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Authors

Li, Yin
Lin, Bingzhen
Agadir, Anissa
et al.

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Molecular Determinants of AHPN (CD437)-Induced Growth Arrest and Apoptosis in Human Lung Cancer Cell Lines

YIN LI,¹ BINGZHEN LIN,¹ ANISSA AGADIR,¹ RU LIU,¹ MARCIA I. DAWSON,² JOHN C. REED,¹
JOSEPH A. FONTANA,³ FRÉDÉRIC BOST,⁴ PETER D. HOBBS,² YUN ZHENG,¹ GUO-QUAN CHEN,¹
BRAHAM SHROOT,⁵ DAN MERCOLA,⁴ AND XIAO-KUN ZHANG^{1*}

The Burnham Institute, Cancer Research Center, La Jolla, California 92037¹; Retinoid Program, SRI International, Menlo Park, California 94025²; Marilyn and Stuart Greenbaum Cancer Center, University of Maryland, Baltimore, Maryland 21201³; Sidney Kimmel Cancer Center, San Diego, California 92121⁴; and Centre International de Recherches Dermatologiques (CIRD), Galderma, Valbonne, France⁵

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6-[3-(1-Adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN or CD437), originally identified as a retinoic acid receptor γ -selective retinoid, was previously shown to induce growth inhibition and apoptosis in human breast cancer cells. In this study, we investigated the role of AHPN/CD437 and its mechanism of action in human lung cancer cell lines. Our results demonstrated that AHPN/CD437 effectively inhibited lung cancer cell growth by inducing G_0/G_1 arrest and apoptosis, a process that is accompanied by rapid induction of c-Jun, nur77, and p21^{WAF1/CIP1}. In addition, we found that expression of p53 and Bcl-2 was differentially regulated by AHPN/CD437 in different lung cancer cell lines and may play a role in regulating AHPN/CD437-induced apoptotic process. On constitutive expression of the c-JunAla(63,73) protein, a dominant-negative inhibitor of c-Jun, in A549 cells, nur77 expression and apoptosis induction by AHPN/CD437 were impaired, whereas p21^{WAF1/CIP1} induction and G_0/G_1 arrest were not affected. Furthermore, overexpression of antisense nur77 RNA in A549 and H460 lung cancer cell lines largely inhibited AHPN/CD437-induced apoptosis. Thus, expression of c-Jun and nur77 plays a critical role in AHPN/CD437-induced apoptosis. Together, our results reveal a novel pathway for retinoid-induced apoptosis and suggest that AHPN/CD437 or analogs may have a better therapeutic efficacy against lung cancer.

Retinoids, the natural and synthetic vitamin A derivatives, regulate a broad range of biological processes, including growth, differentiation, and development, in both normal and neoplastic cells (22, 27). The effects of retinoids are mainly mediated by two classes of nuclear receptors, the retinoic acid receptors (RARs) and retinoid X receptors (RXRs), that are encoded by three distinct genes (α , β , and γ) and are members of the steroid and thyroid hormone receptor superfamily (32, 40, 77). Retinoid receptors modulate the expression of their target genes in response to their natural ligands *trans*-retinoic acid (*trans*-RA) and 9-*cis*-RA as well as a number of synthetic analogs. 6-[3-(1-Adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN or CD437), first identified as a RAR γ -selective retinoid by receptor binding and transcriptional activation assays (3), was recently reported to effectively inhibit the growth and induce apoptosis of a variety of cancer cells (9, 38, 47, 56, 57, 68). One unique feature of AHPN/CD437 is that it can act in a p53-independent mechanism (57). In addition, this novel compound did not appear to require activation of RARs or RXR to exert its effect (57), although the contribution of RAR γ activation to its activity cannot be completely excluded, because RAR γ -selective retinoid agonists have been reported to inhibit cancer cell growth (38, 68). Importantly, the growth-inhibitory and apoptosis-inducing effects of AHPN/CD437 could be observed in *trans*-RA-refractory breast cancer (57) and leukemia (29) cell lines, indicating that it may be representative of a novel class of compounds suitable for treatment of *trans*-RA-resistant cancers. Although AHPN/CD437 has

been shown to regulate expression of a number of genes, including p21^{WAF1/CIP1} (57) and Bcl-X_L (28), in breast cancer cells, how it promotes apoptosis and G_0/G_1 arrest remains largely unknown.

The progression of eukaryotic cells through the cell cycle is a complex process and finely regulated by extracellular stimuli and intracellular checkpoints (30, 58). Factors determining whether cells proliferate or cease dividing and differentiate appear to operate mainly in the G_1 phase of the cell cycle (48), and activation or inactivation of cyclin-dependent kinase (cdk) plays a critical role in the process (30, 48, 58). One cdk inhibitor is p21^{WAF1/CIP1}, which mediates p53-induced growth arrest triggered by DNA damage and arrests cells in G_1 phase (12, 14, 21, 24, 45, 73). Regulation of p21^{WAF1/CIP1} by p53 presumably occurs at the transcriptional level through several putative p53 binding sites in the p21^{WAF1/CIP1} promoter region (13, 24). Recent studies have also revealed a p53-independent induction of p21^{WAF1/CIP1}, which also induces growth arrest in response to a variety of stimuli, including growth factors, tetradecanoyl phorbol acetate, and okadaic acid (31, 39, 49, 61, 75). Regulation of p53-independent expression of p21^{WAF1/CIP1} is of great interest, since it could represent an important approach to control aberrant proliferation of cancer cells in which p53 is often deleted or mutated.

Numerous signals can trigger apoptosis, which is an important mechanism for eliminating aberrant or unwanted cells from an organism (17, 62, 67). Once triggered, apoptosis appears to proceed through a central death pathway in which specific cellular proteases and endonucleases are activated to destroy cells (17, 62, 67). Members of the Bcl-2 family are known to modulate apoptosis in different cell types in response to various stimuli (34, 54). Bcl-2 and Bcl-X_L promote cell survival, while Bax enhances cell death (34, 54). A number of

* Corresponding author. Mailing address: The Burnham Institute, Cancer Research Center, 10901 N. Torrey Pines Rd., La Jolla, CA 92037. Phone: (619) 646-3141. Fax: (619) 646-3195. E-mail: xzhang@burnham-inst.org.

transcriptional factors are also involved in regulating apoptosis, probably through their modulation of downstream target genes leading to the central death pathway. p53 can mediate apoptosis in response to DNA damage and adenovirus E1A (10, 37, 42), whereas c-Myc is involved in the cell death of growth-arrested fibroblasts (16). It was demonstrated recently that c-Jun, a component of the AP-1 complex involved in cell cycle progression, differentiation, and cell transformation (2, 66), could also promote apoptosis in various cell types (5, 7, 11, 15, 19, 23, 33, 41). Moreover, nuclear orphan receptor *nur77* (also known as NGFI-B and TR₃) (8, 25, 43) is highly induced in activation-induced T-cell apoptosis (36, 71). The observation that expression of a dominant-negative *nur77* or antisense *nur77* RNA could inhibit apoptosis (36, 71) indicates that *nur77* is also required for induction of activation-induced T-cell apoptosis. *nur77* is rapidly induced in a variety of cells in response to growth and differentiation signals, such as serum growth factors, nerve growth factor, and phorbol esters (8, 25, 44). It functions as a transcriptional factor by binding to its response element as either a monomer (70) or homodimer (51). In addition, *nur77* can heterodimerize with RXR (18, 50) and another orphan receptor COUP-TF (72) to modulate the binding activities of a variety of RA response elements (72) and their sensitivity to *trans*-RA (72). Sequences required for induction of *nur77* by various stimuli have been identified. There are several potential binding sites for the transcriptional factor AP-1 in the *nur77* promoter (64, 69), suggesting that members of the AP-1 family should participate in *nur77* induction and that *nur77* may be a downstream target to mediate c-Jun-induced apoptosis.

Although the conventional retinoids can effectively regulate the proliferation and differentiation of tracheobronchial epithelial cells (22, 27), their efficacy against lung cancer cells is limited (26, 46). The majority of lung cancer cell lines exhibit resistance to the growth-inhibitory effect of *trans*-RA, in part due to abnormal expression of RAR β , that occurs with high frequency in primary human lung cancer tissues and human lung cancer cell lines (20, 34A, 78). Because AHPN/CD437 effectively inhibited the growth of *trans*-RA-refractory breast cancer cells (57) and leukemia cells (29), we investigated the growth-inhibitory and apoptosis-inducing effects of AHPN/CD437 in lung cancer cell lines and its mechanism of action. Our results demonstrate that AHPN/CD437, by inducing apoptosis and G₀/G₁ arrest, is much more effective than *trans*-RA in inhibiting the growth of all four lung cancer cell lines investigated, including a p53-deficient lung cancer cell line. In addition, we show that induction of G₀/G₁ arrest by AHPN/CD437 is mainly conferred by a rapid induction of p21^{WAF1/CIP1}. Furthermore, we demonstrate that promotion of apoptosis by AHPN/CD437 is largely mediated by induction of c-Jun and *nur77* expression and modulated by inhibition of Bcl-2 activity. Thus, our study reveals a novel c-Jun/*nur77* pathway for retinoid-induced apoptosis in human lung cancer cells. The observation that AHPN/CD437 is a much more potent growth inhibitor than *trans*-RA in lung cancer cell lines including p53-deficient lung cancer cell line suggests that AHPN/CD437 or its analogs may have therapeutic potential against lung cancer or its development.

MATERIALS AND METHODS

Retinoids. All retinoids were dissolved in ethanol before dilution with medium. *trans*-RA was obtained from Sigma (St. Louis, Mo.). AHPN/CD437 was prepared as described previously (57).

Cell culture. The non-small-cell lung cancer (NSCLC) cell lines H292, H460, A549, and SK-MES-1 were obtained from American Type Culture Collection. SK-MES-1 cells were grown in minimal essential medium supplemented with 10% fetal calf serum (FCS), A549 cells were grown in Dulbecco modified Eagle

medium with 10% FCS, and H292 and H460 cells were maintained in RPMI 1640 medium supplemented with 10% FCS.

Growth inhibition assay. For adherent growth inhibition, cells were seeded at 1,000 cells per well in 96-well plates and treated 24 h later with various concentrations of retinoids for different periods of time. The control cells received vehicle (ethanol). Media and retinoids were changed every 48 h. Viable-cell number was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay in which the capacity of cells to convert a tetrazolium salt to a blue formazan product was measured by using a cell proliferation or cytotoxicity assay kit (Promega, Madison, Wis.) (35). Results obtained were confirmed by counting the cells with a hemocytometer.

Apoptosis analysis. Apoptosis analyses were done as previously described (35). For morphological analysis, cells were treated with 10⁻⁶ M AHPN/CD437, trypsinized, washed with phosphate-buffered saline (PBS), fixed with 3.7% paraformaldehyde, and stained with 50 μ g of 4,6-diamidino-2-phenylindole (DAPI) per ml containing 100 μ g of DNase-free RNase A per ml to visualize the nuclei. Stained cells were examined by fluorescence microscopy. For the terminal deoxynucleotidyl transferase (TdT) assay, cells were treated with or without 10⁻⁶ M AHPN/CD437. After treatment, cells were trypsinized, washed with PBS, fixed in 1% formaldehyde in PBS, washed with PBS, resuspended in 70% ice-cold ethanol, and immediately stored at -20°C overnight. Cells were then labeled with biotin-16-dUTP by terminal transferase and stained with avidin-FITC (fluorescein isothiocyanate) (Boehringer Mannheim). The labeled cells were analyzed with a FACScater-Plus flow cytometer. Representative histograms are shown in Fig. 2c, 8b, and 9.

Flow cytometric analysis. Cells were trypsinized and collected by centrifugation at 2,000 rpm for 5 min. The cell pellets were then resuspended in 1-ml portions of PBS and fixed in 70% ice-cold ethanol and kept in a freezer overnight. Fixed cells were centrifuged, washed once in PBS, and then resuspended in 100 μ l of phosphate-citrate buffer (192 parts of 0.2 M Na₂HPO₄ and 8 parts of 0.1 M citric acid [pH 7.8]) for 30 min at room temperature to wash out any degraded DNA from apoptotic cells. The cells were then collected by centrifugation at 2,000 rpm, and the cell pellets were washed twice with PBS and then resuspended in PBS containing 50 μ g of propidium iodide (Sigma) per ml and 100 μ g of DNase-free RNase A (Boehringer Mannheim) per ml. The cell suspension was incubated for 30 min at 37°C and protected against light and then analyzed with a FACScater-Plus flow cytometer.

