Involvement of Mitochondrial and Akt Signaling Pathways in Augmented Apoptosis Induced by a Combination of Low Doses of Celecoxib and *N*-(4-Hydroxyphenyl) Retinamide in Premalignant Human Bronchial Epithelial Cells

Claudia P. Schroeder, Humam Kadara, Dafna Lotan, Jong K. Woo, Ho-Young Lee, Waun Ki Hong, and Reuben Lotan

Department of Thoracic, Head and Neck Medical Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas

Abstract

Celecoxib is being evaluated as a chemopreventive agent. However, its mechanism of action is not clear because high doses were used for *in vitro* studies to obtain antitumor effects. We found that celecoxib inhibited the growth of premalignant and malignant human bronchial epithelial cells with IC_{50} values between 8.9 and 32.7 μmol/L, irrespective of cyclooxygenase-2 (COX-2) expression. Normal human bronchial epithelial cells were less sensitive to celecoxib. Because these concentrations were higher than those attainable *in vivo* (≤5.6 μmol/L), we surmised that combining celecoxib with the synthetic retinoid *N*-(4-hydroxyphenyl) retinamide (4HPR) might improve its efficacy. Treatment of premalignant lung cell lines with combinations of clinically relevant concentrations of celecoxib (≤5 μmol/L) and 4HPR (≤0.25 μmol/L) resulted in greater growth inhibition, apoptosis induction, and suppression of colony formation than did either agent alone. This combination also decreased the levels of Bcl-2, induced the release of mitochondrial cytochrome *c*, activated caspase-9 and caspase-3, and induced cleavage of poly(ADP-ribose)polymerase at concentrations at which each agent alone showed no or minimal effects. Furthermore, combinations of celecoxib and 4HPR suppressed the phosphorylation levels of serine/threonine kinase Akt and its substrate glycogen synthase kinase-3β more effectively than the single agents did. Accordingly, overexpression of constitutively active Akt protected bronchial epithelial cells from undergoing apoptosis after incubation with both celecoxib and 4HPR. These findings indicate that activation of the mitochondrial apoptosis pathway and suppression of the Akt survival pathway mediate the augmented apoptosis and suggest that this combination may be useful for lung cancer chemoprevention. (Cancer Res 2006; 66(19): 9762-70)

Introduction

Lung cancer is the leading cause of cancer-related death worldwide (1). The overall survival rate is poor and has not changed appreciably for several decades despite the introduction of novel agents and combined treatment modalities using surgery, radiotherapy, and chemotherapy. Therefore, new strategies are needed for intervention at early stages of lung carcinogenesis before malignant tumors become clinically evident. One of the promising approaches to accomplishing this goal is cancer chemoprevention (2, 3). In fact, high-risk populations for developing lung cancer, including former and current smokers, represent suitable candidates for chemoprevention trials (4). However, randomized controlled trials using dietary supplementation to prevent lung cancer in smokers have shown rather disappointing results (5) and have highlighted the necessity to find novel agents and combination strategies.

Nonsteroidal, anti-inflammatory drugs have been observed to reduce the relative risk for tobacco-induced lung carcinogenesis in both preclinical and clinical studies (4, 6). The anti-inflammatory action of these drugs is mediated through their inhibitory effect on cyclooxygenases (COX), which are essential enzymes for the synthesis of prostaglandins generated from arachidonic acid (7). In fact, COX-2 isoenzyme is frequently up-regulated in neoplastic tissue of the lung and seems to be associated with a poor prognosis among patients with non–small cell lung cancer, implicating a role in carcinogenesis (8, 9). Celecoxib, the first selective COX-2 inhibitor approved for chemoprevention of colon cancer in patients with familial adenomatous polyposis (10), has also been found to decrease the incidence of esophageal cancer in humans (11), and colon (12), gastric (13), lung (14, 15), mammary (16), oral (17), prostate (18), urinary bladder (19), and skin (20) cancer in various animal models with no associated toxicity. Moreover, a number of studies have shown that celecoxib at clinically feasible concentrations (≤5.6 μmol/L) markedly suppresses the biosynthesis of PGE_2 in COX-2–expressing lung cancer cells (21, 22). However, the fact that much higher doses of celecoxib (≥25 μmol/L) are required for growth inhibition and apoptosis induction in cell culture systems suggests a mode of action independent of COX-2–inhibitory activity and raises questions about the clinical relevance of *in vitro* findings (23). Additionally, data have shown that celecoxib was similarly effective in distinct types of cancer cells that were negative for COX-2 expression (24, 25). In short, the precise molecular mechanisms underlying the antitumor effects of celecoxib is not fully understood.

A strong rationale exists for the use of combinations of agents that act in an additive or synergistic manner by increasing treatment efficacy and/or decreasing drug toxicity (5). We previously showed that *N*-(4-hydroxyphenyl) retinamide (4HPR; fenretinide), a synthetic derivative of retinoic acid, exerts potent proapoptotic effects on a variety of cancer cells (26, 27). Moreover, 4HPR

Note: W.K. Hong is an American Cancer Society Clinical Research Professor. R. Lotan is the incumbent of the Irving and Nadine Mansfield and Robert David Levitt Cancer Research Chair.

Requests for reprints: Reuben Lotan, Department of Thoracic, Head and Neck Medical Oncology, The University of Texas M.D. Anderson Cancer Center; 1515 Holcombe Boulevard, P.O. Box 432, Houston, TX 77030. Phone: 713-792-8467; Fax: 713-745-5656; E-mail: rlotan@mdanderson.org.

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combined with celecoxib inhibited growth and induced apoptosis of non–small cell lung cancer cell lines more efficiently than either agent alone did (25), suggesting further investigations for the treatment of human lung cancer. Supported by findings of preclinical and clinical cancer chemoprevention trials, which have indicated great promise for 4HPR and celecoxib administered as single agents (28, 29), we asked whether the combination of both would enhance their individual effects in an *in vitro* model of tobacco-induced human lung carcinogenesis. We found that celecoxib combined with 4HPR at clinically attainable concentrations inhibited growth and induced apoptosis of premalignant and tumorigenic bronchial epithelial cell lines by activating the mitochondrial apoptosis pathway as well as suppressing the Akt survival pathway.

Materials and Methods

Reagents. Celecoxib (4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-] benzene-sulfonamide) was obtained from GD Searle & Co (Chicago, IL), and 4HPR was kindly provided by Dr. James Zweibel. The stock solutions of celecoxib (0.05 mol/L) and 4HPR (0.01 mol/L), both in DMSO, were stored at −80°C and diluted to the desired concentrations with culture medium before use. Bovine serum albumin, DMSO, EDTA, and SDS were purchased from Sigma Chemical Co. (St. Louis, MO). PBS and trypsin were from Life Technologies Invitrogen Corporation (Carlsbad, CA). All culture plasticwares were obtained from BD Bioscience Labware (Bedford, MA).

