognosis and survival of lung cancer patients have been linked to p53 mutations and to Bcl-2 expression (4–6). The simultaneous occurrence of p53 mutations and Bcl-2 expression may play an important role in lung cancer (7) due to the ability of wild-type p53 to mediate apoptosis (8) and Bcl-2 to prevent this process (9, 10).

These grim facts highlight the urgent need to develop new strategies for the prevention and therapy of lung cancer. One of the approaches that has been explored in recent years is the use of retinoids, a group of natural and synthetic vitamin A derivatives. The relationship between vitamin A and lung cancer is well established based on the following observations: (a) vitamin A deficiency induces squamous metaplasia in the upper aerodigestive tract (11), and this aberrant differentiation is similar to premalignant changes found in heavy smokers (12); (b) several epidemiological studies have suggested a correlation between vitamin A deficiency and increased lung cancer risk.
Induction of Apoptosis in Lung Cell Lines by 4HPR

(13, 14); (c) retinoids reversed squamous metaplasia in the trachea of vitamin A-deficient hamsters (15); (d) retinoids inhibited lung carcinogenesis in experimental animals (16); and (e) retinyl palmitate prevented the development of second primary tumors in lung cancer patients (17). The mechanism of these effects of retinoids is not fully understood; however, it may be related to their ability to regulate the growth and differentiation of tracheobronchial epithelial cells (18–20) and lung carcinoma cell lines (21–24), presumably by binding to nuclear RARs and RXRs and activating gene transcription (25–27) or repressing transcription by other factors, such as activator protein 1 (28).

Many human SCLC and NSCLC cell lines are refractory to the growth-inhibitory effects of ATRA (21–24, 29), potentially limiting the lung cancer-preventive and therapeutic effects of retinoids. Retinoid refractoriness may arise from defects in the expression of nuclear receptors, specifically RARs (21, 24, 30) or cofactors required for transcription (31, 32). Phase II clinical trials with 13-cis-RA (isotretinoin; Ref. 33) or ATRA (34) in advanced NSCLC showed no or minimal activity. These findings discouraged further studies with these natural retinoids as single agents.

Recent reports have demonstrated that the synthetic retinoid 4HPR can induce apoptosis in several human tumor cell types, including neuroblastoma, malignant hematopoietic cell lines, head and neck SCC, cervical carcinoma, and SCLC cell lines (35–41). However, the effect of 4HPR on NSCLC cell lines, which represent the major types of lung cancer, has not been examined. Therefore, in the present study, we compared and contrasted the effect of ATRA and 4HPR on cell growth in 10 NSCLC and 2 SCLC cell lines and in NHBE cells in short-term culture.

Table 1: Characteristics of lung cancer cell lines and normal bronchial epithelial cells used in this study

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Type*</th>
<th>Constitutive p53 status*</th>
<th>Induced by ATRA</th>
<th>p53 status*</th>
<th>ATRA</th>
<th>4HPR</th>
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<tr>
<td>H522</td>
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<td>+</td>
<td>Mt (LOH)</td>
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<td>76.5</td>
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<td>91.0</td>
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* AD, adenocarcinoma; AS, adenosquamous.
* Determined by Northern analysis of total RNA extracted from untreated or ATRA-treated (1 μM, 24 h) cells, except for NHBE cells, in which RT-PCR was used.

Growth inhibition (GI) was determined after a 5-day treatment with 10 μM of either retinoid.

DNA ladder formation was determined using detached cells after treatment with 10 μM of either ATRA or 4HPR. + and −, presence or absence, respectively, of a DNA ladder after 2 or 5 days of treatment.

