Specific Chemopreventive Agents Trigger Proteasomal Degradation of G1 Cyclins: Implications for Combination Therapy

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ABSTRACT

Purpose: There is a need to identify cancer chemoprevention mechanisms. We reported previously that all-trans-retinoic acid (RA) prevented carcinogenic transformation of BEAS-2B immortalized human bronchial epithelial cells by causing G1 arrest, permitting repair of genomic DNA damage. G1 arrest was triggered by cyclin D1 proteolysis via ubiquitin-dependent degradation. This study investigated which chemopreventive agents activated this degradation program and whether cyclin E was also degraded.

Experimental Design: This study examined whether: (a) cyclin E protein was affected by RA treatment; (b) cyclin degradation occurred in derived BEAS-2B-R1 cells that were partially resistant to RA; and (c) other candidate chemopreventive agents caused cyclin degradation.

Results: RA treatment triggered degradation of cyclin E protein, and ALLN, a proteasomal inhibitor, inhibited this degradation. Induction of the retinoic acid receptor agonist (rosiglitazone) each sup-

pression of wild-type cyclin D1 and cyclin E, but ALLN inhibited this degradation. Mutation of threonine 286 stabilized transfected cyclin D1, and mutations of threonines 62 and 380 stabilized transfected cyclin E, despite RA treatment. Specific chemopreventive agents triggered cyclin degradation. Nonclassical retinoids (fenretinide and retinoid X receptor agonists) and a synthetic triterpenoid (2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid) each suppressed BEAS-2B growth and activated this degradation program. However, a vitamin D3 analog (RO-24–5531), a cyclooxygenase inhibitor (indomethacin), and a peroxisome proliferator-activated receptor γ agonist (rosiglitazone) each suppressed BEAS-2B growth, but did not cause cyclin degradation. BEAS-2B-R1 cells remained responsive to nonclassical retinoids and to 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid.

Conclusions: Specific chemopreventive agents activate cyclin proteolysis. Yet, broad resistance did not occur after acquired resistance to a single agent. This provides a therapeutic rationale for combination chemoprevention with agents activating non-cross-resistant pathways.

INTRODUCTION

Carcinogenesis is a chronic and multistep process. Initiation, promotion, and progression stages of carcinogenesis lead to invasive or metastatic malignancies. Each step is a potential target to arrest or reverse carcinogenesis. Cancer chemoprevention intervenes at steps in carcinogenesis before malignancies are clinically evident (1). Clinical validation for this concept was found for tamoxifen, a selective estrogen receptor modulator (2). Additional clinical support came from selective cyclooxygenase-2 inhibition that reduced polyps in familial polyposis coli (3).

The retinoids are natural and synthetic derivatives of vitamin A. Retinoids are studied in cancer chemoprevention because of findings from experimental animal models (4), in vitro models (5), epidemiological observations, and clinical trials, as reviewed (6). Wolbach and Howe (7) first reported a retinoid role in tissue homeostasis in 1925. Vitamin A depletion caused squamous metaplasia, and this was reversed by vitamin A repletion (7). An inverse relationship between incidence of certain cancers and vitamin A levels was uncovered, as reviewed (6). A retinoid role in clinical cancer chemoprevention was supported by results from treatment of preneoplastic lesions such as oral leukoplakia (8), cervical dysplasia (9), and xeroderma pigmentosum (10).

Retinoid clinical trials reported a reduction of certain second primary cancers. Examples included 13-cis-retinoic acid treatment that reduced second aerodigestive tract cancers in resected head and neck cancers (11), and adjuvant retinol palmi-

Received 9/23/03; revised 12/19/03; accepted 1/2/04.

Grant support: NIH and National Cancer Institute Grants RO1-CA 87546 (E. Dmitrovsky) and RO1-CA78814 (M. Sporn); Department of Defense Grants DAMD17-99-1-9168 (M. Sporn) and DAMD17-98-1-8604 (M. Sporn); Lance Armstrong Award (S. Freemantle); American Society of Clinical Oncology (ASCO) Young Investigator Award (K. Dragnev); CHEST Foundation of the American College of Chest Physicians and the LUNGevity Foundation and NIH Grant T32-CA09658 (W. Petty); NIH Grant T32-CA9658 (I. Pitha-Rowe); Oracle Giving Fund (E. Dmitrovsky); Samuel Waxman Cancer Research Foundation (E. Dmitrovsky); National Foundation for Cancer Research (M. Sporn); and Oliver and Jennie Donaldson Trust (M. Sporn).