***In vitro* model of human lung carcinogenesis.** The cell lines used in this study represent an *in vitro* model of human lung carcinogenesis. BEAS-2B is a human bronchial epithelial cell line immortalized using an adenovirus 12-SV40 hybrid virus. The transformed 1198 and the tumorigenic 1170-I cell lines were derived from BEAS-2B by exposure to cigarette smoke condensate *in vivo* after transplantation into nude mice (30). The immortalized 1799 cell line was derived from BEAS-2B cells by *in vivo* transplantation without exposure to cigarette smoke condensate (31). These cell lines were obtained from Dr. Klein-Szanto (Fox Chase Cancer Center, Philadelphia, PA). Normal human bronchial epithelial (NHBE) cells were purchased from Clonetics (San Diego, CA) and used at the second passage only. The non–small cell lung cancer cell line A549 (American Type Cell Culture Collection, Rockville, MD) was included as a positive control for COX-2 expression (25). BEAS-2B and 1799 cells were grown in keratinocyte serum-free medium (K-SFM) containing human recombinant epidermal growth factor (2.5 μg) and bovine pituitary extract (25 mg; Life Technologies Invitrogen Corporation) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The cell lines 1198 and 1170-I were maintained in K-SFM supplemented with 3% fetal bovine serum (FBS) from HyClone Laboratories, Inc. (Logan, UT).

Cell growth studies. The cell lines cultured in the medium described above were seeded into 96-well culture plates (6×10^3 or 2×10^4 per well for confluent NHBE culture) in K-SFM with and without 3% FBS, allowed to adhere overnight at 37°C, followed by treatment with celecoxib, 4HPR, and their combinations for 3 days. Control cultures were incubated with DMSO alone. An automated plate reader (model MR5000, Dynatech Laboratories Inc., Chantilly, VA) was used to estimate cell numbers using the sulforhodamine B assay (32). The inhibition of cell growth was calculated as $(1 - A_t / A_c) \times 100\%$, where A_t and A_c represent absorbencies of treated and control cultures, respectively. Concentration response curves were plotted, and IC_{50} concentrations of celecoxib were calculated by interpolation after 3 days in the presence or absence of 3% FBS.

Colony formation studies. Exponentially growing cells were seeded into six-well culture plates (0.8×10^5/well) overnight before treatment with celecoxib, 4HPR, and their combinations. The medium was removed and replaced with fresh medium containing these agents every 3 days. After 14 days of incubation, the cells were fixed with methanol/acetic acid (3:1, v/v) and stained with crystal violet in methanol (0.5%, v/v) to visualize the colonies. For quantification, each well was divided into eight fields, and the number of colonies ≥1 mm was estimated with a colony counter (Fisher Scientific, Pittsburgh, PA).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Fragmentation of intranucleosomal DNA was evaluated using an apoptosis *in situ* detection kit (Apo-Direct, Phoenix Flow Systems, Inc., San Diego, CA) based on the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) technique (33). After treatment, both adherent and nonadherent cells were harvested by trypsinization, pelleted by centrifugation, fixed with ice-cold ethanol (70%, v/v), and stained according to the protocol of the manufacturer. Fluorimetric measurement and data analysis were done on a Coulter XL flow cytometer (Miami, FL). The percentage of cells that were apoptotic was determined from the proportion of fluorescein-isothiocyanate–positive cells within 10,000 cells analyzed. Two independent experiments were done.

Western blot analysis. Samples containing 50 μg of total cellular protein mixed in sample buffer [0.5 mol/L Tris (pH 6.8), 0.3% glycerol, 0.03% β-mercaptoethanol, 10% SDS, and 0.001% bromphenol blue] were electrophoretically separated through 8% to 12% SDS-polyacrylamide slab gels, followed by transfer onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Briefly, cell monolayers were washed twice with ice-cold PBS and collected in lysis buffer containing 150 mmol/L NaCl, 0.02% NaN_3, 2% Igepal CA-630, 0.5% sodium deoxycholate, 0.2% SDS, and 50 mmol/L Tris-HCl (pH 8.0) supplemented with the protease inhibitors leupeptin (1 μg/mL), aprotinin (1 μg/mL), pepstatin (0.5 μg/mL), and phenylmethylsulfonyl fluoride (100 μg/mL). Protein concentrations were measured using the Bio-Rad protein assay (Bio-Rad Laboratories). After blocking with 3% nonfat dry milk solution in 0.1% (w/v) Tween 20 in PBS, the membranes were probed with antihuman antibodies at appropriate dilutions against COX-2 (Oxford Biomedical Research, Inc., Oxford, MI); caspase-3 (clone 31A1067, Imgenex, San Diego, CA); caspase-8, Bcl-2, Bcl-x_L, Bcl-x_S, and Bax (all from Santa Cruz Biotechnology, Santa Cruz, CA); poly(ADP-ribose) polymerase (PARP); caspase-9; hemagglutinin tag for the recombinant protein (HA-tag 262K); and antibodies included in the phosphorylated Akt pathway sampler kit (all from Cell Signaling Technology, Inc., Charlottesville, VA). Antibody binding was detected with horseradish peroxidase–linked secondary antibody and enhanced chemiluminescence (Amersham Biosciences Corp., Piscataway, NJ). Loading and transferring control was confirmed by probing the membranes with anti-β-actin antibody or staining with Ponceau S solution (Sigma Chemical). Mitochondria and cytosol fractionation was done according to a cytochrome *c* releasing apoptosis assay kit (BioVision, Inc., Mountain View, CA).

Adenoviral vector generation. An adenoviral vector expressing constitutively active Akt (MyrAkt), referred to here as Ad-MyrAkt, and an adenoviral vector expressing empty vector, referred to here as Ad-EV, were amplified as described previously (34). Briefly, 1799 transformed cells were infected at 5×10^3 multiplicity of infection (MOI) with Ad-MyrAkt and Ad-EV, respectively. After 2 hours, the medium was replaced with fresh medium containing celecoxib and 4HPR followed by 2 days of incubation. The adenoviral vector expressing a full-length human Akt1 with the Src myristoylation signal fused in-frame to the c-Akt coding sequence with HA under the control of the cytomegalovirus promoter (Ad5CMV-MyrAkt-HA) was constructed using the pAd-shuttle vector system (35, 36). The presence of MyrAkt-HA was confirmed by Western blot analysis of Akt and HA expression. The activity of Ad5CMV-MyrAkt-HA was assessed by detection of cleavage of PARP (Cell Signaling Technology), activation of caspase-3 (Imgenex), and expression of Bcl-2 (Cell Signaling Technology) using immunoblotting.