MATERIALS AND METHODS

Cell Culture

Ten NSCLCs, including five adenocarcinomas, four SCCs, and one adenosquamous carcinoma cell line, as well as two SCLC cell lines (Table 1), were used in the study (detailed characteristics of these cell lines can be found in Refs. 6 and 42). NSCLC cell lines SK-MES-1, CALU-1, H157, and H226 were purchased from the American Type Culture Collection (Rockville, MD). NSCLC cell lines H522, H596, H1648, H1792, and H1944 (42) were kindly provided by Dr. Adi Gazdar (University of Texas Southwestern Medical Center, Dallas, TX). SCLC cell lines H69 and H146 were provided by Dr. Jack Roth (M. D. Anderson Cancer Center, Houston, TX). The NSCLC cells were grown in monolayer culture, and the SCLC cells were grown in suspension in a 1:1 (volume:volume) mixture of DMEM and Ham's F12 medium containing 5% fetal bovine serum at 37°C in the humidified atmosphere of 5% CO2-95% air. NHBE cells were cultured from an adjacent normal area of bronchi from fresh surgical specimens from patients undergoing lobectomy procedures at M. D. Anderson Cancer Center, and the cells were grown in serum-free keratinocyte growth medium (Life Technologies, Inc.) as described elsewhere (29). In some experiments, the serum-free medium was supplemented with either 5% fetal bovine serum or 0.1% BSA.

Retinoids

ATRA and 13-cis-RA were obtained from Dr. Werner Bollag (Hoffmann-La Roche, Inc., Basel, Switzerland). 4HPR was obtained from Dr. Ronald Lubet (Chemoprevention Branch, National Cancer Institute, Bethesda, MD). These retinoids were dissolved in DMSO at a concentration of 10 μM and stored under N2 atmosphere at −20°C. Aliquots from these stock...
solutions were diluted to the appropriate final retinoid concentrations (1, 5, and 10 μM) with growth medium before each experiment.

**Growth Inhibition Assay**

The cells were plated in 96-well tissue culture plates and treated with ATRA or 4HPR for 5 days. Control cultures received the same amount of DMSO (0.01 to 0.1%) as treated cultures did. Growth inhibition was determined by the crystal violet staining method described by Kuenig et al. (43). Briefly, after a 5-day treatment, cells were fixed with 5% glutaraldehyde in PBS, rinsed with distilled water, and dried. Cells were incubated with 1:1 (volume:volume) 200 mM 3-[cyclohexyloxylamino]-1-propanesulfonic acid buffer (pH 9.5) and 0.2% crystal violet at 25°C for 30 min and then washed and dried. The stain was solubilized with 10% glacial acetic acid, and the absorbance at 590 nm was determined using a microtitrator plate reader. The percentage of growth inhibition was calculated by using the equation % growth inhibition = \((1 - Nt/Nc) \times 100\), where Nt and Nc represent the absorbances of stained cells in treated and control cultures, respectively. All of the experiments were performed in triplicate, and the means ± SD were calculated.

**Apoptosis Assays**

**DNA Ladder Formation.** Cells were incubated for 2 and 5 days in medium supplemented with 10 μM ATRA, 13-cis-RA, or 4HPR. Control cultures received DMSO. The cell cultures were processed as follows: cells floating in medium were collected by centrifugation on days 2 and 5, and the cells that remained attached to the dish on day 5 were detached after a brief trypsin treatment. The cells were resuspended in Tris-EDTA buffer (pH 8.0) and lysed in 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.5% Triton X-100 on ice for 15 min. The lysates were centrifuged at 12,000 × g for 15 min to separate soluble (fragmented) from intact (genomic) DNA. Soluble DNA was treated with RNase A (50 μg/ml) at 37°C for 1 h, followed by treatment with protease K (100 μg/ml) in 0.5% SDS at 50°C for 2 h. The residual material was extracted with phenol-chloroform, precipitated in ethanol, electrophoresed in a 1.8% agarose gel, stained with a solution of 0.5% agarose, and then analyzed by flow cytometry using a FACScan flow cytometer (Epics Profile; Coulter Corp., Hialeah, FL) with a 15-mW argon laser used for excitation at 488 nm. Fluorescence was measured at 570 nm. The kit included a suspension of cells that served as negative and positive controls for apoptosis. Computer analysis of the data provided information on the percentage of apoptotic cells, as well as the proportion of cells in the hypodiploid, G1, S, and G2 phases of the cell cycle.