RNA preparation and Northern blot analysis. For Northern blot analysis, total RNAs were prepared by the guanidine hydrochloride-ultracentrifugation method (72). Samples of about 30 μ g of total RNAs from different cell lines were fractionated on 1% agarose gels, transferred to nylon filters, and probed with the ³²P-labeled probe as previously described (72). To normalize the amount of RNA used, the filters were also probed with β -actin.

Antibodies and Western blots. Cells were lysed in a solution containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1% Triton X-100 and protease inhibitors phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin and pepstatin. Equal amounts of lysates (50 μ g) were boiled in sodium dodecyl sulfate (SDS) sample buffer, resolved by SDS-polyacrylamide gel electrophoresis (12.5% polyacrylamide), and transferred to nitrocellulose. After transfer, the membranes were blocked in TBST (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% Tween 20) containing antibody. The membranes were then washed three times with TBST and then incubated for 1 h at room temperature in TBST containing horseradish peroxidase-linked antiimmunoglobulin. After three washes in TBST, immunoreactive products were detected by chemiluminescence with an enhanced chemiluminescence system (ECL; Amersham). Anti-p21 and anti-c-Jun antibodies were obtained from Santa Cruz Biotechnology Inc., anti-p53 antibody was from Oncogene Inc., and antibodies against Bcl-2, Mcl-1, Bcl-X_L, and Bax were described previously (4). Anti- α -tubulin was used for the control.

Transient-transfection assay. A total of 5 \times 10⁵ cells were seeded in each well of six-well culture plates. A modified calcium phosphate precipitation procedure was used for transient transfection as described elsewhere (74, 76). Briefly, 250 ng of reporter plasmid (-73Col-CAT) and 250 ng of β -galactosidase (β -Gal) expression vector (pCH110; Pharmacia) were mixed with carrier DNA (pBlue-script) to 2.5 μ g of total DNA per well. Chloramphenicol acetyltransferase (CAT) activity was normalized for transfection efficiency to the corresponding β -Gal activity.

Stable transfection. Transfection of the dominant-negative c-Jun into A549 cells and the selection of the stable clones were described previously (6). Briefly, the dominant-negative c-Jun expression plasmid [pLHCXcjun(Ala 63,73)] constructed as previously described (52, 53) was transfected into A549 cells. For a control, the empty vector pLHCX was also used. In both cases, the cells were cotransfected with 200 ng of pMT64AA (bearing the geneticin resistance gene), using Lipofectin (Gibco BRL) at 20 μ l/ml of media. Clone selection was performed by adding G418 (Gibco BRL) to the media a final concentration of 1 mg/ml 3 days after the transfection. After 3 weeks, several clones were isolated by using cloning rings. The expression of the c-JunAla(63,73) protein by the clones was determined by Western analysis using anti-c-Jun antibody. Selected clones were then maintained in media supplemented with G418 (0.5 mg/ml), and only low-passage cells (<10 passages) were used. To construct antisense *nur77* expression vector, cDNA for the *nur77* gene was cloned into pRC/CMV expression vector (Invitrogen, San Diego, Calif.) in an antisense orientation. The

resulting recombinant construct was then stably transfected into A549 or H460 cells by the calcium phosphate precipitation method and screened with G418 (800 mg/ml). The integration of exogenous *nur77* cDNA was determined by Southern blotting, and the effect of antisense *nur77* RNA expression on endogenous *nur77* expression was determined by Northern blotting.

Preparation of nuclear extracts and gel retardation assays. Nuclear extracts were prepared essentially according to the method previously described (72). Briefly, cells growing to about 90% confluence were washed with cold PBS and scraped into PBS by using a rubber policeman. Cells were pelleted by low-speed centrifugation and then resuspended in a buffer containing 10 mM Tris-HCl (pH 7.4), 3 mM CaCl_2 , and 2 mM MgCl_2 . After the cells were pelleted, they were lysed in buffer containing 1% Nonidet P-40 by 10 to 15 strokes by using an ice-cold Dounce homogenizer. Immediately after lysis, nuclei were collected by centrifugation at $2,000 \times g$ and washed once with a buffer containing 10 mM HEPES-KOH (pH 7.9), 1.5 mM MgCl_2 , 10 mM KCl, and 0.5 mM dithiothreitol. Nuclear protein were extracted with a high-salt buffer containing 20 mM HEPES-KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM MgCl_2 , 0.2 mM EDTA, and 0.5 mM dithiothreitol. All the buffers used for the procedure contained protease inhibitors, i.e., 100 μg of PMSF per ml, 1 μg of leupeptin per ml, and 1 μg of aprotinin per ml. When it was necessary, nuclear extracts were concentrated with a Centricon 10 instrument (Millipore). Small aliquots of nuclear proteins were immediately frozen and kept at -80°C until use. To study AP-1 binding, nuclear extracts (5 μg) from different lung cancer cells were analyzed by gel retardation assay for their AP-1 binding activity, using ^{32}P -labeled AP-1 as a probe as described previously (1). The AP-1 binding site used in the experiments was derived from the collagenase promoter (TGACTCA). Labeled DNA probes were purified by gel electrophoresis and used for the gel retardation assay.

RESULTS

AHPN/CD437 is more effective than *trans*-RA in inhibiting the growth of human lung cancer cell lines. Recently, the synthetic retinoid AHPN/CD437 was shown to inhibit the growth and induce apoptosis in breast cancer cell lines in a retinoid receptor- and p53-independent manner (57). Because most human NSCLC cell lines express low levels of RAR β and are refractory to growth inhibition by *trans*-RA (34a, 72, 78), we analyzed whether AHPN/CD437 could inhibit the growth of four NSCLC lines, which show various degrees of *trans*-RA sensitivity (34a). These NSCLC lines, SK-MES-1, H460, A549, and H292, were treated with 10^{-6} M AHPN/CD437 or *trans*-RA for different periods of time, and cell numbers were determined daily by the MTT assay. As shown in Fig. 1a, *trans*-RA, at 10^{-6} M, inhibited the growth of A549 and H460 cells by only 20% after 5 days. AHPN/CD437, however, had a much greater growth-inhibitory effect on these cells, with about 50% inhibition. *trans*-RA did not show a clear inhibitory effect on the growth of SK-MES-1 and H292 cells. However, the growth of these cells was strongly inhibited (about 50%) by AHPN/CD437. Growth inhibition by AHPN/CD437 in these lung cancer cell lines was apparent after 2 days of treatment with 10^{-6} M AHPN/CD437. Dose-response experiments (Fig. 1b) demonstrate that AHPN/CD437 reduced the numbers of H460, SK-MES-1, A549, and H292 cells with 50% inhibitory values of approximately 5×10^{-7} , 4×10^{-7} , 3×10^{-6} , and 8.5×10^{-7} M, respectively, which are much lower than those of *trans*-RA. These data demonstrate that AHPN/CD437 is more effective than *trans*-RA in inhibiting the growth of these four NSCLC lines.

Induction of apoptosis in human lung cancer cell lines by AHPN/CD437. AHPN/CD437 was previously shown to induce apoptosis in human breast cancer cell lines (57). To determine whether apoptosis induction could account for its growth-inhibitory effect observed above, nuclear morphology of AHPN/CD437-treated or -untreated lung cancer cells was studied. For comparison, cells treated with *trans*-RA were also analyzed. Treatment of H460, SK-MES-1, and A549 cells with 10^{-6} M AHPN/CD437 for 2 days caused the classical morphological characteristics of apoptosis, including nuclear condensation and fragmentation (Fig. 2a). By contrast, treatment of H292 cells displayed a normal nuclear morphology similar to that of

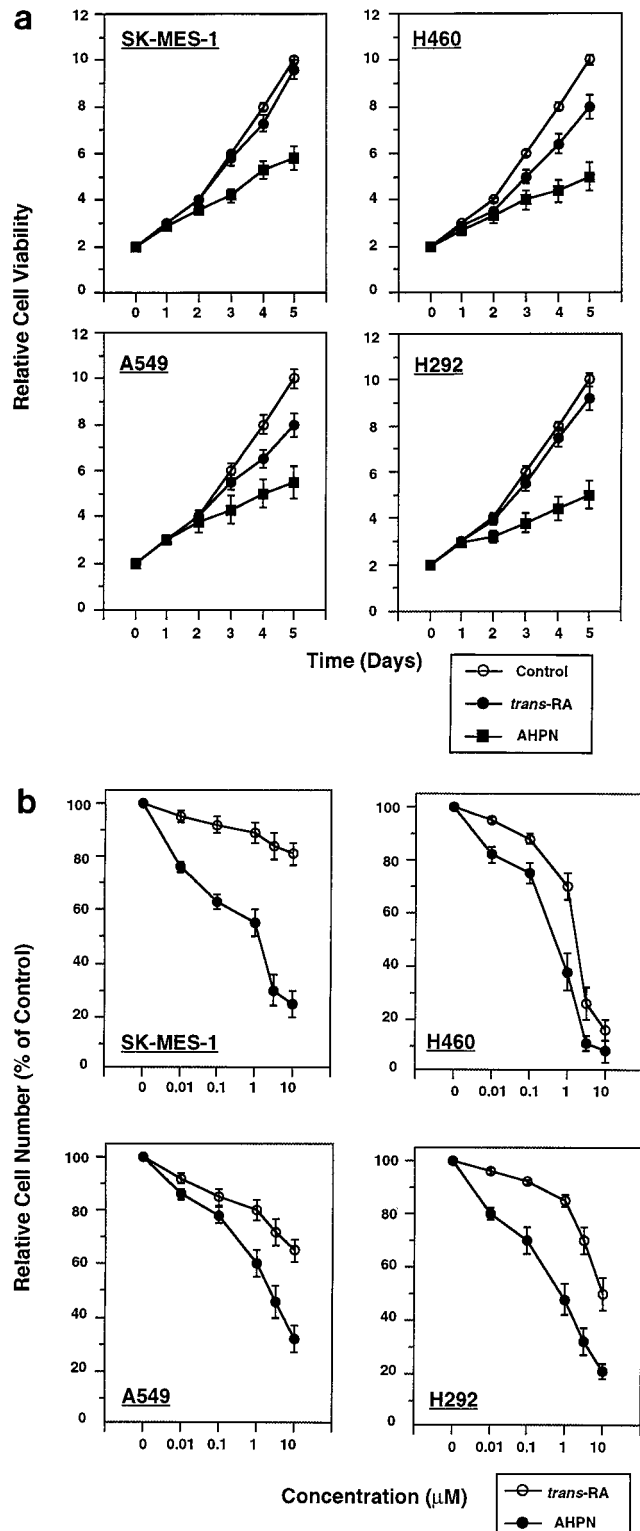


FIG. 1. Growth inhibition by AHPN/CD437 in human lung cancer cell lines. (a) Time course study. The lung cancer cell lines were seeded at a cell density of 1,000 cells/well in 96-well plates. The cells were then treated with 10^{-6} M AHPN/CD437 for the indicated times, and cell viability was then determined by the MTT assay. (b) Effect of AHPN/CD437 concentration on lung cancer cell proliferation. The lung cancer cell lines were treated with the indicated concentration of AHPN/CD437 for 4 days, and cell viability was determined by the MTT assay.