Statistical analysis. Growth studies and the number of colonies were analyzed for statistical significance using Student's two-tailed *t* test with $P \leq 0.05$ and $P \leq 0.005$, respectively. The results represent mean values ± SD of three independent experiments, each done in quadruplicate.

Results

Celecoxib inhibits growth of human premalignant and malignant lung cell lines independent of COX-2. To determine whether the growth-inhibitory effects of celecoxib in an *in vitro*

model of human lung carcinogenesis were associated with the expression of the presumed target enzyme, we analyzed the constitutive COX-2 levels of cells. COX-2 was expressed at a very low level in NHBE cells; was not detected in BEAS-2B, 1799, 1198, or 1170-I cells; and was not induced in 1198 or 1170-I cell lines whether they were cultured in the presence or absence of 3% FBS (Fig. 1*A*). However, the robust expression of COX-2 in A549 lung cancer cells, which were included as a positive control for COX-2, increased by ~20% when grown in the absence of FBS. Furthermore, the constitutive levels of PGE_2 in BEAS-2B, 1198, and A549 cells were 0.2, 0.04, and 2.7 ng/10^6 cells, respectively, as determined by electrospray ionization liquid chromatography tandem mass spectrometry (24), correlated with their differential COX-2 expression. As shown in Fig. 1*B*, celecoxib inhibited the growth of premalignant (BEAS-2B, 1799, and 1198) and tumorigenic 1170-I cell lines with IC_{50} values ranging from 8.9 to 10.1 μmol/L in K-SFM and from 15.9 to 18.7 μmol/L in 3% FBS-containing K-SFM. A549 lung cancer cells were ~2-fold less sensitive to celecoxib than premalignant cell lines irrespective of the presence or absence of serum. NHBE cells, which were seeded at subconfluent density to allow them to proliferate, exhibited a lower sensitivity to celecoxib characterized by IC_{50} values of 23.2 and 62 μmol/L in the absence and presence of 3% FBS, respectively. Confluent cultures of NHBE cells, which better mimic the nonproliferative state *in vivo*, were less sensitive to celecoxib with IC_{50} values of 29.2 and 80 μmol/L in serum-free and serum-containing medium, respectively. Thus, the growth-inhibitory activity of celecoxib seemed to be independent of COX-2 expression and activity. In addition, COX-2 was not modulated in 1198 cells cultured in standard medium or medium supplemented with celecoxib (2.5 and 5 μmol/L), 4HPR (0.25 μmol/L), or both, as shown by immunoblotting (Fig. 1*C*).

Celecoxib plus 4HPR is more potent than either agent alone in inhibiting growth of premalignant and tumorigenic lung cell lines. The effects of low doses of celecoxib in combination with the proapoptotic retinoid 4HPR on cell growth were studied because the IC_{50} values determined in the previous experiment were at least 3-fold higher than peak plasma levels obtained in patients treated with celecoxib (23) and because our recent studies on various non–small cell lung cancer cell lines showed that simultaneous treatment of celecoxib with 4HPR resulted in additive growth suppression (25). As shown in Fig. 2, combinations of 5 μmol/L celecoxib with 0.2 μmol/L 4HPR inhibited cell growth of premalignant and tumorigenic cell lines more effectively than treatment with either agent alone ($P \leq 0.05$ and $P \leq 0.005$ compared with untreated cultures). Furthermore, combinations of 2.5 μmol/L celecoxib and 0.2 μmol/L 4HPR showed greater growth suppression in 1799, 1198, and 1170-I than in BEAS cells. In contrast, NHBE cells grown *in vitro* under either subconfluent or confluent condition seemed to be sensitive to the growth-inhibitory effects of 4HPR (0.2 μmol/L), whereas celecoxib showed no significant activity at concentrations up to 5 μmol/L. Interestingly, combinations of the two agents were less or similarly effective than treatment of NHBE cells with each agent alone.

Celecoxib plus 4HPR suppresses colony formation of premalignant and tumorigenic lung cell lines more effectively than either agent alone. Because the growth-inhibitory effects induced by celecoxib, 4HPR, or both in monolayer cultures were modest, we asked whether treatment with either or both agents could exert greater activity on the formation of colonies, which allows an investigation over a longer period of time. As single