**Northern Blot Analysis for Detection of RARβ mRNA in NSCLC Cells**

Total cellular RNA was purified from retinoid-treated and control cells by the guanidinium thiocyanate method (46). For Northern blot analysis, samples containing 30 μg of cellular RNA were fractionated on 1.2% formaldehyde agarose gels and transferred in 10× SSC (1× SSC: 0.15 M NaCl and 0.015 M sodium citrate) to Duralon-UV membranes by capillary transfer. The pSG5 vector containing a 615-bp EcoRI fragment of RARβ (47) was obtained from Dr. Pierre Chambon (Institute de Génétique et Biologie Moleculaire, Illkirch, Strasbourg, France). The EcoRI/XbaI-cut 340-bp cDNA for GAPDH (48) was used to control for RNA loading. The probes were labeled with [32P]dCTP to a specific activity of approximately 2 × 107 cpm/μl by using random hexanucleotides as primers. The blots were prehybridized and hybridized at 37°C in Rapid-Hyb buffer (Amersham Corp., Arlington Heights, IL) and washed with 2× SSC and 0.1% SDS for 15 min at room temperature followed by 0.1× SSC and 0.1% SDS for 20 min at 60°C. Finally, the blots were dried and placed against an X-ray film (Hyper-film; Amersham) for autoradiography.

**RT-PCR for Detection of RARβ mRNA in NHBE Cells**

RT of RNA isolated from NHBE cells was performed using 1 μg of total RNA and equal volumes of a stock of RT reaction mixture: 5× first-strand buffer [1× first-strand buffer: 50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM MgCl2], 0.1 mM DTT, 1 mM deoxynucleotide triphosphate, 1 unit/μl RNase inhibitor, 2.5 μM random hexamer, and 2.5 units/μl Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD). The reaction was carried out at 42°C for 60 min. PCR was performed on the cDNA product using different primers: RARβ2 (49), 5’-CATGGTTTGGCTTTA- GCA-3’ (sense primer) and 5’-GACCCTTATACACCCCAAG-3’ (antisense primer) to produce a 329-bp oligonucleotide, and GAPDH, 5’-CGTCGGACGCGGCAACATCGCTC-3’ (sense primer) and 5’-GAAGTCACTGGGTTCATTGA-3’ (antisense primer) to produce a 344-bp oligonucleotide. The amplification reaction took place in 10 mM Tris-HCl (pH 8.4), 5 mM KCl, 2 mM MgCl2, 5 units/μl AmpliTag DNA polymerase, and 0.2 μM primers. The reaction used a Perkin-Elmer Corp. thermal cycler with the following program: an initial 3 min of incubation at 94°C, followed by 35 amplification
cycles of 1 min each at 95°C, 60°C, and 70°C. The final extension took place at 72°C for 3 min. Equal volumes of PCR product from each sample were subjected to electrophoresis on a 2% agarose gel, which was then stained with ethidium bromide and photographed under UV illumination.

### Western Blot Analysis of p53 and Bcl-2 Proteins in NSCLC and NHBE Cells

Nuclear and cytoplasmic extracts were prepared from control and 5 µM ATRA- and 4HPR-treated NSCLC cells as described earlier (50). Samples of nuclear or cytoplasmic proteins (80 µg/lane) were electrophoresed in 8% polyacrylamide gels in the presence of 0.1% SDS and transferred to nitrocellulose membranes. The membranes were incubated with mouse IgG monoclonal antibody against p53 (which recognize both wild-type and mutant p53) or against Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA) and washed before incubating them with a peroxidase-conjugated antimouse antibody (Amersham). Immunoreactive bands were detected using an enhanced chemiluminescence reagent (Amersham). The blots were stripped and then incubated with mouse anti-β-actin antibody (Sigma Chemical Co., St. Louis, MO), followed by a second antibody for the assessment of loading of protein in different lanes.

### RESULTS

**Inhibition of the Growth of NSCLC and SCLC cells by ATRA and 4HPR.** Cells were grown in the absence or presence of 1 or 10 µM ATRA or 4HPR for 5 days, and then growth inhibition was assessed. ATRA was ineffective in growth inhibition when used at 1 µM, and even at 10 µM this natural retinoid exhibited limited efficacy against most cell lines. Exceptions were NSCLC cell lines H226 and A549 and SCLC cell line H69, which were inhibited by 40–60% after a 5-day treatment (Fig. 1). In contrast, 4HPR (1 µM) inhibited the growth of the H522 and A549 cell lines by 50% and at 10 µM induced more than 90% inhibition of the growth of most of the NSCLC cell lines (Fig. 1). The two SCLC cell lines (H69 and H146), which were reported previously to be sensitive to 4HPR (41), were somewhat less sensitive than most NSCLC cell lines (Fig. 1). The response of the NSCLC to 4HPR appeared to be independent of p53 status, because lung cancer cell lines with either wild-type or mutant p53 were affected similarly by 4HPR (Table 1).