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Note: M. Sporn is the Oscar M. Cohn Professor.

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nomas (13), and fenretinide (4HPR) decreased second breast cancers (14). In contrast, randomized trials found that β-carotene did not prevent primary lung cancers in high-risk cohorts (15–17). An intergroup randomized trial of 13-cis-retinoic acid also did not prevent second cancers in stage I lung cancer cases (18). A negative clinical interaction occurred in smokers, but benefits were perhaps observed in those who never smoked (18). These findings underscored the need to identify mechanisms for retinoid response or clinical resistance.

Studies in BEAS-2B-immortalized human bronchial epithelial cells uncovered retinoid chemopreventive mechanisms (19). These cells were immortalized with an adenovirus 12-SV40 hybrid virus (20). Exposure to tobacco-associated carcinogens such as nitrosamine-4-(methylnitrosoamino)-1-(3pyridyl)-1-butanol led to malignant transformation of BEAS-2B cells (19). All-trans-retinoic acid (RA) treatment inhibited this effect (19), and this was linked to induced G1 arrest and cyclin D1 degradation that would permit repair of carcinogenic damage (19, 21, 22). Repression of cyclin D1 protein after RA treatment occurred through a ubiquitin-dependent degradation program (21, 22). Retinoic acid receptor (RAR) β and retinoid X receptor (RXR) agonists also activated this program (22). Another mechanism uncovered in this model involved retinoid repression of the epidermal growth factor receptor (23). G1 cyclins and epidermal growth factor receptor are often aberrantly expressed in bronchial preneoplasia and this implicated these species as therapeautic or chemopreventive targets in the lung (24, 25).

Cyclin D1 degradation after retinoid treatment of bronchial epithelial cells was independently confirmed by others (26). Tumor differentiation induced by RA treatment also caused proteolysis of cyclin D1 (27). Microarray analysis after retinoid treatment of acute promyelocytic leukemia cells identified UBE1L, an E1-like ubiquitin-activating enzyme, as an induced species (28). UBE1L is a RA target gene (29) that might activate proteolysis of cyclin D1 (27). Microarray analysis after retinoid treatment of bronchial epithelial cells uncovered retinoid chemopreventive mechanisms (19). These cells were immortalized as described previously (20). These species as therapeutic or chemopreventive targets in the lung (24, 25).

Recombinant GRPE or crude GRPE diets were used to prevent second cancers in stage I lung cancer cases (18). Negative clinical interaction occurred in smokers, but benefits were perhaps observed in those who never smoked (18). These findings underscored the need to identify mechanisms for retinoid response or clinical resistance.

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Immunoblot and Reverse Transcription-PCR Assays. Bronchial epithelial cells were independently treated with DMSO as a vehicle control or with indicated chemopreventive agents for 0–36 h before lysis in 10-cm tissue culture plates (Falcon, Franklin Lakes, NJ) with radioimmunoprecipitation assay buffer containing protease inhibitors, as described (21). Total cellular protein was measured using the Bradford assay. Total protein was subjected to SDS-PAGE before transfer to membranes (Schleicher & Schuell, Inc., Keene, NH). Purchased primary antibodies recognized cyclin D1 (M-20), cyclin E (HE-12), or RARβ (C19; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Appropriate secondary antibodies (Amersham Life Sciences Inc., Arlington Heights, IL) were used. A polyclonal antibody was used to detect actin (C-11; Santa Cruz Biotechnology, Inc.). Murine monoclonal anti-myc (MMS-150P; Covance, Princeton, NJ) or anti-MAA (MMS-101R; Covance) antibodies were used. To inhibit the proteasomal pathway, cells were treated with ALLN (Calbiochem, San Diego, CA) or LLnL (Sigma Chemical Co.) using established techniques (21). Treatments with proteasome inhibitors were typically for 4–12 h at a dosage of 25–100 μM with addition of the vehicle, DMSO, as a control, RA, or the indicated chemopreventive agents at the dosed described treatments and duration treatments. To confirm comparable loading of total protein, Coomassie staining of gels or probing for actin expression was used. To detect RARβ mRNA expression with a reverse transcription-PCR assay, established techniques (29) were used along with the RARβ forward primer: 5′-GGAATGGGGAATGTCACGACTAATG-3′ and reverse primer: 5′-CAGGCGGAGGCGACCAG-3′.