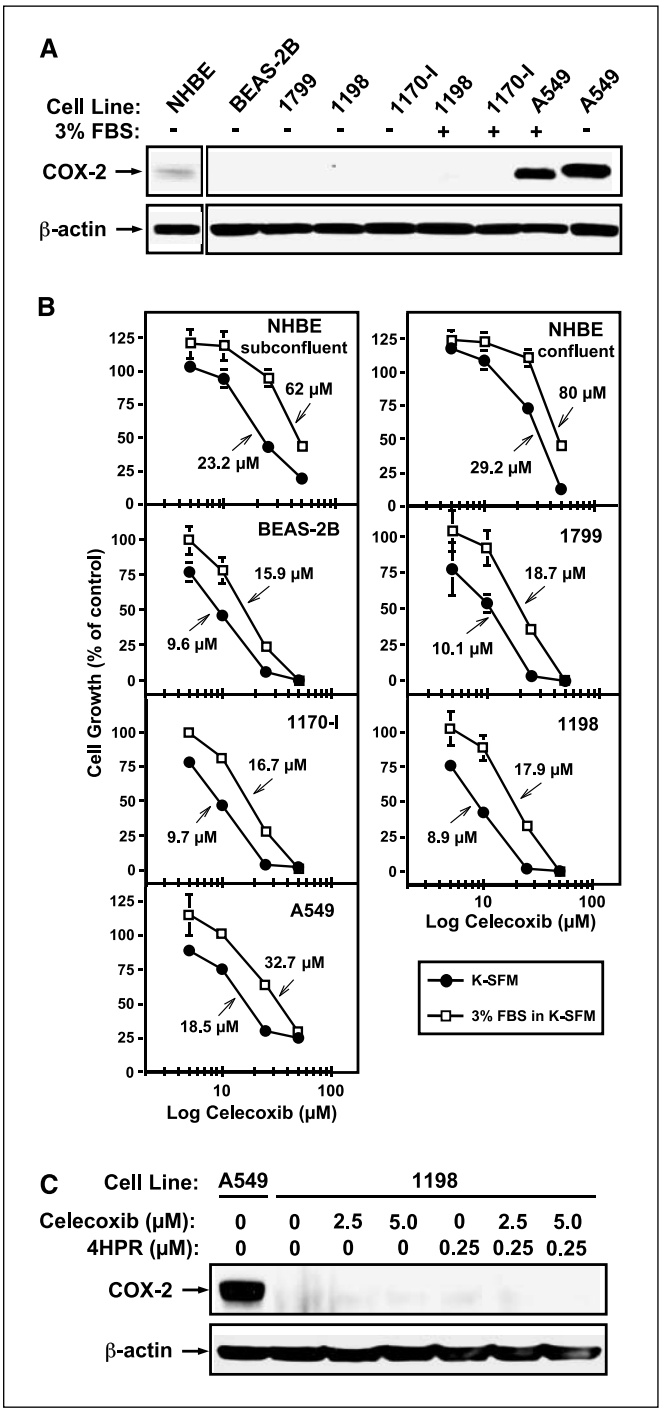

Figure 1. Expression of COX-2 in various premalignant, normal, and malignant human lung cell lines and their growth inhibition by celecoxib. *A,* human normal (NHBE), immortalized (BEAS-2B and 1799), transformed (1198), and tumorigenic (1170-I and A549) epithelial cells were grown in K-SFM supplemented with epidermal growth factor (2.5 μg) and bovine pituitary extract (25 mg) in the presence or absence of FBS (3%). The cells were harvested after 5 days, lysed, and subjected to Western blot analysis (50 μg/lane) using anti-human COX-2 and anti-β-actin (loading control) antibodies. *B,* cells were cultured in K-SFM in the presence or absence of 3% FBS for 3 days. Cell numbers were estimated by the sulforhodamine B assay. *Points,* means from three independent experiments done in quadruplicate; *bars*, SD. The concentrations of celecoxib shown are IC_{50} values calculated from the concentration-response curves of each cell line using DMSO as a control. *C,* expression of COX-2 in 1198 cells treated with celecoxib (2.5 and 5 μmol/L), 4HPR (0.25 μmol/L), and their combinations for 3 days. A549 cells were included as a positive control for COX-2. β-Actin served as the loading control.

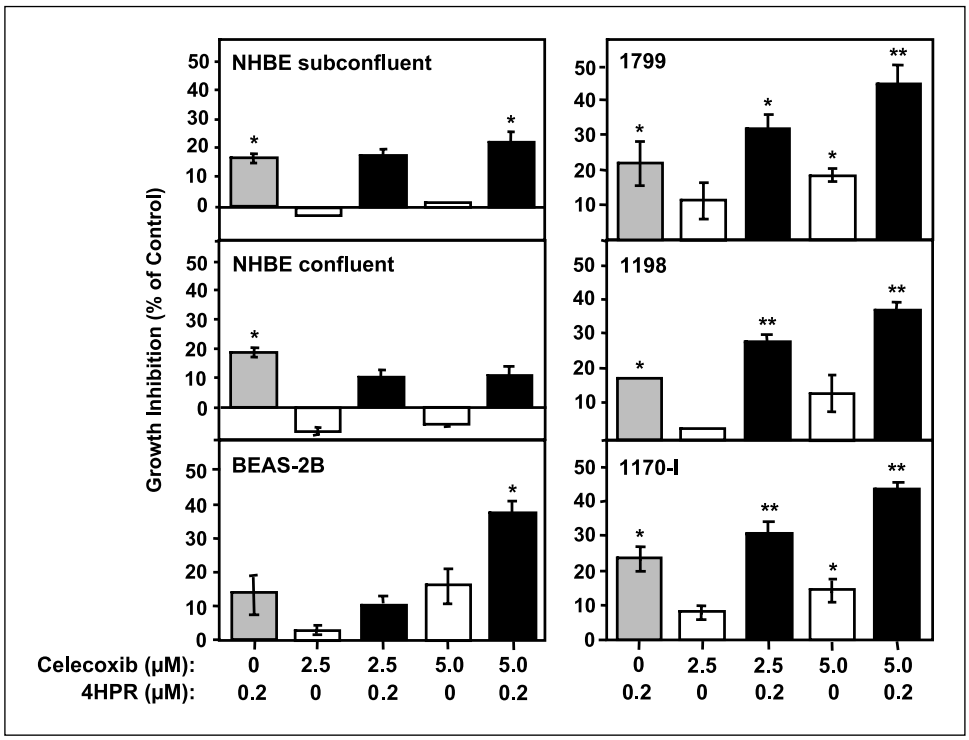

Figure 2. Comparison of the effects of celecoxib, 4HPR, and their combinations on the growth of human bronchial epithelial cell lines representing an *in vitro* model of lung carcinogenesis. Cells were seeded into 96-well culture plates and incubated overnight before treatment with celecoxib (2.5 and 5 μmol/L), 4HPR (0.2 μmol/L), or both agents for 3 days. Cultures incubated with DMSO alone served as a control. Cell numbers were estimated by the sulforhodamine B assay and analyzed for statistical significance using Student's paired *t* test with $P \leq 0.05$ (*) and $P \leq 0.005$ (**) related to control cultures in DMSO, respectively. *Columns*, means from three independent experiments done in quadruplicate; *bars*, SD.

agents, both 4HPR and celecoxib exerted some inhibitory effects on colony formation in all cell lines (Fig. 3). For example, 5 μmol/L celecoxib reduced the mean size of colonies in BEAS-2B cells as well as the size and number of colonies in the other cell lines. The colony-forming ability of 1799, 1198, and 1170-I cells was inhibited by 61%, 45%, and 27%, respectively ($P < 0.05$ for all comparisons), after treatment with 5 μmol/L celecoxib. 4HPR (0.2 μmol/L) was also effective by itself in reducing the size of colonies in BEAS-2B cells, however, without altering their number. On the contrary, 0.2 μmol/L 4HPR suppressed colony formation of 1799, 1198, and 1170-I cells by 36%, 15%, and 57%, respectively ($P < 0.05$, P = NS, and $P < 0.005$). 4HPR at the lower concentration of 0.1 μmol/L significantly inhibited the number of colonies in only 1198 and 1170-I cells by 24% and 38% ($P < 0.05$ and $P < 0.005$), respectively. Combining celecoxib and 4HPR substantially augmented the modest effect of treatment with the single agents. In particular, 5 μmol/L celecoxib plus 0.2 μmol/L 4HPR markedly suppressed colony formation in all cell lines by 86% (BEAS-2B) to 99% (1799, 1198, and 1170-I; $P < 0.005$ for all comparisons). Even 0.1 μmol/L 4HPR plus 5 μmol/L celecoxib significantly reduced the number of colonies in 1799, 1198, and 1170-I cells by 92%, 79%, and 63% ($P < 0.005$ for all comparisons), respectively, while decreasing the size of colonies in BEAS-2B cells without altering their number (Fig. 3).