NSCLC cells treated with 10 µM 4HPR for 3 days changed their morphology, and some became rounded. Eventually, many cells detached from the dishes (data not shown). ATRA failed to induce similar changes in the morphology of the cells. There were no changes in the morphology of the cells after a 3-day treatment with 1 µM of either retinoid (data not shown).

**Effect of ATRA and 4HPR on the Growth of NHBE Cells.** NHBE cells were growth inhibited by both ATRA and 4HPR; however, they were more sensitive to the latter retinoid (Fig. 1). These cells were more sensitive to 1 µM 4HPR when they were grown in serum-free medium supplemented with BSA than in medium supplemented with serum. NHBE cells were more sensitive to 10 µM ATRA than any of the lung carcinoma cell lines were (Fig. 1), in agreement with our previous report (29). NHBE cells exposed to 4HPR exhibited morphological changes that included extension of cell processes by some cells and rounding of other cells (data not shown).

Induction of Apoptosis by ATRA and 4HPR in Lung Cancer Cells. The effects of retinoids on induction of apoptosis in the lung carcinoma cells were examined using several methods. DNA fragmentation leading to the formation of a DNA ladder in agarose gels was observed in 5 of the 10 4HPR-treated NSCLC cell lines (H522, H1648, H1792, A549, and CALU-1; Table 1 and Fig. 2). In most of these cells (H1792, CALU-1, H549, and H522), soluble DNA was detected after 2 days of 4HPR treatment but not after a similar treatment with ATRA. After 5 days, a DNA ladder was also observed in some cultures treated with either ATRA or 13-cis-RA. Attached H1792 cells exhibited a more extensive ladder formation after 4HPR treatment than after treatment with the other retinoids (Fig. 2). No DNA ladders were observed in the two SCLC cell lines (data not shown).

 Examination of PI-stained nuclei under the fluorescence microscope revealed much smaller nuclei in 4HPR-treated H1944 adenocarcinoma cells than in either control or ATRA-treated cells, presumably due to extensive nuclear condensation (Fig. 3) that can occur during apoptosis. This effect was much less pronounced in other 4HPR-treated NSCLC cells (data not shown).

DNA content and apoptosis in all of the lung cancer cell lines were analyzed by flow cytometry of cells stained with PI and the TUNEL method, respectively. The PI-stained cells showed a population of cells with hypodiploid DNA content that increased 2–8-fold in five of the eight 4HPR-treated cell lines.
Effects of ATRA and 4HPR on DNA fragmentation. Cells were treated with DMSO (Control) or 10 μM ATRA, 13-cis-RA, or 4HPR. Floating cells were collected after 2 and 5 days, and the cells that remained attached after 5 days were also collected. Soluble DNA was extracted from the floating and attached cell populations and subjected to electrophoresis in a 2% agarose gel. The gel was stained with ethidium bromide and photographed under UV illumination.

Fig. 2

shown in Table 2 and Fig. 4, left columns. Retinoid treatment induced no consistent changes in DNA content distribution in NSCLC cell lines. Furthermore, the cell cycle population changes induced by 4HPR differed among these cell lines.

Apoptosis was detected by the TUNEL assay in most of the NSCLC cell lines after 4HPR treatment, and examples of the most and least sensitive cells are shown in Fig. 4, A and B, respectively (Fig. 4, middle and right columns). The H522 cells were analyzed 1 day after 4HPR treatment, because a 5-day treatment resulted in lysis of all of the cells. Also, cells were treated with 1 or 5 but not 10 μM 4HPR to prevent extensive apoptosis, which would result in too few cells for analysis by flow cytometry. Treatment of H522 adenocarcinoma cells, which express a mutant p53, with 5 μM ATRA for 1 day increased apoptotic cells from 12.6% in control to 29.2%. In contrast, a similar treatment with 4HPR resulted in apoptosis of 94.2% of the cells (Fig. 4A). The H1944 adenocarcinoma cells, which express a wild-type p53, were less affected by both retinoids; they were resistant to ATRA and showed 31.7 and 47.6% apoptosis after treatment with 4HPR for 3 and 5 days, compared with 16.6% apoptosis in untreated control cells cultured for 5 days (Fig. 4B, middle and right columns; Table 2). The SCLC H146 cells were also resistant to ATRA but were sensitive to 4HPR. These cells were less sensitive to 4HPR than H522 cells in that no increases in apoptotic cells were observed after a 3-day treatment, and a longer treatment (5 days) was required to increase apoptosis to 85%, a level that was achieved after 1 day of treatment of the H522 cells (Fig. 4C). However, the H146 cells showed an increase in apoptotic cells after a 5-day treatment with 1 μM 4HPR, whereas the NSCLC cells were not affected by the lower 4HPR concentration. A previous report demonstrated that H146 cells were induced to undergo extensive apoptosis after a 7-day treatment with 1 μM 4HPR (41). Four of the five adenocarcinoma cell lines were induced to undergo apoptosis by 4HPR as detected by the DNA ladder or the TUNEL method, whereas only one of the four SCC cell lines has undergone apoptosis (Tables 1 and 2).