Transfection of Cyclin Species. Mutant cyclin D1 was engineered with a threonine to alanine transversion at residue 286, as described previously (27). Mutant cyclin E species were engineered including cyclin E T62A (threonine to alanine sub-
stition at residue 62) or cyclin E T62A/T380A (threonine to alanine transversions at residues 62 and 380). These were generated using wild-type cyclin E (19) provided by Dr. Andrew Koff (Memorial Sloan-Kettering Cancer Center, New York, NY) or a mutant cyclin E T380A species (with a threonine to alanine transversion at residue 380) provided by Dr. Bruce Clurman (Fred Hutchinson Cancer Center, Seattle, WA). In vitro mutagenesis and the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) were used to engineer mutant cyclin E species using either wild-type cyclin E or mutant cyclin E T380A expression plasmids. Primer sequences used to generate the T62A cyclin E mutation were: 5′-ACCCTGCTCCTGTATCCCCGCGCCTGACAAAGAAGATGA-3′ for the forward primer and 5′-TCATCTTCTTTGTACGCCGCGGGAATCAGGAGCaGggt-3′ for the reverse primer. Site-specific mutations were confirmed by DNA sequencing.

Independent transient transfection experiments were performed using BEAS-2B cells. This was accomplished using vectors expressing wild-type cyclin D1 (21), cyclin D1 T286A (27), wild-type cyclin E (19), mutant cyclin E T62A, mutant cyclin E T380A, or mutant cyclin E T62A/T380A species that were introduced into BEAS-2B cells using Effectene (Qiagen, Valencia, CA), and established transfection techniques (23). Transfectants were treated either with DMSO as vehicle, RA, or RAR antagonist ALLN (50 μM) treatment (data not shown). In contrast, RA (4 μM) treatment reduced expression of these transfected cyclins, as shown in Fig. 3. Treatment with the proteasome inhibitors LLnL (50 μM) and 100 μM (data not shown) or ALLN (50 μM) inhibited repression of cyclin D1 and cyclin E proteins, after RA treatment. This indicated a link between proteasome-dependent degradation of these cyclins and RA response of human bronchial epithelial cells.

Mechanisms involved in this repression were examined. Transient transfection conditions for BEAS-2B cells were optimized previously using an Effectene (Qiagen)-based transfection procedure that reproducibly achieved efficient transfection of these cells (23). RA (4 μM) treatment reduced expression of these transfected cyclins, as shown in Fig. 3. Treatment with the proteasome inhibitors LLnL (50 μM) and 100 μM (data not shown) or ALLN (50 μM) inhibited repression of cyclin D1 and cyclin E proteins, after RA treatment. This indicated a link between proteasome-dependent degradation of these cyclins and RA response of human bronchial epithelial cells.

To determine the relationship between retinoid-mediated growth suppression and cyclin proteolysis, partially RA-resistant BEAS-2B-R1 cells were derived from BEAS-2B cells. Features of BEAS-2B and BEAS-2B-R1 lines were compared in Table 1. Retinoid resistance was a rare event in that only a single RA-resistant BEAS-2B-R1 human bronchial epithelial cell line was derived. As depicted, RA resistance was infrequently detected in BEAS-2B cells. Cyclin degradation refers to RA induced degradation of cyclin D1 and cyclin E proteins.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>RA response</th>
<th>Resistance frequency</th>
<th>Cyclin degradation</th>
<th>G1 arrest</th>
</tr>
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<tbody>
<tr>
<td>BEAS-2B</td>
<td>Sensitive</td>
<td>≤5 × 10⁶</td>
<td>Sensitive</td>
<td>Present</td>
</tr>
<tr>
<td>BEAS-2B-R1</td>
<td>Resistant</td>
<td>N.A.</td>
<td>Resistant</td>
<td>Inhibited</td>
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* N.A., not applicable.

Cyclin E was also repressed by RA treatment, as displayed in Fig. 1. Cyclin D1 repression preceded that of cyclin E, as shown in Fig. 1. Whereas reduction of cyclin D1 protein occurred within 8 h of RA treatment, the decline in cyclin E occurred later and was not typically detected before 16 h after RA treatment, as seen in Fig. 1. These effects on cyclin D1 and cyclin E depended on the RA dosage, because repression was more evident at the 4 μM than 2 μM RA dosages (data not shown).