Celecoxib plus 4HPR induces apoptosis in BEAS-2B and 1198 cells more efficiently than either agent alone. Treatment of BEAS-2B and 1198 cells with up to 5 μmol/L celecoxib had negligible effects on the induction of apoptosis as determined by the TUNEL assay (Fig. 4*A* and *B*). On the other hand, treatment with 0.25 μmol/L 4HPR increased the amount of apoptotic BEAS-2B and 1198 cells to 10 ± 2.4% and 18 ± 1.7%, respectively. However, combinations of 0.25 μmol/L 4HPR and celecoxib (≤5 μmol/L) significantly augmented apoptosis compared with treatment with either agent alone ($P \leq 0.05$ and $P \leq 0.005$ compared with untreated culture). For example, incubation with 5 μmol/L celecoxib plus 0.25 μmol/L 4HPR caused ~60% apoptotic BEAS-2B and 1198 cells, respectively.

Effects of celecoxib plus 4HPR on apoptosis-related proteins in BEAS and 1198 cells. The expression of proteins related to apoptosis was applied to confirm apoptotic events induced by the combination of celecoxib and 4HPR in BEAS-2B and 1198 cells. As a result, we obtained a decrease in the expression levels of procaspase-3 and procaspase-9, indicating caspase activation concomitant with an increase in cleavage of the 113 kDa PARP to the 89 kDa fragment (Fig. 4*C* and *D*). These effects were most profound in cells treated with a combination of 5 μmol/L celecoxib and 0.25 μmol/L 4HPR. Notably, in BEAS-2B cells, 4HPR alone diminished the level of procaspase-3 and increased the cleavage of PARP. No changes were detected in the expression levels of caspase-8 in either cell line. The level of the antiapoptotic protein Bcl-2 decreased and that of the proapoptotic molecule Bcl-X_S increased in both cell lines in response to combined treatment, whereas the expression of the proapoptotic protein Bax and the antiapoptotic protein Bcl-X_L was unaltered regardless of treatment. We further examined the effects of celecoxib and 4HPR on the mitochondrial pathway by analysis of cytochrome *c* release, which contributes to caspase-3 and caspase-9 activation, resulting in the degradation of PARP and subsequent cleavage of internucleosomal DNA. Treatment of 1198 cells with 5 μmol/L celecoxib plus 0.25 μmol/L 4HPR markedly induced cytochrome *c* release from the mitochondria into the cytosol (Fig. 4*D, bottom*). Altogether, these TUNEL and Western blot data confirm the apoptosis-inducing effects of combined celecoxib and 4HPR treatment.

Celecoxib plus 4HPR induces apoptosis partly by suppressing the Akt signaling pathway in 1799 cells. To determine whether combinations of celecoxib and 4HPR can alter the Akt signaling pathway in 1799 transformed human bronchial epithelial cells, we investigated the effects of these agents alone and in combination on the activation of Akt and its downstream molecule

glycogen synthase kinase-3β (GSK-3β). The constitutively high levels of phosphorylated Akt and phosphorylated GSK-3β were marginally decreased in response to 5 μmol/L celecoxib or 0.25 μmol/L 4HPR alone, but greatly decreased by the combination of these two agents. Likewise, the expression of the antiapoptotic protein Bcl-2 only declined after incubation with celecoxib in combination with 4HPR. No alterations appeared in the expression of Akt and GSK-3α/β after treatment with celecoxib and/or 4HPR compared with DMSO control cultures (Fig. 5*A* and *B*).

To further examine whether expression of constitutively active Akt can protect cells from undergoing apoptosis, we infected 1799 cells with adenoviral vector containing MyrAkt tagged with hemagglutinin for the recombinant protein (MyrAkt-HA) before treatment with celecoxib and 4HPR and compared the levels of apoptosis-related proteins in those cells with cells infected with adenoviral vector control (Ad5CMV). Successful infection is illustrated by the appearance of a single protein band by an antibody against HA-tag and a second band by an anti-Akt antibody (Fig. 5*C, top two panels*). As expected, these bands were not detected in cells infected with at Ad5CMV only. The 1799 cells expressing MyrAkt-HA were less sensitive than the cells infected with control vector to the apoptosis-inducing effects of celecoxib plus 4HPR as indicated by unaltered expressions of procaspase-3, Bcl-2, and decreased levels of PARP cleavage (Fig. 5*C*).

Discussion

Celecoxib has been shown to regress colorectal adenomas in patients with familial adenomatous polyposis relative to placebo, which resulted in the Food and Drug Administration approving celecoxib for adjunctive management of this disease (10). The presumed mechanism of action of celecoxib is selective inhibition of COX-2, an enzyme responsible for the metabolic conversion of arachidonic acid to prostaglandins, which play important roles in inflammation, cell proliferation, cell survival, and carcinogenesis (7). Moreover, the induction of COX-2 represents an early event in cancer development, and its expression seems to be associated with a poor prognosis in various types of cancer, including lung