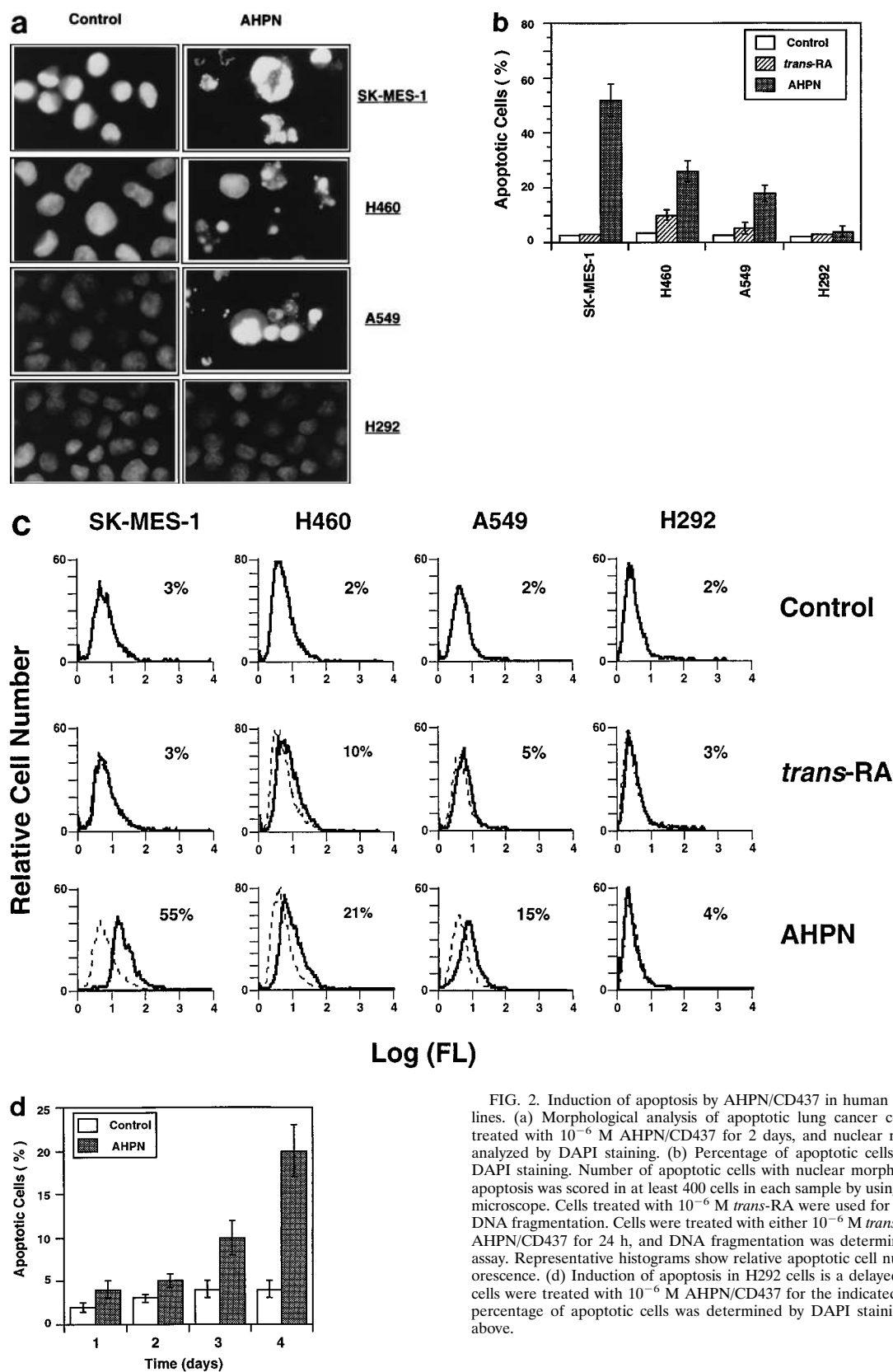


FIG. 2. Induction of apoptosis by AHPN/CD437 in human lung cancer cell lines. (a) Morphological analysis of apoptotic lung cancer cells. Cells were treated with 10^{-6} M AHPN/CD437 for 2 days, and nuclear morphology was analyzed by DAPI staining. (b) Percentage of apoptotic cells determined by DAPI staining. Number of apoptotic cells with nuclear morphology typical of apoptosis was scored in at least 400 cells in each sample by using a fluorescence microscope. Cells treated with 10^{-6} M *trans*-RA were used for comparison. (c) DNA fragmentation. Cells were treated with either 10^{-6} M *trans*-RA or 10^{-6} M AHPN/CD437 for 24 h, and DNA fragmentation was determined by the TdT assay. Representative histograms show relative apoptotic cell numbers. FL, fluorescence. (d) Induction of apoptosis in H292 cells is a delayed process. H292 cells were treated with 10^{-6} M AHPN/CD437 for the indicated times, and the percentage of apoptotic cells was determined by DAPI staining as described above.

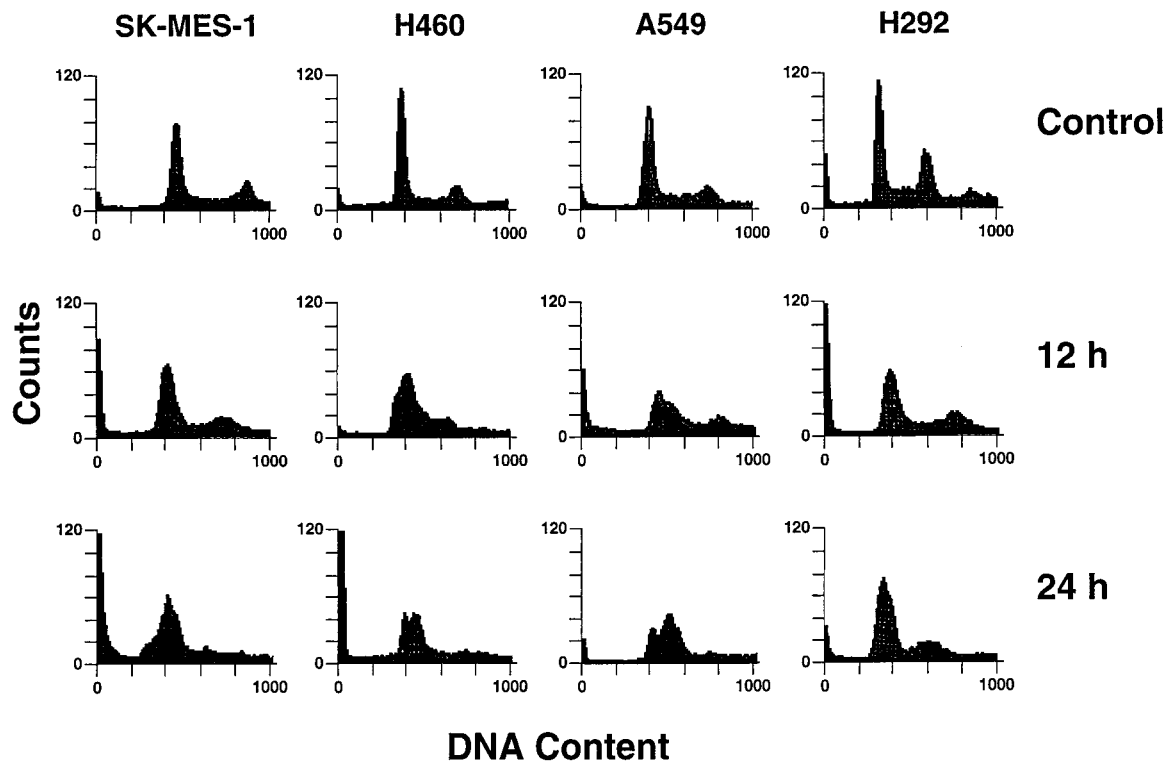


FIG. 3. DNA content analysis by flow cytometry in human lung cancer cell lines. Cells were treated with 10^{-6} M AHPN/CD437 for the indicated times, stained with propidium iodide, and analyzed by flow cytometry. The DNA content is presented as relative fluorescence. Cells in G_0/G_1 phase are in the first peak, and cells in G_2/M phase are in the second peak. Cells in S phase are in the area between the G_0/G_1 and G_2/M phase peaks. Quantitation of the cell cycle distribution is presented in Table 1.

untreated cells. When apoptotic cells were scored based on morphological criteria (Fig. 2b), about 52% of SK-MES-1, 26% of H460, and 17% of A549 cells displayed apoptotic morphology in response to AHPN/CD437. Under the same conditions, *trans*-RA did not show a clear apoptosis-inducing effect on these cell lines, except that a slight induction (10%) of apoptotic cells was observed in H460 cells. AHPN/CD437-induced apoptosis was studied further by the TdT assay. As shown in Fig. 2c, extensive DNA fragmentation was observed in AHPN/CD437-treated SK-MES-1, H460, and A549 cells, but not in H292 cells when they were treated for 1 day. To determine whether apoptosis in H292 cells was a delayed process, H292 cells were treated with 10^{-6} M AHPN/CD437 for longer periods of time and apoptosis was studied by nuclear morphology changes. As shown in Fig. 2d, apoptosis was also observed in H292 cells, when these cells were exposed to 10^{-6} M AHPN/CD437 for 3 days or longer.

Induction of G_0/G_1 arrest by AHPN/CD437 in human lung cancer cell lines. Because AHPN/CD437 was previously re-

ported to inhibit breast cancer cell growth by inducing G_0/G_1 arrest (57), we then investigated whether AHPN/CD437 could also induce G_0/G_1 arrest in NSCLC lines. The DNA content analysis (Fig. 3) showed that various NSCLC lines underwent a stable G_0/G_1 arrest after AHPN/CD437 treatment. The entry of these cells into S phase was suppressed, while G_0/G_1 phase increased as early as 12 h after treatment with 10^{-6} M AHPN/CD437 (Table 1). Interestingly, H292 cells that exhibited a delayed apoptotic process in response to AHPN/CD437 (Fig. 2), also showed G_0/G_1 arrest similar to those of other cell lines. Thus, AHPN/CD437 can induce G_0/G_1 arrest in human lung cancer cell lines independently of their apoptotic process.

Induction of p21^{WAF1/CIP1} and p53 by AHPN/CD437 in human lung cancer cell lines. To obtain insight into the molecular mechanism by which AHPN/CD437 induces G_0/G_1 arrest in human NSCLC lines, we examined the effect of AHPN/CD437 on gene expression. Since p21^{WAF1/CIP1} is known to play an important role in growth arrest that follows exposure to various stimuli (12, 14, 21, 24, 45, 73) and is highly induced by

TABLE 1. Cell cycle distribution of human lung cancer cell lines

Cell cycle phase	Distribution in cell cycle (%) ^a											
	SK-MES-1			H460			A549			H292		
	Cont.	12 h	24 h	Cont.	12 h	24 h	Cont.	12 h	24 h	Cont.	12 h	24 h
G_0/G_1	55	69	81	58	77	80	62	74	79	52	63	74
S	22	15	11	19	14	12	26	18	17	21	11	8
G_2/M	23	16	8	23	9	8	12	8	4	27	26	18

^a Cell cycle distribution of cells after treatment with 1 μ M AHPN. Values represent the results of flow cytometry experiments. Representative histograms are shown in Fig. 3. Cont., control.

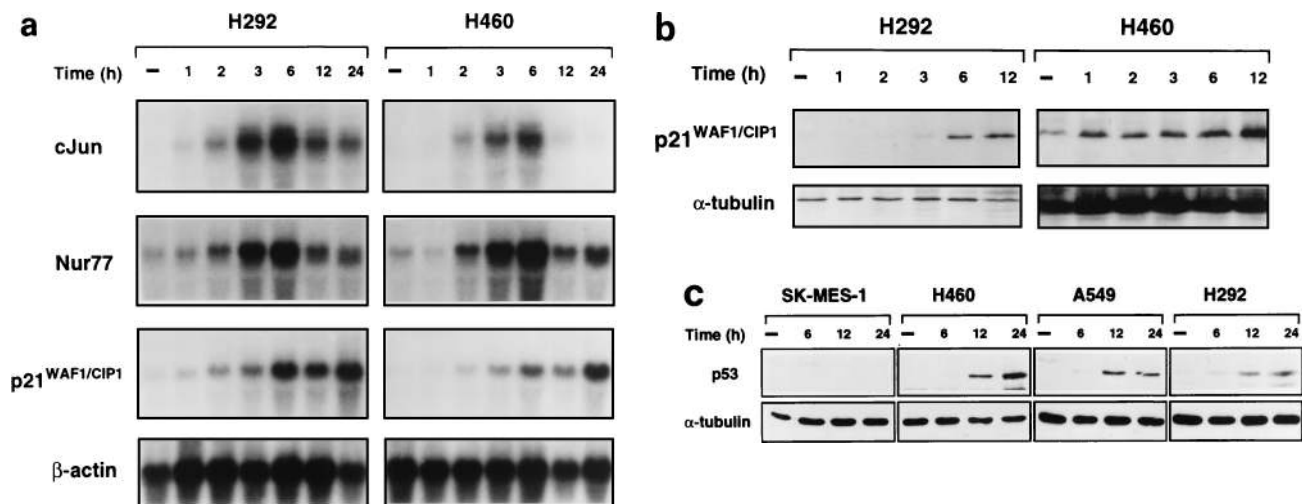


FIG. 4. Regulation of gene expression by AHPN/CD437 in human lung cancer cell lines. (a) Northern blot analysis. H292 and H460 cells were treated with 10^{-6} M AHPN/CD437 for the indicated times, and total RNAs were prepared and analyzed for the expression of the indicated gene by Northern blotting. Expression of the β -actin was used to ensure that equal amounts of RNA were used. (b) Western blot analysis of *p21^{WAF1/CIP1}* expression. Cell extracts were prepared from H292 or H460 cells treated with 10^{-6} M AHPN/CD437 for the indicated times and analyzed for the expression of *p21^{WAF1/CIP1}* by Western blot analysis using a monoclonal anti-*p21^{WAF1/CIP1}* antibody. Expression of the α -tubulin was used to control protein loading. (c) Western blot analysis of p53 expression in SK-MES-1, H460, A549, and H292 cells. Cell extracts were prepared from the indicated cell lines treated with 10^{-6} M AHPN/CD437 for the indicated times and analyzed for the expression of p53 by Western blot analysis. Expression of α -tubulin was used as a control. —, not treated with AHPN/CD437.