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In contrast to parental BEAS-2B cells, BEAS-2B-R1 cells continued to proliferate in culture, despite 4 μM RA treatment. After RA treatment, a dose-dependent repression of growth occurred in BEAS-2B cells, as depicted in Fig. 2A. This retinoid effect was inhibited in BEAS-2B-R1 cells, even after 3 days of RA-treatment, as confirmed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay shown in Fig. 2A. As expected from prior work (19), BEAS-2B cells exhibited G1 arrest after RA (4 μM) treatment (data not shown). In contrast, in BEAS-2B-R1 cells, G1 arrest was inhibited when these cells were cultured with RA for 30 h (−RA, G1 % 41.2 ± 1.3 SD as compared with +RA, G1 % 41.5 ± 1.8 SD). RA treatment no longer appreciably repressed expression of either cyclin D1 or cyclin E proteins in BEAS-2B-R1 cells, as shown in Fig. 2B. Prior work revealed that RA treatment prominently induced RARβ protein in BEAS-2B cells (22). RARβ mRNA expression...
was examined using a reverse transcription-PCR assay, and RARβ was deregulated in BEAS-2B-R1 cells after RA treatment (data not shown). RARβ immunoblot expression was also deregulated, as shown in Fig. 2C. Whereas RARβ protein was induced in BEAS-2B cells after RA treatment, this was not detected in BEAS-2B-R1 cells.

Mechanisms involved in retinoid repression of G1 cyclins were studied. Wild-type or mutant (cyclin D1 T286A) cyclin D1 species and wild-type or mutant (cyclin E T62A, cyclin E T380A, and cyclin E T62A/T380A) cyclin E species were individually transfected into BEAS-2B cells. As shown in Fig. 3, when wild-type cyclin D1 or wild-type cyclin E was transfected into BEAS-2B cells, RA treatment repressed expression of these species. Treatment with the proteasome inhibitor ALLN inhibited this repression. When the mutant cyclins were individually transfected into BEAS-2B cells, marked effects on stability of these species were observed after RA treatment. After RA treatment, repression of endogenous cyclin D1 (data not shown) and transfected wild-type cyclin D1 species was observed in Fig. 3. In contrast, transfected cyclin D1 T286A was stabilized in BEAS-2B cells after RA treatment. These findings implicated a direct role for threonine 286 in regulating stability of cyclin D1 in these cells after RA treatment. This extends prior work indicating a role for threonine 286 in phosphorylation-dependent regulation of cyclin D1 (34) by showing that stability after RA treatment also depended on this residue.

Different results were obtained when mutant cyclin E species were transfected into BEAS-2B cells. RA treatment repressed transfected wild-type cyclin E. This was antagonized by treatment with a proteasomal inhibitor. Double mutation of cyclin E (cyclin E T62A/T380A) inhibited this degradation, as shown in Fig. 3. Transfected cyclin E species with a single mutation (cyclin E T62A or cyclin E T380A) did not affect stability of cyclin E as prominently as detected for transfected cyclin E T62A/T380A species (data not shown). These findings were consistent with prior evidence for these threonine residues affecting cyclin E stability (35).

These findings established that RA treatment triggered cyclin degradation in human bronchial epithelial cells and raised the prospect that this was a specific mechanism. To confirm this, experiments were conducted using other candidate chemopreventive agents. Results were compared with findings obtained after RA treatment. Prior work revealed that specific retinoid receptor agonists activated cyclin D1 proteolysis. Retinoids that activated RARγ/H9252 or RXR triggered cyclin D1 proteolysis through a proteasome-dependent pathway, whereas RARα or RARγ agonists did not cause this effect (22). Prior work was extended by examining whether agents that activated other pathways would also induce cyclin proteolysis or repress growth of human bronchial epithelial cells.