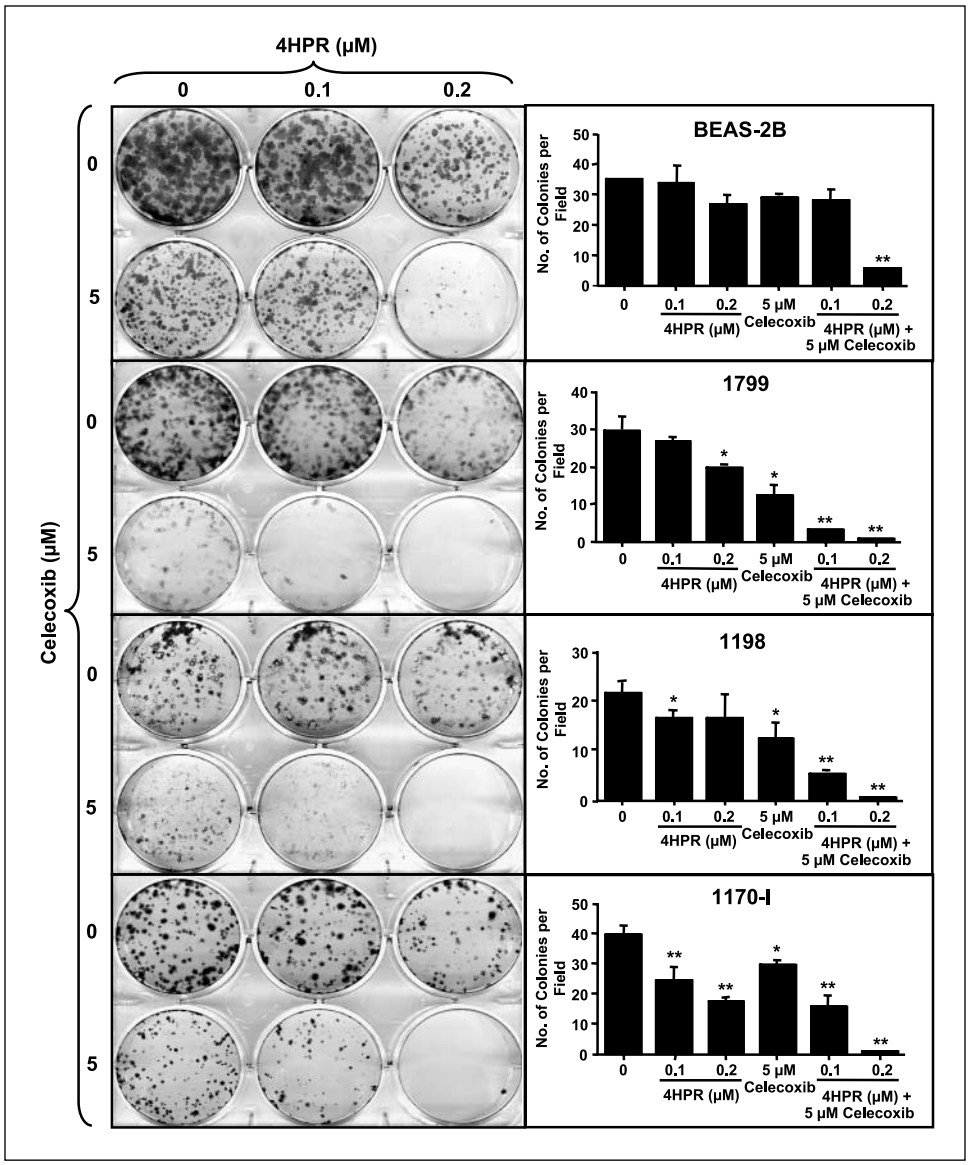

Figure 3. Effects of celecoxib, 4HPR, and their combinations on the colony-forming ability of human bronchial epithelial cell lines representing an *in vitro* model of lung carcinogenesis. Exponentially growing cells were seeded into six-well culture plates and treated with celecoxib (5 μmol/L), 4HPR (0.1 and 0.2 μmol/L), and their combinations for 14 days. *Columns,* number of colonies for each cell line calculated from quadruplicate determinations; *bars*, SD. $P \leq 0.05$ (*) and $P \leq 0.005$ (**) compared with cultures in DMSO (*leftmost column*) by Student's paired *t* test.

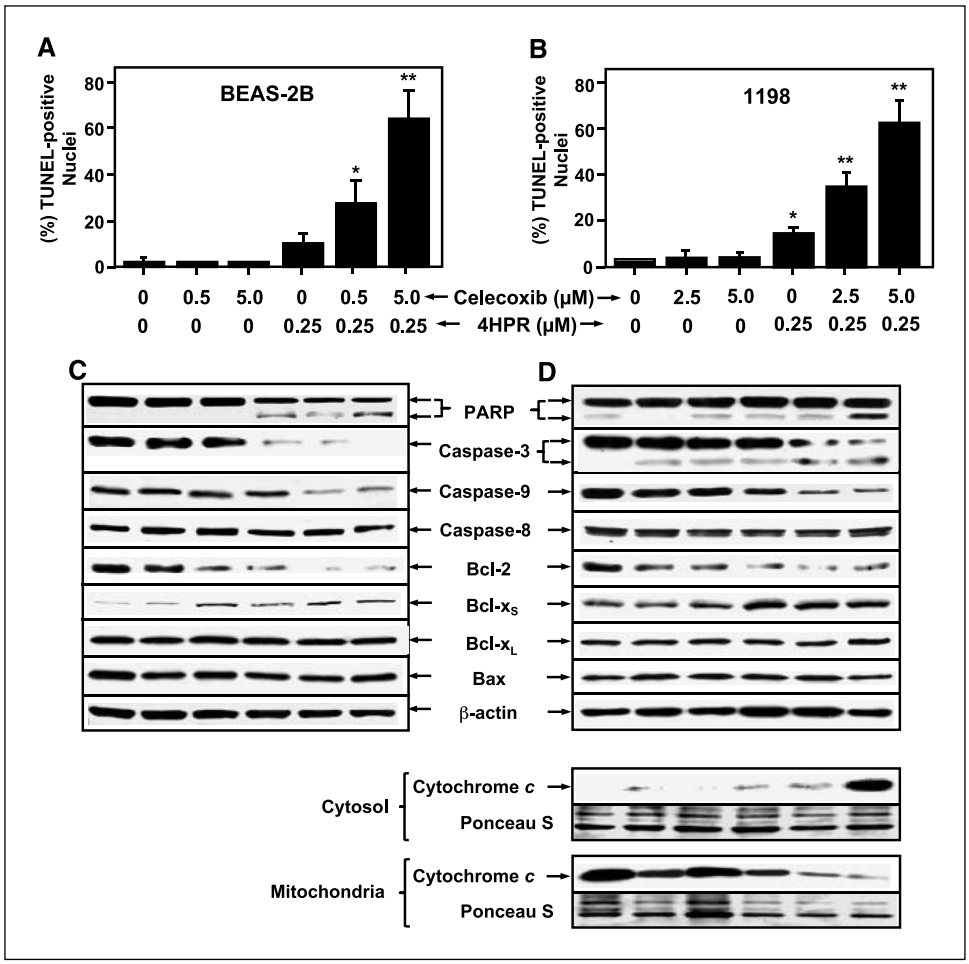

Figure 4. Effects of celecoxib, 4HPR, and their combinations on the induction of apoptosis and expression of apoptosis-related proteins in BEAS-2B and 1198 bronchial epithelial cell lines. *A* and *B,* the cells were treated with celecoxib (≤5 μmol/L), 4HPR (0.25 μmol/L), or both for 3 days before being harvested and analyzed by TUNEL assay. *Columns,* percentage of TUNEL-positive nuclei representative of two independent experiments and calculated for statistical significance using Student's two-tailed *t* test with $P \leq 0.05$ (*) and $P \leq 0.005$ (**), respectively. *C* and *D,* cells treated as above were harvested, lysed, and subjected to Western blot analysis using antibodies against the indicated proteins. The release of cytochrome *c* into the cytosol of 1198 cells was determined by Western blotting after fractionation of mitochondria and cytosol. β-Actin and Ponceau S staining were used as loading controls.

cancer (8, 9, 18). The fact that celecoxib at clinically achievable concentrations markedly decreased the PGE_2 production in COX-2–expressing cells supports its use as a cancer therapeutic and chemopreventive agent (17, 18, 20–24).