Induction of Apoptosis by ATRA and 4HPR in NHBE Cells. To compare the response of NHBE cells and lung carcinoma cells to ATRA and 4HPR, the normal cells, which usually are maintained in serum-free medium, were cultured in medium supplemented with 5% FCS. The NHBE cells showed an increase in apoptotic cells from 2.3% in controls to 38.2% in cultures treated with 5 μM 4HPR for 3 days (Fig. 4D). This response was lower than that of the H522 cells but higher than that of the H1944 cells.
Induction of Apoptosis in Lung Cell Lines by 4HPR

Treatment with ATRA increased RARα mRNA levels in H157 and failed to do so (Fig. 5A). Because the H522 cells were extremely sensitive: no cells were left after >2 days of treatment with 4HPR, and the control NHBE cells grown in the presence of 5% serum were growth inhibited after >3 days in culture, even in the absence of retinoids.

We examined the expression and modulation of p53 and Bcl-2 protein levels in NSCLC cells. Table 1 shows that ATRA treatment of NSCLC cell lines has demonstrated that ATRA can modulate p53 (56–59) and Bcl-2 (60–63) levels at the mRNA and protein levels. ATRA was able to increase RARα, as found previously (29), 4HPR failed to increase RARα mRNA levels in these or other cells and decreased the mRNA level in the H1944 cells (Fig. 5A). As reported previously (29), the level of RARα mRNA in NHBE cells was below detection by Northern blotting of total RNA; therefore, we used RT-PCR and found that, whereas ATRA was able to increase RARα, as found previously (29), 4HPR failed to do so (Fig. 5B). RARα, RARγ, and RXRα were detected by Northern blotting in all of the cell lines, albeit at varying levels, and their levels were not modulated by either ATRA or 4HPR (data not shown).

Expression and Induction of RARβ in NSCLC cells. Some reports linked RARβ expression with increased growth inhibition and apoptosis after ATRA treatment of breast carcinoma cells (51–55). Therefore, we analyzed RARβ expression by Northern blotting in the 10 NSCLC cell lines grown in the absence or presence of 1 μM ATRA for 24 h. Table 1 shows that RARβ mRNA was detected in three of the cell lines (H522, H1792, and H1944). Analysis of other NSCLC cell lines revealed basal and ATRA-induced RARβ expression that was similar to that in previous reports (data not shown; Refs. 21–24).

Treatment with ATRA increased RARβ mRNA levels in H157 cells (Fig. 5A). However, 4HPR failed to increase RARβ mRNA levels in these or other cells and decreased the mRNA level in the H1944 cells (Fig. 5A).

As reported previously (29), the level of RARβ mRNA in NHBE cells was below detection by Northern blotting of total RNA; therefore, we used RT-PCR and found that, whereas ATRA was able to increase RARβ, as found previously (29), 4HPR failed to do so (Fig. 5B). RARα, RARγ, and RXRα were detected by Northern blotting in all of the cell lines, albeit at varying levels, and their levels were not modulated by either ATRA or 4HPR (data not shown).

Expression and Modulation of p53 and Bcl-2 Protein Levels in NHBE Cells and NSCLC Cells. We examined the effects of ATRA and 4HPR on the levels of the p53 and Bcl-2 proteins, which are related to apoptosis, in the NSCLC and NHBE cells, because previous studies with various tumor cell lines have demonstrated that ATRA can modulate p53 (56–59) and Bcl-2 (60–63) levels at the mRNA and protein levels.