BEAS-2B cells were treated with RA, and results were compared with those obtained after treatment with a PPARγ agonist (rosiglitazone), a cyclooxygenase inhibitor (indomethacin; data not shown), a synthetic vitamin D agonist (RO-24–5531), a triterpenoid, CDDO (31), the nonclassical retinoid, 4HPR (36–40), or a rexinoid that activated the RXR pathway (data not shown; Ref. 22). Treatment with each of these agents caused growth suppression of BEAS-2B cells in the growth conditions shown in Fig. 4A. Of these agents, only CDDO,
4HPR, and RXR agonists (22) repressed cyclin D1 protein expression, as shown in Fig. 4B. Cyclin D1 proteolysis was triggered by specific chemopreventive agents. Immunoblot analysis for cyclin D1 was performed on lysates from cells obtained from the experiment depicted in Fig. 4A. Cyclin repression was inhibited by cotreatment with the proteasome inhibitor, ALLN, as shown in Fig. 4B. A dose response for each agent was performed, and the dosages displayed in Fig. 4B each had induced growth suppression without appreciable cytotoxicity.

BEAS-2B-R1 cells were examined to determine whether acquired resistance to one pharmacological agent conferred resistance to another agent that activated a similar chemopreventive pathway. A nonclassical retinoid (4HPR) and a triterpenoid (CDDO) still repressed cyclin D1 expression (as shown in Fig. 4C), although these cells were resistant to RA treatment. 4HPR can activate nuclear retinoid receptor-dependent and -independent mechanisms (36–39), whereas CDDO acts through distinct mechanisms (31). 4HPR and CDDO were individually able to suppress growth of BEAS-2B-R1 cells, as shown in Fig. 4D.

**DISCUSSION**

Carcinogenic transformation of human bronchial epithelial cells was inhibited by RA treatment (19). A mechanism proposed for this chemoprevention involved proteasome-dependent degradation of cyclin D1 (21, 22). This caused G1 arrest that could permit repair of carcinogenic damage to genomic DNA. The study extended prior work by reporting that proteasome-dependent degradation of cyclin E also occurred after RA treatment of human bronchial epithelial cells. Cyclin repression was inhibited after RA treatment of BEAS-2B-R1 cells. Repression of both cyclin D1 and cyclin E might be required for optimal growth suppression of these cells. Specific chemopreventive agents activated repression of these cyclins. Of the examined agents, only RA, certain nonclassical retinoids, or the triterpenoid, CDDO, triggered this proteolysis, as shown in Fig. 3. This provided a basis for combination cancer chemoprevention using an optimal retinoid combined with another agent that activated another therapeutic pathway.

One prediction was that RA-resistant human bronchial epithelial cells would deregulate expression of G1 cyclins. This was confirmed by results displayed in Fig. 2, where partially RA-resistant BEAS-2B-R1 cells no longer repressed cyclin expression. Cyclin deregulation was associated with aberrant RARβ expression. This implicated disruption of RARβ signaling with deregulated expression of these cyclins. Examination of BEAS-2B-R1 cells established that broad resistance to chemopreventive agents was not conferred. Cyclin D1 repression still occurred in these cells after treatment with a nonclassical retinoid or CDDO, as shown in Fig. 4.

These findings are pertinent to clinical cancer chemoprevention. Combination therapy would be indicated in clinical cancer chemoprevention (40). This permits each agent to be administered at clinically tolerable dosages, perhaps at lower dosages than when administered as single agents. Each agent included as part of a combination chemopreventive regimen could be chosen to target distinct therapeutic pathways, optimizing cooperative effects. A strategy to enhance clinical interactions would use agents that interact with a critical oncogenic target. Cyclin D1 is an attractive target to consider, because engineered transgenic models indicate its important role in carcinogenesis (41). Findings reported here are relevant to cancer chemoprevention, because recent studies using an animal model as well as analysis of a clinical trial directly implicated cyclin D1 as a marker or mediator of chemopreventive response.
These preclinical and clinical studies provide independent validation of prior work (21, 22, 24). Evidence also exists for epidermal growth factor receptor as a target for lung cancer chemoprevention (23). It is notable that cyclin D1 was a common target for the retinoid and epidermal growth factor receptor pathways (23), although different mechanisms regulated cyclin D1 through these pathways. A combination chemopreventive regimen might be developed to repress cyclin expression through non-cross-resistant pathways.