However, recent studies have suggested that the activity of celecoxib *in vivo* may not be exclusively mediated by a COX-2–dependent pathway (23, 24). For example, Sinicrope et al. (37) reported that celecoxib administered at 400 mg twice daily over 6 months induced polyp regression in patients with familial adenomatous polyposis by modulating *in vivo* rates of cell proliferation and apoptosis without evidences for suppression of PGE_2 in either normal tissue or adenomas. Furthermore, the concentrations of celecoxib needed to exert antitumor activity (e.g., apoptosis) *in vitro* are at least 10-fold higher than those required to inhibit COX-2 activity *in vivo*, raising the question whether the mechanisms identified *in vitro* are applicable to tumor responses *in vivo* (23–25). In addition, experimental studies have consistently shown that the sensitivity of various cancer cells to celecoxib is not related to their COX-2 expression status (24, 25).

We have used an *in vitro* model of human lung carcinogenesis to investigate the effects of clinically relevant concentrations of celecoxib in combination with the synthetic retinoid 4HPR. This model consists of distinct bronchial epithelial cell lines derived from SV40 large T-antigen immortalized BEAS-2B cells after exposure to cigarette smoke condensate and has proven useful in studying the process of carcinogenic transformation and the efficacy of cancer chemopreventive agents (30, 31). Our results indicate that cell lines constituting this model showed no detectable COX-2 expression, had corresponding low levels of PGE_2, and were nonetheless sensitive to the growth-inhibitory activity of celecoxib, such as A549 cancer cells, which constitutively expressed COX-2. Because the IC_{50} values for celecoxib were higher than those achievable in clinical trials (23), we decided to combine celecoxib with the proapoptotic retinoid 4HPR. Our data show that treatment of premalignant and tumorigenic cell lines with celecoxib or 4HPR at clinically relevant concentrations exhibited only modest inhibitory effects (<20%) on cell growth. However, incubation of these cell lines with 5 μmol/L celecoxib plus 0.2 μmol/L 4HPR revealed additive effects on growth suppression in monolayer cultures determined after 3 days of incubation.

The preclinical evaluation of therapeutic and chemopreventive drugs typically involves the comparison of the effects on premalignant and normal cells to determine whether a "therapeutic window" exists. Clearly, chemopreventive agents are required to have a better safety profile than therapeutic drugs because they may be administered over a prolonged period of time. Both celecoxib (10, 37) and 4HPR (29, 38) have been found to have low or no side effects in humans at doses that lead to plasma levels comparable with concentrations we used in this study and even considerably higher in the case of 4HPR (38). Therefore, we anticipated finding low or no effects of these agents on NHBE cells. However, celecoxib inhibited the growth, especially of subconfluent

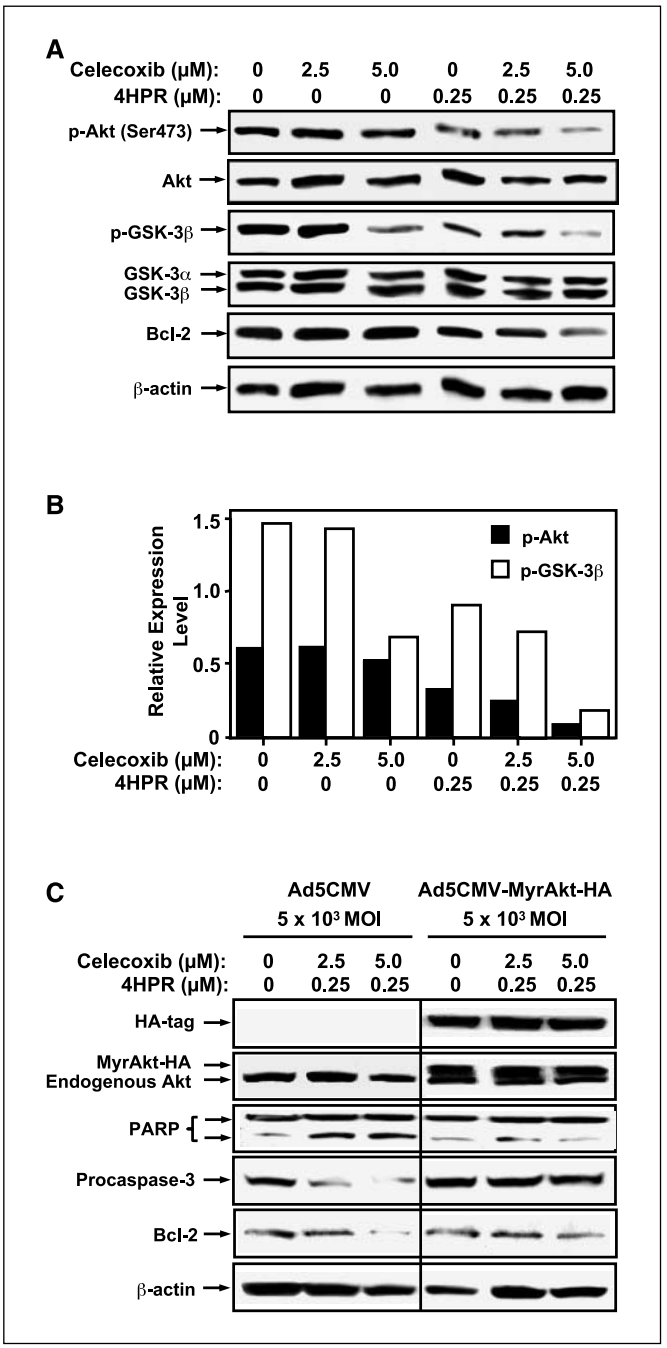

Figure 5. Effects of celecoxib, 4HPR, and their combinations on the Akt survival pathway in 1799 human transformed bronchial epithelial cells. *A,* after 2 days of treatment with celecoxib and 4HPR, protein extracts (50 μg/lane) prepared from the cells were subjected to Western blot analysis to determine the expression of phosphorylated Akt (Ser^{473}), phosphorylated GSK-3β, Akt, GSK-3α/β, and Bcl-2. *B,* the blots were scanned using SF Launcher v2.0.5, and the densities of p-Akt (Ser^{473}) and p-GSK-3β were quantified in relation to the corresponding total protein expression and normalized to β-actin by NIH image 1.58 software. *C,* effects of constitutively active Akt on the response of 1799 bronchial epithelial cells to apoptosis induced by celecoxib and 4HPR. The cells were infected at 5×10^3 MOI of adenoviral vector control (Ad5CMV) or adenoviral vector containing myristoylated Akt (Ad5CMV-MyrAkt-HA) in keratinocyte SFM for 1 day before incubation with celecoxib/4HPR for an additional 2 days. Successful infection is illustrated by the appearance of a single protein band by an antibody against HA-tag and a second band by an anti-Akt antibody (*top two panels*). As expected, these bands were not detected in cells infected with at Ad5CMV. Apoptosis-inducing activity was determined by the expression of procaspase-3, Bcl-2, and cleavage of PARP. β-Actin served as a control for protein loading.