AHPN/CD437 in breast cancer cells (57), we studied whether *p21^{WAF1/CIP1}* was also induced in NSCLC lines by AHPN/CD437. We initially determined the expression of *p21^{WAF1/CIP1}* at mRNA levels in various NSCLC lines treated with 10^{-6} M AHPN/CD437 for different periods of time (Fig. 4a). Treatment with AHPN/CD437 caused a marked up-regulation of *p21^{WAF1/CIP1}* message in H292 and H460 cells (Fig. 4a). Induction of *p21^{WAF1/CIP1}* message occurred as early as 1 h after exposure to 10^{-6} M AHPN/CD437, and an increase of about 35-fold was observed after 24 h of treatment in these cells. Levels of *p21^{WAF1/CIP1}* protein were also examined by Western blotting using an anti-*p21^{WAF1/CIP1}* monoclonal antibody (Fig. 4b). The amounts of the 21-kDa protein in H460 and H292 cell lines correlated with the levels of *p21^{WAF1/CIP1}* mRNA. Similar observations were also made in SK-MES-1 and A549 cells (data not shown). Thus, induction of *p21^{WAF1/CIP1}* may be responsible for AHPN/CD437-induced G_0/G_1 arrest.

Expression of *p21^{WAF1/CIP1}* is mediated by both p53-dependent and -independent mechanisms (13, 24, 31, 39, 49, 61). We, therefore, studied whether induction of *p21^{WAF1/CIP1}* and G_0/G_1 arrest in NSCLC lines was due to up-regulation of p53. H460, A549, and H292 cells express wild-type p53, while SK-MES-1 cells contain mutated p53 (55). Western blot analysis using an anti-p53 antibody showed that levels of p53 protein were rapidly induced by AHPN/CD437 in H460, A549, and H292 cells (Fig. 4c). In contrast, we did not observe any induction or expression of p53 protein in SK-MES-1 cells. These data suggest that induction of p53 may contribute to expression of *p21^{WAF1/CIP1}* in H460, A549, and H292 cells. However, the observation that p53 was not expressed in SK-MES-1 cells also indicates that induction of *p21^{WAF1/CIP1}* and G_0/G_1 arrest by AHPN/CD437 can be mediated by a p53-independent mechanism.

Induction of c-Jun and nur77 by AHPN/CD437 in human lung cancer cell lines. Because nur77 is involved in regulating apoptotic process (36, 71), we then examined whether nur77 was induced by AHPN/CD437 in NSCLC lines. Northern blot analysis demonstrated that nur77 message was highly induced by AHPN/CD437 in H292 and H460 cells (Fig. 4a), as well as

in SK-MES-1 and A549 cells (data not shown). Induction of nur77 was very fast, occurring as early as 2 h after exposure to AHPN/CD437, with maximal induction observed after 6 h of treatment, which then gradually decreased after 12 h of treatment.

Activation of nur77 is mainly mediated by the presence of multiple AP-1 binding sites in its promoter (64, 69). We next determined whether c-Jun, a component of AP-1, was up-regulated by AHPN/CD437. Figure 4a shows that c-Jun was rapidly induced by AHPN/CD437 in H292 and H460 cells. Time course analysis demonstrated that induction of c-Jun transcript was apparent with only 1 h of exposure to 10^{-6} M AHPN/CD437. Similar to the induction of nur77, treatment with AHPN/CD437 for 6 h resulted in a maximal induction of c-Jun, which then gradually decreased after prolonged treatments. The similarity in the induction patterns of c-Jun and nur77 suggests that c-Jun may be involved in the regulation of nur77 expression.

To further determine AHPN/CD437-induced AP-1 activities, we measured AP-1 binding and transactivation activities in H460 and H292 cells. Nuclear proteins from cells treated with or without 10^{-6} M AHPN/CD437 for 6 h were analyzed for their AP-1 binding activity by using the AP-1 binding site as a probe. Figure 5a demonstrates that nuclear proteins from AHPN/CD437-treated cells formed a much stronger binding complex with the AP-1 binding site than those from nontreated cells, suggesting that AHPN/CD437 could induce AP-1 binding activities. We next determined AHPN/CD437-induced AP-1 transactivation activity in H460 and H292 cells by a transient-transfection assay, using -73Col-CAT as a reporter. The reporter contains the CAT gene linked with the collagenase promoter that contains an AP-1 binding site, and the reporter is often used to measure AP-1 activities (74). As shown in Fig. 5b, when the -73Col-CAT reporter was transfected into H460 or H292 cells, we observed a strong induction of reporter transcription when cells were treated with AHPN/CD437. The induction of reporter activities was AHPN/CD437 concentration dependent. Thus, AHPN/CD437 can induce AP-1 activity in human lung cancer cells.

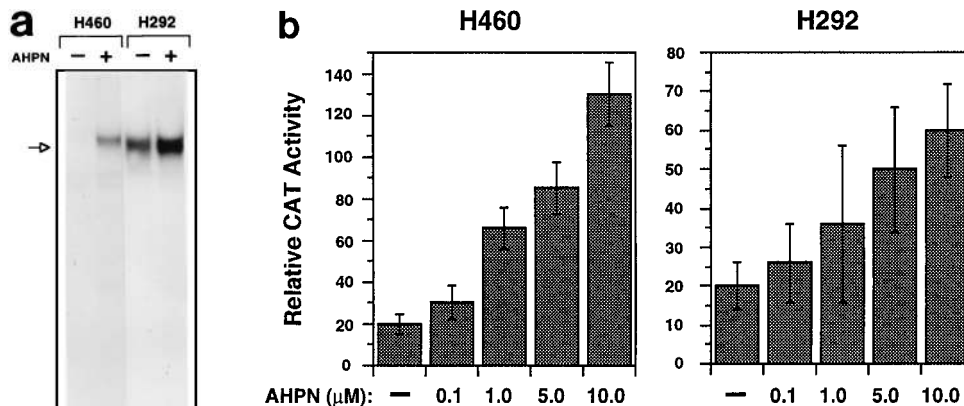


FIG. 5. Effect of AP-1 activity by AHPN/CD437 in human lung cancer cell lines. (a) Induction of AP-1 binding activity by AHPN/CD437 in H460 and H292 lung cancer cells. Nuclear proteins were prepared from H460 or H292 cells treated with 10^{-6} M AHPN/CD437 for 6 h (+) or not treated with AHPN/CD437 (-). Following this preincubation, the reaction mixtures were incubated with 32 P-labeled AP-1 probe and analyzed by the gel retardation assay. The arrow points to the position of the AP-1 binding complex. (b) Induction of AP-1 activity by AHPN/CD437 in H460 and H292 cells. The -73Col-CAT reporter was transfected into H460 or H292 cells. After transfection, the cells were treated with the indicated concentration of AHPN/CD437 and harvested 24 h later. CAT activities were then determined. β -Gal activities were measured to normalize transfection efficiency.

Regulation of Bcl-2 expression by AHPN/CD437 in lung cancer cell lines. Members of the Bcl-2 family are involved in the regulation of the apoptotic process (34, 54). To determine their involvement in AHPN/CD437-induced apoptosis in human NSCLC lines, expression of several members of the Bcl-2 family in response to AHPN/CD437 was examined by Western blotting (Fig. 6). When the expression of Bcl-2 was analyzed, we did not detect any expression of Bcl-2 in apoptosis-sensitive A549 cells (Fig. 6a). Bcl-2 was expressed in apoptosis-sensitive SK-MES-1 and H460 cells. However, its expression levels were dramatically inhibited when the cells were treated with 10^{-6} M AHPN/CD437. Inhibition of Bcl-2 expression could be observed when the cells were treated for 12 h, and after a 24-h treatment, expression of Bcl-2 was almost completely inhibited. In contrast, Bcl-2 was highly expressed and its expression could not be clearly inhibited by AHPN/CD437 in H292 cells. When other members of the Bcl-2 family were studied, we found that Mcl-1, Bcl-X_L, and Bax were highly expressed in H460 cells (Fig. 6b) and the other NSCLC lines (data not shown), and their expression was not clearly affected by AHPN/CD437 treatment except that a slight induction of Bax was observed after 24 h of treatment (Fig. 6b). These data suggest that the lack of Bcl-2 in A549 cells or its inhibition in SK-MES-1 and H460 cells may render lung cancer cells more susceptible to the apoptosis-inducing effect of AHPN/CD437.

c-Jun-mediated nur77 induction is critical for AHPN/CD437-induced apoptosis. Our observation that c-Jun induction by AHPN/CD437 preceded nur77 and p21^{WAF1/CIP1} induction suggested that c-Jun might play a critical role in the induction of nur77 and p21^{WAF1/CIP1} and subsequent growth arrest or apoptosis by AHPN/CD437. We, therefore, stably transfected a dominant-negative c-Jun [c-JunAla(63,73)] cDNA into A549 cells. c-JunAla(63,73), where the two serine residues at positions 63 and 73 are replaced by two alanine residues, functions as a dominant inhibitor of endogenous c-Jun and has been successfully used in a number of previous studies (6, 52, 53, 59, 60). A stable clone that expressed a high level of c-JunAla(63,73) was analyzed for its effect on AP-1 transactivation activity by a transient-transfection assay using the -73Col-CAT reporter. As shown in Fig. 7a, the -73Col-CAT reporter was highly activated by AHPN/CD437 in paren-

tal A549 cells or A549 stably expressing an empty control vector (A549/vec). However, treatment of A549 cells that expressed the dominant-negative c-Jun (A549/dnJun) with AHPN/CD437 did not show any clear effect on collagenase promoter activity. We also examined the effect of the dominant-negative c-Jun on AHPN/CD437-induced endogenous c-Jun expression by Western blotting (Fig. 7b). Expression of

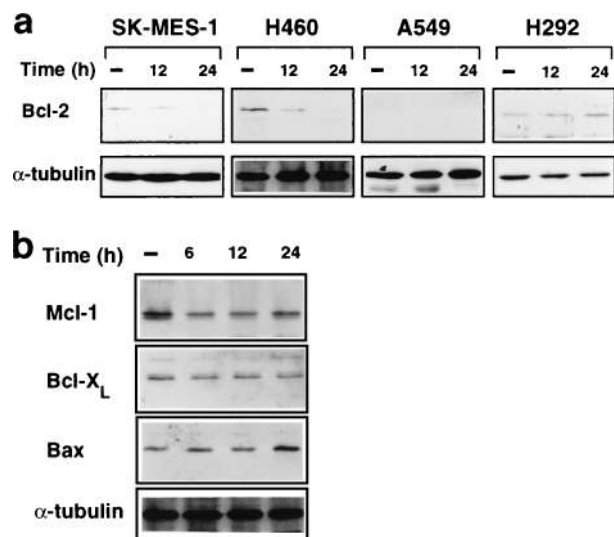


FIG. 6. Effect on expression of members of the Bcl-2 family genes in human lung cancer cell lines by AHPN/CD437. (a) Western blot analysis of Bcl-2 expression. Cell extracts were prepared from SK-MES-1, H460, A549, or H292 cells treated with 10^{-6} M AHPN/CD437 for the indicated times and analyzed for the expression of Bcl-2 by Western blot analysis using a polyclonal anti-Bcl-2 antibody. (b) Western blot analysis of Bcl-2 family genes in H460 lung cancer cell line. Cell extracts were prepared from H460 cells treated with 10^{-6} M AHPN/CD437 for the indicated times and analyzed for the expression of Mcl-1, Bcl-X_L, and Bax genes by Western blot analysis using a polyclonal anti-Mcl-1, anti-Bcl-X_L, or anti-Bax antibody. Expression of the α -tubulin was used as a control. -, not treated with AHPN/CD437.