The p53 protein was detected in cells reported to have a wild-type p53 (H1944 and A549) but in only one (H596) of the eight NSCLC cell lines reported to have a mutant p53 (Fig. 6; Table 1). The lack of detectable p53 protein in many of the NSCLC cells is due to the presence of stop codon and frameshift mutations in their p53 gene. ATRA decreased p53 levels in A549 and H596, which express wild-type and mutated p53, respectively, whereas 4HPR, which failed to do so (Fig. 6). Both retinoids increased p53 levels (presumably wild type) in NHBE cells.

Bcl-2 was not detected in NHBE cells but was detected in all of the NSCLC cells. A549 expressed a lower Bcl-2 level than the other cell lines (Fig. 6). ATRA decreased the levels of Bcl-2 protein in H596, H1944, and A549, whereas 4HPR decreased Bcl-2 in A549, H226, H1648, and SK-MES-1. There appears to be no clear relationship between the ability of 4HPR or ATRA to modulate p53 or Bcl-2 and their ability to induce apoptosis. For example, neither retinoid affected Bcl-2 in the most sensitive H522 cells.

**DISCUSSION**

Programmed cell death is a physiological mechanism by which organisms eliminate cells during embryonic development and in the adult to counterbalance cell division for homeostatic regulation of tissue mass. The term “apoptosis” was coined by Kerr et al. (64) to describe this process, which is characterized by cell shrinkage, chromatin condensation, nuclear segmentation, and internucleosomal degradation of DNA. Apoptosis can be induced by a variety of external and intracellular signals, including those that induce terminal differentiation or DNA damage. This induction may constitute a protective antineoplas-

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**Table 2** Effects of ATRA and 4HPR on DNA content distribution and apoptosis in lung carcinoma cell lines and normal bronchial epithelial cells

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<th>Treatment (μM)</th>
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<td>16.0</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>ATRA (5)</td>
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<td>21.2</td>
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<td>4HPR (5)</td>
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<td>66.6</td>
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<tr>
<td>ATRA (1)</td>
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<td>68.5</td>
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<td>Control</td>
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<td>32.4</td>
</tr>
<tr>
<td>4HPR (5)</td>
<td>3.1</td>
<td>68.5</td>
</tr>
</tbody>
</table>

Cell lines: SK-MES-1: small cell lung carcinoma (SCLC); H146: SCLC; H522, H157, H1648, H1944: NSCLC cell lines; NHBE: normal bronchial epithelial cells.

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"a Cells were treated with either ATRA or 4HPR at 1 or 5 μM for 5 days, except for H522 and NHBE cells that were treated for 1 and 3 days, respectively, because the H522 cells were extremely sensitive: no cells were left after >2 days of treatment with 4HPR, and the control NHBE cells grown in the presence of 5% serum were growth inhibited after >3 days in culture, even in the absence of retinoids.

"b Determined by flow cytometry of propidium iodide-stained cell nuclei. HD, hypodiploid; G1, diploid; G2, tetraploid; S, DNA synthesizing fraction.

"c Determined by the TUNEL assay.
Fig. 4  Effects of ATRA and 4HPR on cell cycle and apoptosis in malignant and normal lung cells. Cells were treated with the indicated retinoids at the concentrations and duration indicated. The cells were then stained with PI for DNA content analysis and with fluorescein-labeled dUTP to label DNA fragments by the TUNEL method as described in “Materials and Methods.” **Left columns,** data on DNA content distribution; **middle columns,** fluorescence of cells labeled by the TUNEL method; **right columns,** fluorescence of viable and apoptotic cells (represented by dots below and above, respectively, a demarcation line determined by a standard cell line provided with the labeling kit). **A,** H522 cells; **B,** H1944 cells; **C,** H146 cells; **D,** NHBE cells; **WT,** wild type; **MU,** mutant.

tic mechanism to eliminate DNA-damaged cells, the replication of which may fix mutations and result in malignant transformation (65). Carcinogenesis is often associated with a decreased tendency to undergo apoptosis in response to certain physiological stimuli and cytotoxic agents. Therefore, agents that can induce apoptosis or restore the ability to undergo apoptosis in premalignant and malignant cells are expected to be effective in cancer prevention and treatment (65, 66). Recently, several reports have demonstrated that certain retinoids, in addition to exerting cytostatic effects on tumor cells in vitro (67), also induce apoptosis in various cell types during normal development and in cultured untransformed and tumor cells (35–41, 60–63, 68).