proliferating NHBE cultures, albeit with lower potency than it did inhibit premalignant and tumorigenic cell proliferation. Interestingly, at low concentrations (2.5 and 5 μmol/L), celecoxib failed to inhibit NHBE cell growth, whereas it suppressed the growth of their premalignant and tumorigenic counterparts. 4HPR (0.2 μmol/L) showed significant but low inhibition compared with untreated cultures; however, combinations with celecoxib did not augment this effect. Thus, these agents appear to exert a selective effect on premalignant and tumorigenic cells compared with NHBE cells. Notably, the use of NHBE cells as a control is not straightforward because these cells are cultured under conditions where they are stimulated to proliferate, i.e., K-SFM supplemented with epidermal growth factor and bovine pituitary extract, hence emulate hyperplasia rather than normal bronchial epithelium. In fact, the proliferative index of histologically normal lung epithelium from nonsmokers *in vivo* is 0.16 ± 0.15% and 1.26 ± 1.17% in biopsies obtained from smokers (39) as opposed to 4.8% to 36% in premalignant lesions such as dysplasia (39, 40). Therefore, we investigated the effects of the agents on both subconfluent (proliferating) and confluent (quiescent) NHBE cells. Indeed, we found that confluent NHBE cells were less inhibited by high concentrations of celecoxib or low concentrations of celecoxib combined with 4HPR than premalignant and tumorigenic cells.

Combinations of low doses of both celecoxib and 4HPR resulted in a more dramatic inhibitory activity on the formation of colonies, where cells were seeded at low density and treated for 14 days, compared with growth inhibition in cultures at higher density measured after 3 days of incubation. We observed that BEAS-2B cells exhibited lower sensitivity to combinations of celecoxib and 4HPR than 1799, 1198, and 1170-I cells did (Figs. 2 and 3). The reason for such difference is unclear. However, it is noteworthy that the three more sensitive cell lines were derived from BEAS-2B cells after growing as xenotransplants in nude mice for 6 months (30). This *in vivo* passage may have been accompanied by selective pressure that resulted in some distinction from the parental cell line, which was immortalized with SV40 large T-antigen. Recently, we found that various human bronchial epithelial cell lines immortalized with hTERT and CDK4 (41) also lack COX-2 expression yet exhibit higher sensitivity to the combination of celecoxib and 4HPR than to either agent alone (data not shown). Thus, we conclude that the sensitivity of premalignant bronchial epithelial cell lines to this combination is not restricted to SV40 large T-antigen immortalized cells.

To gain insight into the mechanisms by which celecoxib in combination with 4HPR enhanced the apoptosis of human bronchial epithelial cell lines, as observed with the TUNEL assay, we examined the expression levels of several apoptosis-related proteins. Our data indicate that celecoxib plus 4HPR activates the mitochondrial apoptosis pathway as evidenced by suppression of the antiapoptotic protein Bcl-2, increase in the proapoptotic protein Bcl-x_S, release of apoptogenic cytochrome *c* into the cytosol, activation of caspase-9 and caspase-3, and cleavage of PARP. However, no changes were detected in the expression levels of Bcl-x_L, Bax, or caspase-8. On the basis of our initial findings that none of these cell lines expressed COX-2, we concluded that the growth-inhibitory and apoptosis-inducing effects of celecoxib in combination with 4HPR are mediated by COX-2–independent mechanisms. Numerous studies have shown that celecoxib induces apoptosis in a variety of cell types, but only at concentrations between 50 and 100 μmol/L (23, 25, 42–44). The mechanism ascribed to these effects was based on activation of the

mitochondrial signaling pathway, as indicated by breakdown of the mitochondrial membrane potential, release of cytochrome *c*, activation of caspase-9 and caspase-3, and cleavage of PARP via a Bcl-2/Bcl-x_L–independent pathway in rat cholangiocarcinoma (42), human lymphoma (43), and prostate carcinoma (44) cell lines. Correspondingly, various reports have delineated the proapoptotic effects of 4HPR at concentrations above 3 μmol/L by a mechanism that includes augmented generation of reactive oxygen species from the mitochondria with subsequent release of cytochrome *c*, activation of caspase-9 and caspase-3, and cleavage of PARP without altering Bcl-2 and Bax (25–27). However, ectopic overexpression of Bcl-2 did not protect T-cell acute lymphoblastic leukemia cells from apoptosis induced by 4HPR but markedly delayed its onset (45). Nonetheless, in those cells, 4HPR induced apoptosis via a mitochondrial (reactive oxygen species mediated) pathway that involves the obligatory contributions of the proapopotic Bcl-2 family members Bax and/or Bak (46).

It is well established that alterations to the Akt signaling pathway are frequent in human malignancies that result in enhanced resistance to apoptosis through multiple mechanisms (47). In fact, previous studies on rat cholangiocarcinoma (42), human prostate (44), hepatocellular (48), and colon cancer (49) cells have shown that celecoxib inhibits Akt phosphorylation in association with induction of apoptosis via the mitochondrial pathway. However, these effects required high concentrations of celecoxib that are not attainable *in vivo*. In contrast, our data indicate that incubation of premalignant bronchial epithelial cells with clinically relevant concentrations of celecoxib combined with 4HPR augments apoptosis by reducing phosphorylation levels of Akt and its direct downstream substrate GSK-3β. This conclusion was supported by further experiments showing that overexpression of constitutive active Akt protects, at least partly, premalignant cells from undergoing apoptosis induced by celecoxib plus 4HPR. Taken together, our findings strongly warrant additional evaluation of the efficacy of this combination in chemoprevention and therapy of lung cancer in animal models and eventually in clinical trials.

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Involvement of Mitochondrial and Akt Signaling Pathways in Augmented Apoptosis Induced by a Combination of Low Doses of Celecoxib and *N*-(4-Hydroxyphenyl) Retinamide in Premalignant Human Bronchial Epithelial Cells

Claudia P. Schroeder, Humam Kadara, Dafna Lotan, et al.

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