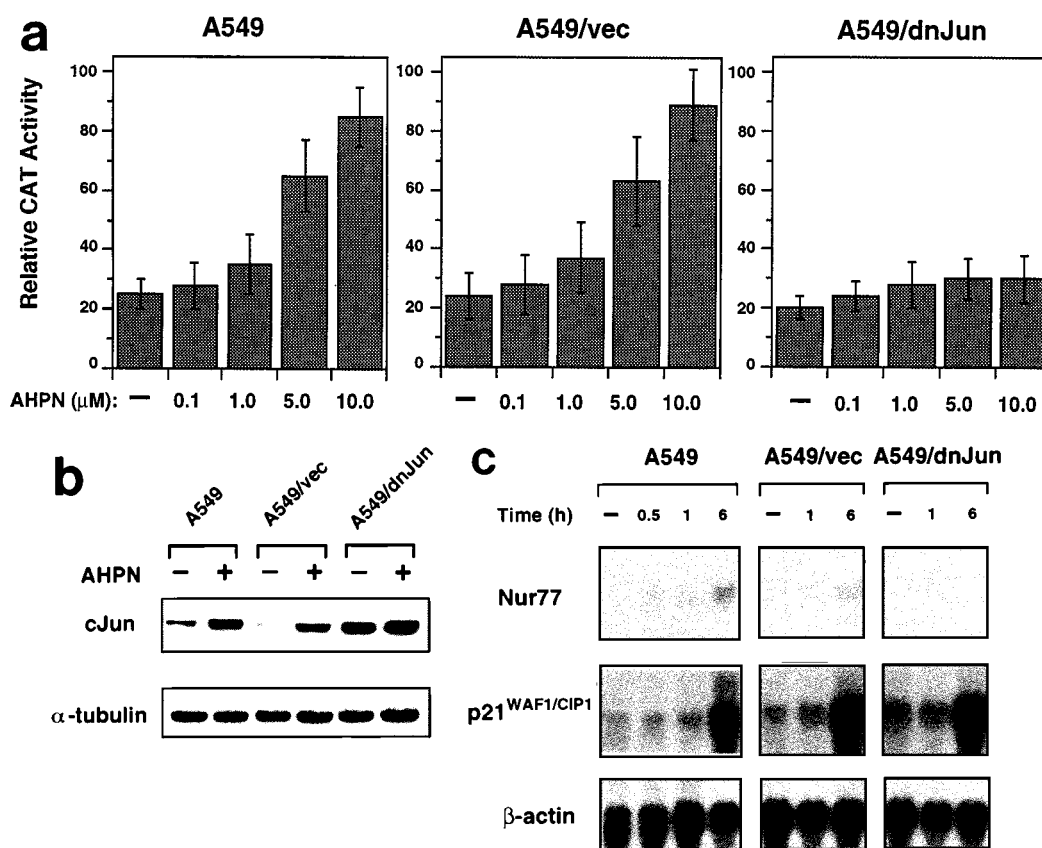


FIG. 7. Effect of dominant-negative c-Jun on AHPN/CD437-induced AP-1 transactivation activity and induction of nur77 and p21^{WAF1/CIP1} by AHPN/CD437 in A549 cells. (a) Inhibition of AHPN/CD437-induced AP-1 activity by dominant-negative c-Jun expression in A549 cells. The -73Col-CAT reporter was transfected into A549, A549/vec, and A549/dnJun cells. After transfection, the cells were treated with the indicated concentration of AHPN/CD437 and harvested 24 h later. CAT activities were then determined. β-Gal activities were measured to normalize transfection efficiency. (b) Inhibition of AHPN/CD437-induced c-Jun expression of dominant-negative c-Jun expression. Cells were treated with 10⁻⁶ M AHPN/CD437 for 4 h (+) and analyzed for the expression of c-Jun by Western blotting using anti-c-Jun antibody (Santa Cruz Biotechnology Inc.). (c) Effect of dominant-negative c-Jun expression on nur77 and p21^{WAF1/CIP1} expression. Indicated cells were treated with 10⁻⁶ M AHPN/CD437 for the indicated times, and total RNAs were prepared and analyzed for the expression of the indicated gene by Northern blotting. Expression of the β-actin was used to control RNA loading. -, not treated with AHPN/CD437.

c-Jun in A549 and A549/vec cells was strongly induced by AHPN/CD437. A549/dnJun expressed an increased level of immunoreactive c-Jun as reported previously (6). However, treatment of A549/dnJun cells with AHPN/CD437 only slightly increased the immunoreactive c-Jun level. These data suggest that AHPN/CD437-induced AP-1 activity was largely inhibited by expression of the dominant-negative c-Jun, probably through interference with the endogenous activated c-Jun. We next examined whether nur77 or p21^{WAF1/CIP1} was induced by AHPN/CD437 in the A549/dnJun clone. nur77 was strongly induced in A549 and A549/vec cells when they were treated with 10⁻⁶ M AHPN/CD437 for 6 h. However, the same treatment failed to induce nur77 in A549/dnJun cells (Fig. 7c). These data demonstrate that overexpression of the dominant-negative c-Jun inhibits the induction of nur77 by AHPN/CD437. When expression of p21^{WAF1/CIP1} was analyzed, we observed a similar induction pattern by AHPN/CD437 in A549, A549/vec, and A549/dnJun cells (Fig. 7c), indicating that expression of the dominant-negative c-Jun did not have any effect on p21^{WAF1/CIP1} expression. Thus, induction of c-Jun activity by AHPN/CD437 is critical for nur77 expression, but not for p21^{WAF1/CIP1} expression.

We next determined whether loss of AP-1 activities and nur77 expression in the dominant-negative c-Jun stable clone affected AHPN/CD437-induced growth arrest or apoptosis. As

shown in Fig. 8a, AHPN/CD437 strongly inhibited the growth of the A549/vec clone, similar to that observed in the parental A549 cells. However, the growth-inhibitory effect of AHPN/CD437 was largely reduced in the A549/dnJun stable clone, with only 12% growth inhibition by 10⁻⁶ M AHPN/CD437 compared to 35% growth inhibition in the control stable clone. We then examined whether inhibition of AP-1 and nur77 expression by the dominant-negative c-Jun decreased AHPN/CD437-induced apoptosis by the TdT assay (Fig. 8b). Treatment of 10⁻⁶ M AHPN/CD437 induced about 15% of apoptotic cells in A549 cells and 17% in A549/vec cells. However, the same treatment failed to induce apoptosis in the A549/dnJun clone. These data therefore demonstrate that induction of c-Jun by AHPN/CD437 is critical for AHPN/CD437-induced apoptosis. We also studied the effect of dominant-negative c-Jun expression on G₀/G₁ arrest by cell cycle analysis (Fig. 8c). A549, A549/vec, and A549/dnJun all underwent G₀/G₁ arrest in response to treatment with 10⁻⁶ M AHPN/CD437 (Table 2). Thus, inhibition of c-Jun and nur77 expression did not affect AHPN/CD437-induced G₀/G₁ arrest, suggesting that p21^{WAF1/CIP1}, rather than c-Jun and nur77, is critical for AHPN/CD437-induced G₀/G₁ arrest in these lung cancer cells.

In order to provide more-direct evidence for the involvement of nur77 in mediating c-Jun-induced apoptosis, we stably

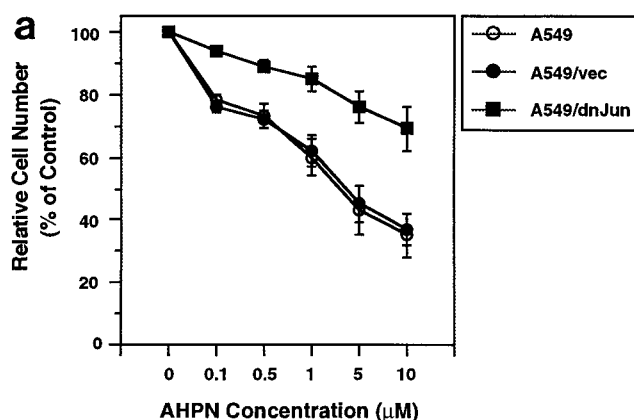
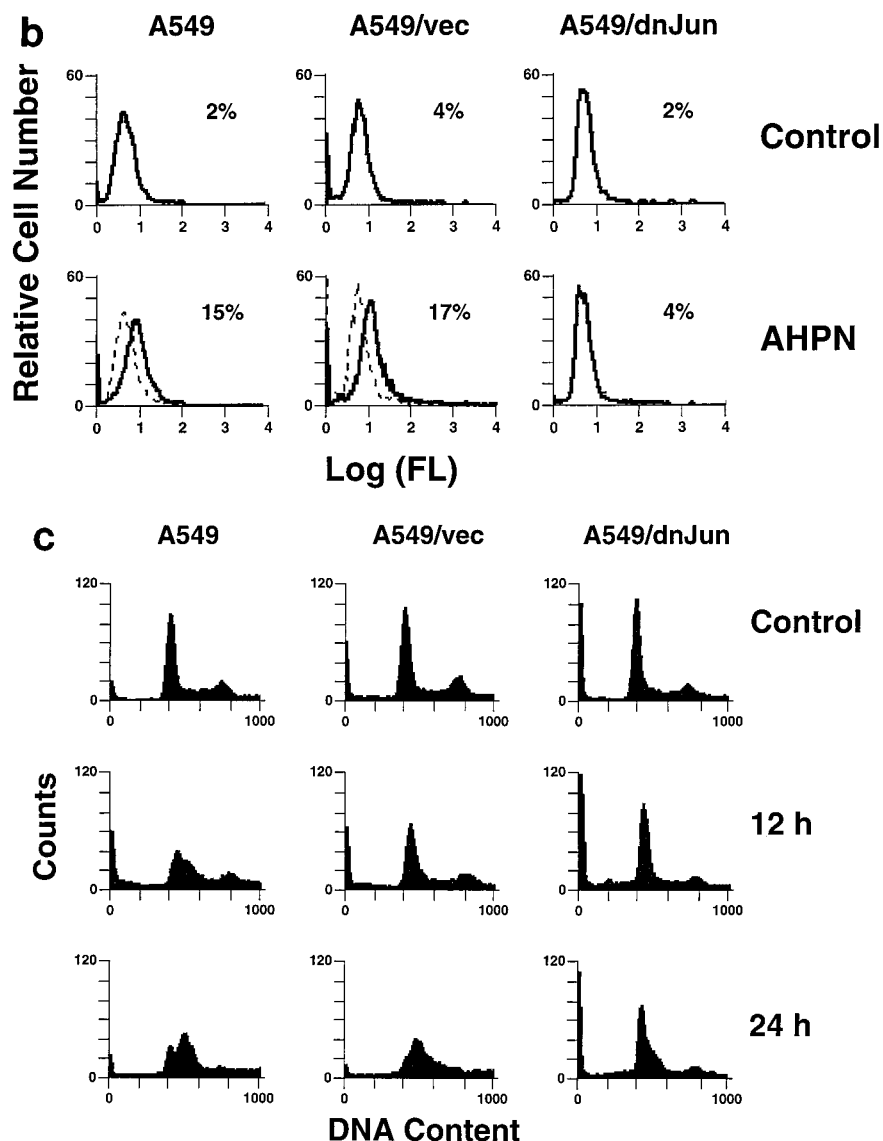