In this report, we describe for the first time the ability of both ATRA and 4HPR to induce apoptosis in NSCLC cell lines. Although this result is not surprising in view of the previous reports by others and our own group that 4HPR could induce apoptosis in a variety of tumor cell types, the findings have a potential clinical importance because of the magnitude of the lung cancer problem and the fact that NSCLCs constitute the majority of lung cancers. 4HPR was more effective than ATRA in growth inhibition, especially at 10 μM. Although this con-
Induction of Apoptosis in Lung Cell Lines by 4HPR

Concentration is about 10-fold higher than the peak plasma level in patients receiving 200 mg of 4HPR daily, it may be relevant in vivo, because some tissues can concentrate the retinoid, as evidenced by the presence of >10 μM 4HPR in the nipple discharge of a woman who had taken 200 mg/day oral 4HPR for 15 days (69). It is noteworthy that dose-escalation studies with 4HPR in cancer patients (both adults and children) are currently underway to attempt to increase the blood level of 4HPR.

The induction of apoptosis by 4HPR was detected by DNA ladder formation earlier (2 days) than by ATRA (5 days). In addition, some cell lines (e.g., CALU-1 and H522) were sensitive to 4HPR-induced but resistant to ATRA-induced apoptosis. NHBE cells were more sensitive to 10 μM ATRA than NSCLC and SCLC cell lines, whereas the sensitivity of NHBE and NSCLC cell lines to 4HPR was similar. Thus, it appears that, whereas progression in lung carcinogenesis is associated with decreased sensitivity to ATRA, there is no decrease in sensitivity to 4HPR. The sensitivity of NHBE cells to growth inhibition may be much greater in vitro than in vivo, because the serum-free growth medium includes mitogenic substances (e.g., bovine pituitary extract), and serum-containing medium includes various growth factors. This may increase the proportion of proliferating cells in vitro relative to in vivo and a corresponding increase in apoptosis. Notwithstanding this assumption, our results indicate that NHBE cells exhibit less apoptosis by the TUNEL assay than lung adenocarcinoma but more than SCCs. Among the NSCLC cell lines, adenocarcinomas and SCCs showed similar growth inhibition by ATRA and 4HPR; however, there appear to be differences in the mechanisms leading to this inhibition. Specifically, DNA ladder formation and degree of apoptosis determined by the TUNEL assay were detected in most adenocarcinoma cell lines, but only one of the 4HPR-treated SCC cell lines showed a DNA ladder. Among the various apoptosis assays that we have used, the TUNEL assay appeared to be the most sensitive one, followed by analysis of DNA ladder formation. However, this applied primarily to the adenocarcinoma cells, because DNA content distribution analysis indicated that the hypodiploid population increased in SCC cell lines even more than in adenocarcinoma cell lines, suggesting that some of the SCC cell lines may undergo DNA degradation by a mechanism that does not involve the typical internucleosomal cleavage detected by the DNA ladder and TUNEL assays. It is also possible that SCCs undergo necrosis rather than apoptosis in the presence of 4HPR.

Although the mechanism underlying the difference in the responses of adenocarcinomas and SCCs is unclear, it does not appear to be related to either the p53 or the Bcl-2 status. Likewise, there were no consistent changes in cell cycle distribution of treated cells, suggesting no relationship between cell cycle modulation and apoptosis by 4HPR.