Mechanisms responsible for RA-dependent cyclin degradation were examined. Prior work indicated that specific cyclin D1 and cyclin E residues regulated ubiquitin-dependent degradation of these species (27, 34, 44–46). Threonine 286 present in the PEST domain of cyclin D1 was implicated in retinoid-mediated degradation of this cyclin (21, 27). Removal of the PEST domain of cyclin D1 rendered this species resistant to degradation, despite RA treatment (21). Transfection of mutant cyclin D1 T286A stabilized this species and inhibited its degradation by RA treatment, as shown in Fig. 3. This indicated the direct involvement of this residue in retinoid-dependent degradation of cyclin D1.

Other mutations were involved in ubiquitin-dependent degr-
radiation of cyclin E (44, 45). A double mutation of cyclin E affecting threonines 62 and 380 stabilized cyclin E after RA treatment of transfected BEAS-2B cells, as displayed in Fig. 3. This finding was consistent with involvement of the human F-box protein, hCdc4 (35), that might target cyclin E for proteolysis by chemopreventive agents. This protein is homologous to Archipelago in Drosophila and was mutated in some cancer cell lines (47, 48). Future work should determine whether this species would be affected by treatment with these chemopreventive agents.

Aberrant cyclin D1 or cyclin E expression occurs early during lung carcinogenesis. Overexpression of these cyclins was frequent in bronchial preneoplasia and squamous cell lung cancers (24). These species could represent chemopreventive or therapeutic targets as well as surrogate markers for clinical response in the lung. A novel finding of this study was that cyclin D1 and cyclin E were coordinately regulated by specific chemopreventive agents. This argues for a common mechanism for their regulation. Microarray analysis identified UBE1L as a retinoic acid target gene that triggered degradation of PML/RARα, a key step involved in RA response in acute promyelocytic leukemia (28, 29). Future work should determine whether this or another RA-induced species signals cyclin degradation in human bronchial epithelial cells.

Retinoids treat certain premalignant lesions and reduce incidence of some second malignancies (8–14). Yet, a randomized trial with 13-cis-retinoic acid was not clinically beneficial, especially in smokers (18). One mechanism mediating these clinical effects could involve proteasome-dependent cyclin degradation. Inability to activate this pathway could account for clinical resistance to carotenoids or classical retinoids (15–18). Potential beneficial chemopreventive effects were observed in those who did not smoke, but were treated with 13-cis-retinoic acid (18). Perhaps 13-cis-retinoic acid activated cyclin degradation in these cases.

A difference between the bronchial epithelium of smokers and nonsmokers could involve RARβ expression. RARβ repression was frequent in lung cancers (49). Repression might result from methylation-induced silencing (50) that would cause resistance to retinoids that signal growth suppression through RARβ. BEAS-2B-R1 cells had acquired RARβ repression and were partially resistant to RA but sensitive to nonclassical retinoids. Signals downstream of RARβ such as cyclin proteolysis were still engaged by chemopreventive agents that bypassed RARβ repression. One appealing strategy to overcome clinical resistance to classical retinoids in this setting would use a pharmacological agent that directly activated critical signals downstream of RARβ. An alternative approach would use a classical retinoid with a chromatin-remodeling agent to overcome RARβ silencing and thereby engage RARβ-dependent pathways.

In summary, this study found that proteasome-dependent degradation of G1 cyclins was a cancer chemopreventive mechanism activated by specific agents. The importance of this in regulating cellular growth was confirmed by finding that derived RA-resistant human bronchial epithelial cells were also resistant to this proteolysis, despite RA treatment. In contrast, these cells remained sensitive to other chemopreventive agents that still caused cyclin degradation. Frequent aberrant expression of cyclins (24) in bronchial preneoplasia and lung cancers implicated these species as targets for chemoprevention or markers of clinical response. Future clinical trials should determine which regimen optimally activates this cancer chemoprevention mechanism.

ACKNOWLEDGMENTS

We thank Dr. Andrew Koff (Memorial Sloan-Kettering Cancer Center, New York, NY) for providing the wild-type cyclin E vector and Dr. Bruce Clurman (Fred Hutchinson Cancer Center, Seattle, WA) for providing the mutant cyclin E species affecting threonine residue 380. We thank Dr. Thomas Hermann (Ligand, La Jolla, CA) for providing bexarotene, Dr. Milan Uskokovic (Hoffmann-LaRoche, Basel, Switzerland) for providing RO-24-5531, and Dr. Timothy M. Willson (Glaxo-SmithKline, Research Triangle, NC) for providing rosiglitazone.

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