FIG. 8. Effect of dominant-negative c-Jun expression on growth inhibition, apoptosis induction, and G_0/G_1 arrest by AHPN/CD437 in A549 cells. (a) Effect on cell proliferation by AHPN/CD437. Cells were seeded at 1,000 cells per well in 96-well plates and treated with the indicated concentrations of AHPN/CD437 for 4 days. The number of viable cells was determined by the MTT assay. (b) Effect on apoptosis induction by AHPN/CD437. Cells were treated with 10^{-6} M AHPN/CD437 for 24 h, and apoptosis induction was analyzed by the TdT assay. Representative histograms show relative apoptotic cell numbers. FL, fluorescence. (c) Effect on G_0/G_1 arrest by AHPN/CD437. Cells were treated with 10^{-6} M AHPN/CD437 for the indicated times, stained with propidium iodide, and analyzed by flow cytometry. The DNA content is presented as relative fluorescence. Quantitation of the cell cycle distribution is presented in Table 2.



expressed antisense *nur77* cDNA in A549 cells. A stable clone that expressed the antisense *nur77* RNA (A549/A-nur77) was analyzed for apoptosis in response to AHPN/CD437. As shown in Fig. 9a, AHPN/CD437-induced apoptosis in A549/A-nur77

cells was largely impaired, whereas the response to AHPN/CD437 of a control clone that expressed the empty vector (A549/vec) was similar to that of the parental A549 cells. To further demonstrate the involvement of *nur77*, we stably ex-

TABLE 2. Effect on cell cycle of AHPN/CD437 in A549/dnJun cells

Cell cycle phase	Distribution in cell cycle (%) ^a								
	A549			A549/vec			A549/dnJun		
	Cont.	12 h	24 h	Cont.	12 h	24 h	Cont.	12 h	24 h
G ₀ /G ₁	62	74	79	62	76	78	66	74	76
S	26	18	17	26	15	16	24	18	16
G ₂ /M	12	8	4	12	9	6	10	8	8

^a Cell cycle distribution of cells after treatment with 1 μ M AHPN. Values represent the results of flow cytometry experiments. Representative histograms are shown in Fig. 8c. Cont., control.

pressed antisense *nur77* RNA in H460 cells. Two stable clones (H460/A-nur77#14 and H460/A-nur77#15) that expressed antisense *nur77* RNA showed few apoptotic cells (4%) when they were treated with AHPN/CD437 for 24 h, whereas H460 cells and a control clone that expressed the empty vector (H460/vec) exhibited 21 and 20% apoptotic cells, respectively (Fig. 9b). These data suggest that *nur77* is an important downstream target of c-Jun in AHPN/CD437-induced apoptotic process.

DISCUSSION

AHPN/CD437, originally identified as a RAR γ -selective retinoid (3), inhibits the growth and induces the apoptosis of breast cancer (57, 68), cervical cancer (9, 47), melanoma (56), and leukemia (29) cells. In this study, we demonstrate that AHPN/CD437, through induction of apoptosis and G₀/G₁ arrest, is also a more potent inhibitor than *trans*-RA on the growth of four human NSCLC lines, including a p53-deficient cell line. Our results show that induction of G₀/G₁ arrest is mainly due to induction of p21^{WAF1/CIP1}. Importantly, we found that induction of c-Jun and *nur77* plays a critical role in AHPN/CD437-induced apoptosis.

AHPN/CD437 induced G₀/G₁ arrest in four NSCLC lines investigated (Fig. 3 and Table 1). Our results demonstrate that induction of p21^{WAF1/CIP1} is likely responsible for AHPN/CD437-induced G₀/G₁ arrest. p21^{WAF1/CIP1} was highly induced by AHPN/CD437 in lung cancer cell lines (Fig. 4a and b), similar to that observed in human breast cancer cell lines (57). It occurred very rapidly, within 1 h of the AHPN/CD437 addition (Fig. 4), suggesting that p21^{WAF1/CIP1} may be one of the AHPN/CD437 primary response genes. p21^{WAF1/CIP1} is a potent inhibitor of cdk complexes and is induced in response to DNA damage and in senescent cells (14, 21, 24, 45, 73). Recent evidence indicates that p21^{WAF1/CIP1} arrests the cell cycle in G₀/G₁ by preventing the phosphorylation of critical cdk substrates required for cell cycle progression (14, 21, 24, 45, 73). Although expression of c-Jun and *nur77* was also rapidly induced by AHPN/CD437 (Fig. 4a), our observation that inhibition of c-Jun and *nur77* expression by a dominant-negative c-Jun did not affect G₀/G₁ arrest in A549 cells (Fig. 8) suggests that c-Jun and *nur77* are unlikely to be involved in AHPN/CD437-induced cell cycle arrest. Thus, the marked increase of p21^{WAF1/CIP1} provides a likely explanation for the action of AHPN/CD437 in inducing G₀/G₁ arrest and preventing cell cycle progression.

Induction of p21^{WAF1/CIP1} is mainly mediated by p53-dependent and -independent mechanisms (13, 24, 31, 39, 49, 61). Our results (Fig. 4c) suggest that induction of p53 by AHPN/CD437 may contribute to its inducing effect on p21^{WAF1/CIP1} expression in certain lung cancer cell lines. However, the facts that SK-MES-1 cells lack wild-type p53 (55) and did not show p53 induction upon AHPN/CD437 treatment (Fig. 4c) suggest that p21^{WAF1/CIP1} induction by AHPN/CD437 can be mediated by a p53-independent mechanism. Such a p53-independent mech-

anism of AHPN/CD437 was previously demonstrated in breast cancer cells (57). Since most lung cancer cell lines lack wild-type p53 (63), aberrant cell proliferation resulting from mutated or deleted p53 could be attenuated by AHPN/CD437.

Human NSCLC cells underwent extensive apoptosis in response to AHPN/CD437, as evidenced by typical morphological changes and by DNA degradation in the TdT assay (Fig. 2). Our results demonstrate that apoptosis induction by AHPN/CD437 involves regulation of genes for c-Jun, *nur77*, p53, and Bcl-2. Induction of c-Jun is probably the critical step. It occurs very fast, within 1 h of addition of AHPN/CD437 (Fig. 4). Expression of a transdominant inhibitor of c-Jun that inhibited AHPN/CD437-induced c-Jun expression and AP-1 transactivation activities (Fig. 7a and b) inhibited AHPN/CD437-induced apoptosis (Fig. 8b), but not AHPN/CD437-induced G₀/G₁ arrest (Fig. 8c) in A549 cells. These results convincingly demonstrate that c-Jun is a critical mediator of AHPN/CD437-induced apoptosis. In addition, they indicate that AHPN/CD437-induced G₀/G₁ arrest and apoptosis are separate signaling responses. The involvement of c-Jun in the apoptotic progress is supported by a recent report that an increase of c-Jun activation is sufficient to trigger apoptotic cell death in NIH 3T3 fibroblasts (5). Moreover, strong and prolonged induction of c-Jun has also been reported in response to stress-inducing stimuli, including UV and ionizing irradiation, growth factor deprivation, and anticancer agents that can trigger apoptosis (7, 11, 15, 19, 23, 33, 41).

The apoptotic activity of AHPN/CD437 is also mediated by nuclear orphan receptor *nur77*. *nur77* was highly induced by AHPN/CD437 in all four NSCLC lines investigated, and induction occurred when cells were exposed to AHPN/CD437 for as short a time as 2 h (Fig. 4). The involvement of *nur77* is shown by our observation that inhibition of its expression by c-JunAla(63,73) completely abolished AHPN/CD437-induced apoptosis in A549 cells (Fig. 8b). Moreover, inhibition of *nur77* expression by overexpression of antisense *nur77* RNA largely impaired the ability of AHPN/CD437 to induce apoptosis in both A549 and H460 cells (Fig. 9). The apoptotic effect of *nur77* was originally demonstrated by the findings that it was highly induced during T-cell receptor-mediated apoptosis in T-cell hybridoma (36, 71) and that expression of dominant-negative or antisense *nur77* inhibited apoptosis (36, 71). Since *nur77* is involved in the transcriptional control of its target genes, it is likely that *nur77* may be responsible for induction of as yet unidentified genes executing cell death.

nur77 is likely one of the important downstream targets of c-Jun during AHPN/CD437-induced apoptotic process. This is based on our observation that induction of c-Jun preceded *nur77* induction by AHPN/CD437 (Fig. 4). c-Jun was induced by AHPN/CD437 within 1 h of exposure of H292 and H460 cells, while *nur77* message was induced after a 2-h treatment (Fig. 4). In addition, induction patterns of both genes were very similar, with a maximal induction after 6 h of treatment,

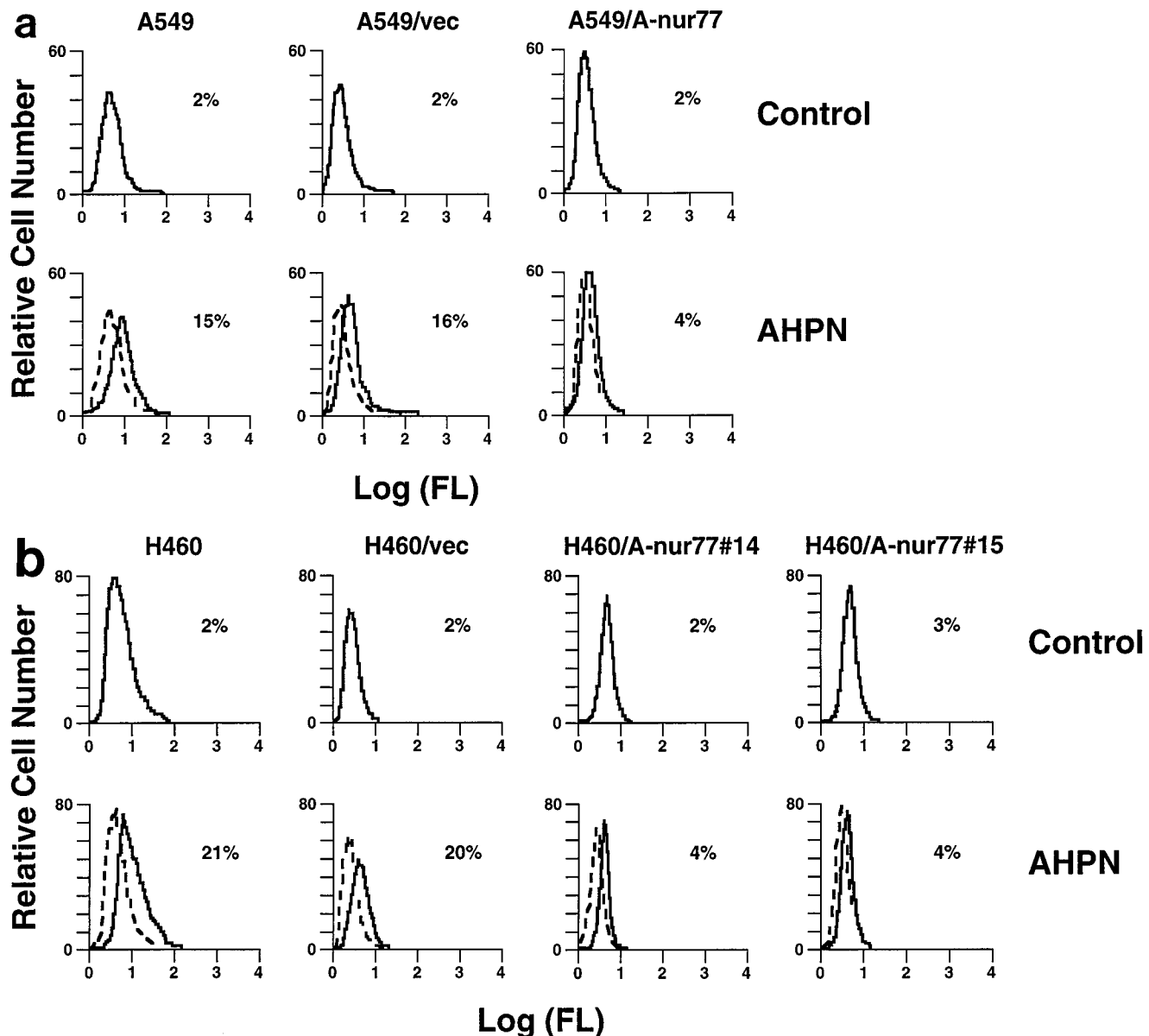


FIG. 9. Effect of antisense *nur77* RNA expression on apoptosis induction by AHPN/CD437 in lung cancer cells. (a) The stable A549 clones that express antisense *nur77* RNA (A549/A-nur77) or the empty vector (A549/vec) and the parental A549 cells were treated with 10^{-6} M AHPN/CD437 for 24 h, and apoptosis induction was analyzed by the TdT assay. (b) The stable H460 clones that express antisense *nur77* RNA (H460/A-nur77) or the empty vector (A549/vec) and the parental H460 cells were treated with 10^{-6} M AHPN/CD437 for 24 h, and apoptosis induction was analyzed by the TdT assay. Representative histograms show relative apoptotic cell numbers. FL, fluorescence.

followed by a gradual decrease with prolonged AHPN/CD437 treatment (Fig. 4). Moreover, expression of the dominant-negative c-Jun that inhibited AHPN/CD437-induced c-Jun expression (Fig. 7b) and AP-1 activity (Fig. 7a) impaired *nur77* induction by AHPN/CD437 (Fig. 7c). Induction of *nur77* expression by c-Jun is most likely mediated by several AP-1 binding sites present in the *nur77* promoter (64, 69). How AHPN/CD437 induces c-Jun transcription requires further investigation. Increase of *c-jun* mRNA production by various stimuli is highly regulated by phosphorylation of c-Jun or activated transcription factor 2 that bind to the AP-1 binding sites present in the *c-jun* promoter (65). The rapid induction of c-Jun by AHPN/CD437 suggests that the effect of AHPN/CD437 may be mediated by a kinase or phosphatase that modifies transcriptional factors required for c-Jun induction.