Previous reports have suggested that RARβ expression may be associated with apoptosis (51, 53, 68). ATRA-induced apoptosis in mouse limb bud core mesenchymal cells was linked to RARβ induction (68), and transfection of RARβ into cultured breast carcinoma cell lines (51, 53) enhanced apoptosis. RARβ expression was also associated with lung carcinogenesis. Spe-
specifically, suppression of RARβ mRNA levels was found in >50% of NSCLC in vivo (30) and in the majority of NSCLC and SCLC cell lines (Table 1; Refs. 20–24). Mice expressing antisense RARβ transgene showed spontaneous lung carcinomas (70), and transfection of RARβ into CALU-1 lung SCC cells suppressed their tumorigenicity in nude mice (71). Because RARβ2 promoter contains a RA response element, the expression of this receptor can be up-regulated in many cell types by ATRA (72–74). However, most lung cancer cell lines do not show an increase in RARβ level after ATRA treatment (20–24), presumably due to aberrations in the expression or function of cofactors required for RARβ2 gene transcription (31, 32). Among the 10 NSCLC cell lines that we have studied, only one (H157) showed RARβ induction by ATRA. NHBE cells also exhibited RARβ induction by ATRA, as reported previously (29). In contrast, 4HPR failed to induce RARβ in any of our tumor cell lines, even when the cells were treated with 10 μM 4HPR for up to 2 days (data not shown). This result is similar to previous reports on the failure of 4HPR to induce RARβ in mammary tumor cell lines (73). 4HPR did not induce RARβ in NHBE cells, as opposed to induction of RARβ during senescence of normal breast epithelial cell (73). Apparently, the effects of 4HPR on the growth and apoptosis of NSCLC and NHBE cells are independent of RARβ constitutive expression and induction.

To begin to understand the mechanisms by which ATRA and 4HPR induce apoptosis, we analyzed their effects on the levels of two genes that have been implicated either in mediating apoptosis (p53) or in protecting against apoptosis (Bcl-2). The level of wild-type p53 increases after exposure of certain cells to growth factors or to agents that cause DNA damage, and this may lead to both cell growth arrest and apoptosis (74, 75). Conversely, deficiency of wild-type p53 may lead to resistance to induction of apoptosis (76). ATRA was reported to decrease the amount of p53 mRNA and protein in F9 embryonal carcinoma cells (56, 58) and in neuroblastoma cells (57). Nonetheless, ATRA increased the transcriptional activity of p53 in F9 cells (58). No changes in p53 levels have been found in ATRA-treated P19 embryonal carcinoma cells (62) or in 4HPR-treated leukemia cells (38) or SCLC cells (41). In contrast, ATRA increased the stability of wild-type p53 in certain NSCLC cell lines (59). We found that ATRA increased p53 protein levels in NHBE cells and decreased p53 levels in H966 and H549 cell lines. However, the induction of apoptosis by 4HPR in NSCLC cell lines did not seem to be related to the status or modulation of p53.

ATRA decreased Bcl-2 levels in certain HL-60 myeloid leukemia cell lines (60, 61) and P19 embryonal carcinoma cells (62) and induced apoptosis in these cells. Overexpression of Bcl-2 inhibited ATRA-induced apoptosis of embryonal carcinoma cells (62) and delayed the induction of apoptosis by 4HPR (38); however, no changes in endogenous Bcl-2 have been found in either malignant hematopoietic cells (38) or SCLC cells (41) treated with 4HPR. Recently, 4HPR was reported to increase Bcl-2 in HL-60 leukemia cells (63). We found that ATRA and 4HPR decreased the level of Bcl-2 protein in three NSCLC cell lines; however, there was no correlation between this effect and the ability of the retinoids to induce apoptosis in the corresponding cells, because apoptosis was induced even in cells in which the Bcl-2 level was unaffected by the retinoids.

The mechanism by which 4HPR induces apoptosis in NSCLC cells remains to be elucidated. It appears to be independent of p53, Bcl-2, RARβ, and the retinoid receptor activation pathway. Recently, we have demonstrated that 4HPR can induce reactive oxygen species in cervical carcinoma cells (77). Preliminary studies indicate that this mechanism is responsible for the induction of apoptosis in only one of five of the NSCLC cell lines; thus, other mechanisms may operate in the majority of NSCLC cell lines. 4HPR has been found to be an effective chemopreventive agent in experimental animals (16, 78, 79) and has exhibited some efficacy in clinical trials of breast cancer prevention (77, 78). Eliminating tumor cells by inducing apoptosis is a promising approach to cancer therapy (66). Therefore, our demonstration that 4HPR can induce apoptosis in NHBE and NSCLC cells indicates that it may have some effectiveness in cancer prevention and therapy. Clinical trials with 4HPR (e.g., with bronchial metaplasia patients at M. D. Anderson Cancer Center) are in progress to test this hypothesis.

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