Although induction of *nur77* is required for AHPN/CD437-induced apoptosis in lung cancer cell lines, *nur77* was also rapidly and highly induced by AHPN/CD437 in H292 cells (Fig. 4) that, however, showed a delayed apoptotic process (Fig. 2). Thus, the susceptibility to undertake apoptotic process in response to *nur77* signaling is dependent on the cellular context. This is true for other cell-death-inducing stimuli, where the sensitivity to apoptosis frequently depends on the cell type and the availability of external or internal survival factors (17, 62, 67). Our results demonstrate that one of the factors that contribute to the sensitivity of lung cancer cells to apoptosis-inducing effect of AHPN/CD437 is Bcl-2. Bcl-2 is known to induce cell survival in a variety of cell types (34, 54). Expression of Bcl-2 was strongly inhibited by AHPN/CD437 in SK-MES-1 and H460 cells that underwent a rapid apoptosis in

response to AHPN/CD437 and was not observed in A549 cells that were also very sensitive to the apoptosis-inducing effect of AHPN/CD437 (Fig. 6). In contrast, Bcl-2 was highly expressed and its expression was not clearly affected by AHPN/CD437 in H292 cells (Fig. 6). These data suggest that Bcl-2 may play an important role in the regulation of the onset of AHPN/CD437-induced apoptosis.

p53, which is known to be responsible for apoptosis triggered by oncogenes, ionizing radiation and certain anticancer drugs (67), was also highly induced by AHPN/CD437 in several NSCLC lines (Fig. 4c). It was previously reported that the apoptosis-inducing effect of p53 was in part due to its activation of the death gene *bax* (44). Indeed, Bax expression was slightly enhanced by AHPN/CD437 in lung cancer cells (Fig. 6b). This suggests that p53 may have a role in the modulation of AHPN/CD437-induced apoptosis in certain NSCLC lines. However, the observation that p53 is not expressed in SK-MES-1 cells (Fig. 4c) which underwent extensive apoptosis (Fig. 2) suggests that p53 may not be necessarily required for AHPN/CD437 to induce apoptosis in lung cancer cells.

In conclusion, AHPN/CD437 can effectively induce apoptosis and G₀/G₁ arrest in human lung cancer cell lines. Induction of apoptosis is mediated by a c-Jun-nur77 pathway and is modulated by levels of p53 and Bcl-2 that can be regulated by AHPN/CD437 in a cell type-specific manner, whereas induction of G₀/G₁ arrest is conferred by up-regulation of p21^{WAF1/CIP1}. Our demonstration that AHPN/CD437 effectively induces growth arrest and apoptosis in different lung cancer cell lines and recent reports that similar effects occur in breast cancer (57) and leukemia (29) cells and that several AHPN/CD437 analogs profoundly inhibited cancer cell growth (38) suggest that AHPN/CD437 may represent a group of compounds with potential therapeutic activity against both retinoid-sensitive and -insensitive cancers. Our findings that AHPN/CD437 utilizes multiple anticancer pathways in different cell types and that its effect does not require p53 may have important implications in cancer treatment, especially against lung cancer in which p53- or retinoid receptor-dependent growth arrest and apoptosis may be impaired due to high frequencies of p53 mutations (63) and abnormal expression of retinoid receptor (20, 35a, 72, 78).

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REFERENCES

- Agadir, A., Y. F. Shealy, D. L. Hill, and X.-K. Zhang. 1997. Retinyl methyl ether down-regulates activator protein 1 transcriptional activation in breast cancer cells. *Cancer Res.* 57:3444-3450.
- Angel, P., and M. Karin. 1991. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim. Biophys. Acta* 1072:129-157.
- Bernard, B. A., J.-M. Bernardon, C. Delescluse, B. Martin, M.-C. Lenoir, J. Maignan, B. Charpentier, W. R. Pilgrim, U. Reichert, and B. Shroot. 1992. Identification of synthetic retinoids with selectivity for human nuclear retinoic acid receptor γ . *Biochem. Biophys. Res. Commun.* 186:977-983.
- Bodrug, S. E., C. Aime-Sempe, T. Sato, S. Krajewski, M. Hanada, and J. C. Reed. 1995. Biochemical and functional comparisons of Mcl-1 and Bcl-2 proteins: evidence for a novel mechanism of regulating Bcl-2 family protein function. *Cell Growth Differ.* 2:173-182.
- Bossy-Wetzel, E., L. Bakiri, and M. Yaniv. 1997. Induction of apoptosis by the transcription factor c-Jun. *EMBO J.* 16:1695-1709.
- Bost, F., R. McKay, N. Dean, and D. Mercola. 1997. The Jun kinase/stress-activated protein kinase pathway is required for epidermal growth factor stimulation of growth of human A549 lung carcinoma cells. *J. Biol. Chem.* 272:33422-33430.
- Bullock, G., S. Ray, J. Reed, T. Miyashita, A. M. Ibrado, Y. Huang, and K. Bhalla. 1995. Evidence against a direct role for the induction of *c-jun* expression in the mediation of drug-induced apoptosis in human acute leukemia cells. *Clin. Cancer Res.* 1:559-564.
- Chang, C., and J. Kokontis. 1988. Identification of a new member of the steroid receptor superfamily by cloning and sequence analysis. *Biochem. Biophys. Res. Commun.* 155:971-977.
- Chao, W. R., P. D. Hobbs, L. Jong, X.-K. Zhang, Y. Zheng, Q. Wu, B. Shroot, and M. I. Dawson. 1997. Effects of receptor class- and subtype-selective retinoids and an apoptosis-inducing retinoid on the adherent growth of the NIH:OVCAR-3 ovarian cancer cell line in culture. *Cancer Lett.* 115:1-7.
- Clarke, A. R., C. A. Purdie, D. J. Harrison, R. G. Morris, C. C. Bird, M. L. Hooper, and A. H. Wyllie. 1993. Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature* 362:849-852.
- Colotta, F., N. Polentarutti, M. Sironi, and A. Mantovani. 1992. Expression and involvement of c-fos and c-jun protooncogenes in programmed cell death induced by growth factor deprivation in lymphoid cell lines. *J. Biol. Chem.* 267:18278-18283.
- Deng, C., P. Zhang, J. W. Harper, S. J. Elledge, and P. Leder. 1995. Mice lacking p21^{WAF1/CIP1} undergo normal development, but are defective in G1 checkpoint control. *Cell* 82:675-684.
- El-Deiry, W., J. W. Harper, P. M. O'Connor, V. E. Velculescu, C. E. Canman, J. Jackman, J. A. Pietenpol, M. Burrell, D. E. Hill, Y. Wang, K. G. Wiman, W. E. Mercer, M. B. Kastan, K. W. Kohn, S. J. Elledge, K. W. Kinzler, and B. Vogelstein. 1994. WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res.* 54:1169-1174.
- El-Deiry, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler, and B. Vogelstein. 1993. WAF1, a potential mediator of p53 tumor suppression. *Cell* 75:817-825.
- Estus, S., W. J. Zaks, R. S. Freeman, M. Gruda, R. Bravo, and E. M. Johnson. 1994. Altered gene expression in neurons during programmed cell death: identification of c-jun as necessary for neuronal apoptosis. *J. Cell Biol.* 127:1717-1728.
- Evan, G. I., A. H. Wyllie, C. S. Gilbert, T. D. Littlewood, H. Land, M. Brooks, C. M. Waters, L. Z. Penn, and D. C. Hancock. 1992. Induction of apoptosis in fibroblasts by c-myc protein. *Cell* 69:119-128.
- Fisher, D. E. 1994. Apoptosis in cancer therapy: crossing the threshold. *Cell* 78:539-542.
- Forman, B. M., K. Umeson, J. Chen, and R. M. Evans. 1995. Unique response pathways are established by allosteric interactions among receptors. *Cell* 81:541-550.
- Freeman, A. J., J. A. Vrana, R. M. Tombes, H. Jiang, S. P. Chellappan, P. B. Fisher, and S. Grant. 1997. Effects of antisense p21 WAF1/CIP1/MDA6 expression on the induction of differentiation and drug-mediated apoptosis in human myeloid leukemia cells HL-60. *Leukemia* 11:504-513.
- Gebert, J. F., N. Moghal, J. V. Frangioni, D. J. Sugarbaker, and B. G. Neel. 1991. High frequency of retinoic acid receptor β abnormalities in human lung cancer. *Oncogene* 6:1859-1868.
- Gu, Y., C. W. Turek, and D. O. Morgan. 1993. Inhibition of CDK2 activity in vivo by an associated 20KD regulatory subunit. *Nature* 366:707-710.
- Gudas, L. J., M. B. Sporn, and A. B. Roberts. 1994. Cellular biology and biochemistry of the retinoids, p. 443-520. *In* M. B. Sporn, A. B. Roberts, and D. S. Goodman (ed.), *The retinoids*. Raven Press, New York, NY.
- Ham, J., C. Babij, J. Whitfield, C. M. Pfarr, D. Lallemand, M. Yaniv, and L. L. Rubin. 1995. A c-jun dominant negative mutant protects sympathetic neurons against programmed cell death. *Neuron* 14:927-939.
- Harper, J. W., G. R. Adami, N. Wei, K. Keyomarsi, and S. J. Elledge. 1993. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* 75:805-816.
- Hazel, T. G., D. Nathans, and L. F. Lau. 1988. A gene inducible by serum growth factors encodes a member of the steroid and thyroid hormone receptor superfamily. *Proc. Natl. Acad. Sci. USA* 85:8444-8448.
- Hennekens, C. H., J. E. Buring, J. E. Manson, M. Stampfer, B. Rosner, N. R. Cook, C. Belanger, F. LaMotte, J. M. Gaziano, P. M. Ridker, W. Willett, and R. Peto. 1996. Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms and cardiovascular disease. *N. Engl. J. Med.* 334:1145-1149.
- Hong, W. K., and L. M. Itri. 1994. Retinoids and human cancer, p. 597-630. *In* M. B. Sporn, A. B. Roberts, and D. S. Goodman (ed.), *The retinoids*. Raven Press, New York, NY.
- Hsu, C. K. A., A. K. Rishi, X.-S. Li, M. I. Dawson, U. Reichert, B. Shroot, and J. A. Fontana. 1997. Bcl-X_L expression and its downregulation by a novel retinoid in breast carcinoma cells. *Exp. Cell Res.* 232:17-24.
- Hsu, C. A., A. K. Rishi, X.-S. Li, T. M. Gerald, M. I. Dawson, C. Schiffer, U. Reichert, B. Shroot, G. C. Poirer, and J. A. Fontana. 1997. Retinoid induced

- apoptosis in leukemia cells through a retinoic acid nuclear receptor-independent pathway. *Blood* 89:4470-4479.
30. Hunter, T., and J. Pines. 1997. Cyclins and cancer II: cyclin D and CDK inhibitors come of age. *Cell* 79:573-582.
 31. Jiang, H., J. Lin, Z. Su, F. R. Collart, E. Huberman, and P. B. Fisher. 1994. Induction of differentiation in human promyelocytic HL-60 leukemia cells activates p21^{WAF1/CIP1} expression in the absence of p53. *Oncogene* 9:3397-3406.
 32. Kastner, P., M. Mark, and P. Chambon. 1995. Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? *Cell* 83:859-869.
 33. Kim, R., and W. T. Beck. 1994. Differences between drug-sensitive and -resistant human leukemic CEM cells in c-jun expression, AP-1 DNA-binding activity, and formation of Jun/Fos family dimers, and their association with internucleosomal DNA ladders after treatment with VM-26. *Cancer Res.* 54:4958-4966.
 34. Korsmeyer, S. J. 1995. Regulators of cell death. *Trends Genet.* 11:101-105.
 - 34a. Li, Y., M. I. Dawson, A. Agadir, M.-D. Lee, L. Jong, P. D. Hobbs, and X.-K. Zhang. 1998. Regulation of RAR β expression by RAR- and RXR-selective retinoids in human lung cancer cell lines: effect on growth inhibition and apoptosis induction. *Int. J. Cancer* 75:88-95.
 35. Liu, Y., M.-O. Lee, H.-G. Wang, Y. Li, Y. Hashimoto, M. Klaus, J. C. Reed, and X.-K. Zhang. 1996. Retinoic acid receptor β mediates the growth-inhibitory effect of retinoic acid by promoting apoptosis in human breast cancer cells. *Mol. Cell. Biol.* 16:1138-1149.
 36. Liu, Z.-G., S. W. Smith, K. A. McLaughlin, L. M. Schwartz, and B. A. Osborne. 1994. Apoptotic signals delivered through the T-cell receptor of a T-cell hybrid require the immediate-early gene *nur77*. *Nature* 367:281-284.
 37. Lowe, S. W., and H. E. Ruley. 1993. Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. *Genes Dev.* 7:535-545.
 38. Lu, X.-P., A. Fanjul, N. Picard, M. Pfahl, D. Rungta, K. Nared-Hood, B. Carter, J. Piedrafit, S. Tang, E. Fabbri, and M. Pfahl. 1997. Novel retinoid-related molecules as apoptosis inducers and effective inhibitors of human lung cancer cells *in vivo*. *Nat. Med.* 3:686-690.
 39. Macleod, K. F., N. Sherry, G. Hannon, D. Beach, T. Tokino, K. Kinzler, B. Vogelstein, and T. Jacks. 1995. p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. *Genes Dev.* 9:935-944.
 40. Mangelsdorf, D. J., and R. M. Evans. 1995. The RXR heterodimers and orphan receptors. *Cell* 83:841-850.
 41. Manome, Y., R. Datta, N. Taneja, T. Shafman, E. Bump, R. Hass, R. Weichselbaum, and D. Kufe. 1993. Coinduction of c-jun gene expression and internucleosomal DNA fragmentation by ionizing radiation. *Biochemistry* 32:10607-10613.
 42. Merritt, A. J., C. S. Potten, C. J. Kemp, J. A. Hickman, A. Balmain, D. P. Lane, and P. A. Hall. 1994. The role of p53 in spontaneous and radiation-induced apoptosis in the gastrointestinal tract of normal and p53-deficient mice. *Cancer Res.* 54:614-617.
 43. Milbrandt, J. 1988. Nerve growth factor induces a gene homologous to the glucocorticoid receptor gene. *Neuron* 1:183-188.
 44. Miyashita, T., and J. C. Reed. 1995. Tumor suppressor p53 is a direct transcriptional activator of the human *bax* gene. *Cell* 80:293-299.
 45. Noda, A., S. F. Ning, O. M. Venable, O. M. Pereira-Smith, and J. R. Smith. 1994. Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. *Exp. Cell Res.* 211:90-98.
 46. Omenn, G. S., G. E. Goodman, M. D. Thornquist, J. Balmes, M. R. Cullen, A. Glass, J. P. Keogh, F. L. Meyskens, B. Valanis, J. H. Williams, S. Barnhart, and S. Hammar. 1996. Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. *N. Engl. J. Med.* 334:1150-1155.
 47. Oridate, N., M. Higuchi, S. Suzuki, B. Shroot, W. K. Hong, and R. Lotan. 1997. Induction of apoptosis in human C33A cervical carcinoma cells by the synthetic retinoid 6-[3-(1-adamantyl)- α -hydroxyphenyl]-2-naphthalene carboxylic acid AHPN/CD437. *Int. J. Cancer* 70:484-487.
 48. Pardee, A. B. 1989. G1 events and regulation of cell proliferation. *Science* 246:603-608.
 49. Parker, S. B., G. Eichele, P. Zhang, A. Rawls, A. T. Sands, A. Bradley, E. N. Olson, J. W. Harper, and S. J. Elledge. 1995. p53-independent expression of p21^{Cip1} in muscle and other terminally differentiating cells. *Science* 267:1024-1027.
 50. Perlmann, T., and L. Jansson. 1995. A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and Nurrl. *Genes Dev.* 9:769-782.
 51. Philips, A., S. Lesage, R. Gingras, J.-H. Maira, Y. Gauthier, P. Hugo, and J. Drouin. 1997. Novel dimeric Nur77 signaling mechanism in endocrine and lymphoid cells. *Mol. Cell. Biol.* 17:5946-5951.
 52. Potapova, O., H. Fakhrai, S. Baird, and D. Mercola. 1996. Platelet-derived growth factor-B/v-sis confers a tumorigenic and metastatic phenotype to human T98G glioblastoma cells. *Cancer Res.* 56:280-286.
 53. Potapova, O., A. Haghighi, F. Bost, C. Liu, M. J. Birrer, R. Gjerset, and D. Mercola. 1997. The Jun kinase/stress-activated protein kinase pathway functions to regulate DNA repair and inhibition of the pathway sensitizes tumor cells to cisplatin. *J. Biol. Chem.* 272:14041-14044.
 54. Reed, J. C. 1994. Bcl-2 and the regulation of programmed cell death. *J. Cell Biol.* 124:1-6.
 55. Robinson, L. A., L. J. Smith, M. P. Fontaine, H. D. Kay, C. P. Mountjoy, and S. J. Pirruccello. 1995. c-myc antisense oligodeoxynucleotides inhibit proliferation of non-small cell lung cancer. *Ann. Thorac. Surg.* 60:1583-1591.
 56. Schadendorf, D., M. A. Kern, M. Artuc, H. L. Pahl, T. Rosenbach, I. Fichtner, W. Nurnberg, S. Stuting, E. von Stebut, M. Worm, A. Makki, K. Jurgovsky, G. Kolde, and B. M. Henz. 1996. Treatment of melanoma cells with the synthetic retinoid AHPN/CD437 induces apoptosis via activation of AP-1 *in vitro*, and causes growth inhibition in xenografts *in vivo*. *J. Cell Biol.* 135:1889-1898.
 57. Shao, Z.-M., M. I. Dawson, X. S. Li, A. K. Rishi, M. S. Sheikh, Q.-X. Han, J. V. Ordenez, B. Shroot, and J. A. Fontana. 1995. p53 independent G₀/G₁ arrest and apoptosis induced by a novel retinoid in human breast cancer cells. *Oncogene* 11:493-504.
 58. Sherr, C. J. 1994. G1 phase progression: cycling on cue. *Cell* 79:551-555.
 59. Smeal, T., B. Binetruy, D. A. Mercola, M. Birrer, and M. Karin. 1991. Oncogenic and transcriptional cooperation with Ha-Ras requires phosphorylation of c-Jun on serines 63 and 73. *Nature* 354:494-496.
 60. Smeal, T., B. Binetruy, D. Mercola, A. Grover-Bardwick, G. Heidecker, U. R. Rapp, and M. Karin. 1992. Oncoprotein-mediated signalling cascade stimulates c-Jun activity by phosphorylation of serines 63 and 73. *Mol. Cell. Biol.* 12:3507-3513.
 61. Steinman, R. A., B. Hoffman, A. Iro, C. Guillof, D. A. Liebermann, and M. E. el-Housseini. 1994. Induction of p21 WAF-1/CIP1 during differentiation. *Oncogene* 9:3389-3396.
 62. Steller, H. 1995. Mechanisms and genes of cellular suicide. *Science* 267:1445-1449.
 63. Takahashi, T., M. M. Nau, I. Chiba, M. J. Birrer, R. K. Rosenberg, M. Vinocour, M. Levitt, H. Pass, A. F. Gazdar, and J. D. Minna. 1989. p53: a frequent target for genetic abnormalities in lung cancer. *Science* 246:491-494.
 64. Uemura, H., A. Mizokami, and C. Chang. 1995. Identification of a new enhancer in the promoter region of human TR3 orphan member of steroid receptor superfamily. *J. Biol. Chem.* 270:5427-5433.
 65. van Dam, H., D. Wilhelm, I. Herr, A. Steffen, P. Herrlich, and P. Angel. 1995. ATF-2 is preferentially activated by stress-activated protein kinases to mediate c-jun induction in response to genotoxic agents. *EMBO J.* 14:1798-1811.
 66. Vogt, P., and T. J. Bos. 1990. Jun: oncogene and transcription factor. *Cancer Res.* 55:1-35.
 67. White, E. 1996. Life, death, and the pursuit of apoptosis. *Genes Dev.* 10:1-15.
 68. Widschwendter, M., G. Daxenbichler, Z. Culig, S. Michel, A. G. Zeimet, M. G. Mortl, A. Widschwendter, and C. Marth. 1997. Activity of retinoic acid receptor- γ selectively binding retinoids alone and in combination with interferon- γ in breast cancer cell lines. *Int. J. Cancer* 71:497-504.
 69. Williams, G. T., and L. F. Lau. 1993. Activation of the inducible orphan receptor gene *nur77* by serum growth factors: dissociation of immediate-early and delayed-early responses. *Mol. Cell. Biol.* 13:6124-6136.
 70. Wilson, T. E., T. J. Fahrner, M. Johnston, and J. Milbrandt. 1991. Identification of the DNA binding site for NGFI-B by genetic selection in yeast. *Science* 252:1296-1300.
 71. Woronicz, J. D., B. Cainan, V. Ngo, and A. Winoto. 1994. Requirement for the orphan steroid receptor *nur77* in apoptosis of T-cell hybridomas. *Nature* 367:277-281.
 72. Wu, Q., Y. Li, R. Liu, A. Agadir, M.-O. Lee, Y. Liu, and X.-K. Zhang. 1997. Modulation of retinoic acid sensitivity in lung cancer cells by a dynamic balance of *nur77* and COUP-TF orphan receptors and their heterodimerization. *EMBO J.* 16:1656-1669.
 73. Xiong, Y., G. J. Hannon, H. Zhang, D. Casso, R. Kobayashi, and D. Beach. 1993. p21 is a universal inhibitor of cyclin kinases. *Nature* 366:701-704.
 74. Yang-Yen, H. F., X.-K. Zhang, G. Graupner, M. Tzukerman, B. Sakamoto, M. Karin, and M. Pfahl. 1991. Antagonism between retinoic acid receptors and AP-1: implications for tumor promotion and inflammation. *New Biol.* 3:1206-1219.
 75. Zeng, Y.-X., and W. S. El-Deiry. 1996. Regulation of p21^{WAF1/CIP1} expression by p53-independent pathways. *Oncogene* 12:1557-1564.
 76. Zhang, X.-K., B. Hoffmann, P. B. Tran, G. Graupner, and M. Pfahl. 1992. Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. *Nature* 355:441-446.
 77. Zhang, X.-K., and M. Pfahl. 1993. Regulation of retinoid and thyroid hormone action through homodimeric and heterodimeric receptors. *Trends Endocrinol. Metab.* 4:156-162.
 78. Zhang, X.-K., Y. Liu, M.-O. Lee, and M. Pfahl. 1994. A specific defect in the retinoic acid receptor associated with human lung cancer cell lines. *Cancer Res.* 54:5663-5669.