**RB Status as a Determinant of Response to UCN-01 in Non-Small Cell Lung Carcinoma**

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**ABSTRACT**

7-Hydroxystaurosporine (UCN-01), a protein kinase inhibitor in clinical development, demonstrates potent antineoplastic activity. To determine whether specific genetic abnormalities would modulate the response to UCN-01, a model of human non-small cell lung carcinoma (NSCLC) cell lines with differential abnormalities of p16CDKN2, RB, and p53 was used for these studies. Cell growth was measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay, and cell cycling was studied using flow cytometric analysis of DNA content. Changes in protein levels and phosphorylation were assessed by Western blotting and measurement of the RB protein by immunofluorescent staining. In cell lines expressing wild-type RB, arrest of cells in G1, and a reduction of cells in S phase. p16CDKN2-null cells showed similar growth inhibition to normal fetal lung fibroblasts. UCN-01-induced growth arrest was accompanied by induction of p21CDKN1 and G1 arrest. The RB-null cell line H596 showed increased G1 arrest compared with the parent cells. We conclude that UCN-01-induced G1 arrest can occur in cells null for p53 and p16CDKN2, and that RB status influences the ability of UCN-01 to induce a G1 arrest. These data suggest that the molecular profile of cell cycle regulating genes in individual tumors may predict responsiveness and provide insight into optimal therapeutic application of this new antineoplastic agent.

**INTRODUCTION**

The elucidation of the molecular events involved in the regulation of cell cycle progression has led to identification of a complex web of interacting genes and their protein products. Prominent within this web are the tumor suppressor genes RB and p53 (also known as TP53; Refs. 1–3). In cycling cells, activated complexes of G1 phase cyclins and Cdk phosphatases result in the release of transcription factors necessary for the onset of and progression through S-phase (2 – 6). In contrast, Cdk inhibitors, such as p16CDKN2 (Mts1) and p21CDKN1 (Waf1/Cip1), interfere with cyclin/Cdk activity, leading to cell cycle arrest at the G1 checkpoint (7 – 10). For example, the p53 pathway is activated in response to DNA damage, resulting in p53-dependent induction of p21CDKN1 and G1 arrest (9, 10). Mutations in cell cycle genes are common to most cancer types, including NSCLCs, suggesting potentially exploitable differences in checkpoint arrest between normal and malignant cells (11 – 13).

Novel antineoplastic agents that modulate cell cycling are in development. One such agent is the protein kinase inhibitor, UCN-01. UCN-01 exhibits potent in vitro and in vivo antitumor activity against a broad range of murine tumors, human cancer cell lines, and xenografts and is in early Phase I clinical trials (14 – 17). UCN-01 has also been shown to potentiate the anticancer activity of cisplatin and other DNA-damaging agents (16 – 19).

UCN-01 originally characterized as an inhibitor of PKC (20). However, its anticancer activity is more likely to result from modulation of cell cycle progression than the direct effect of PKC inhibition. Experiments comparing the effects of UCN-01 and GF 109203X, a selective PKC inhibitor, demonstrated that although both compounds inhibited PKC activity,
UCN-01 had a disproportionally greater effect on cell growth inhibition (21). Initial studies have indicated that UCN-01 treatment results in inhibition of Cdk2, Cdk4, and Cdk6, hypophosphorylation of Rb, and the accumulation of cells in G1 (15, 22–24). Loss of Cdk activity may result from either direct inhibition by UCN-01 or induction of the Cdk inhibitors p21CDKN1 and p27KIP1 (24).

Molecular alterations that contribute to tumorigenesis by disrupting normal cell cycle regulation may also confer selective resistance to classic chemotherapeutic agents, many of which modulate the cell cycle. We hypothesized that the antineoplastic activity of UCN-01 is dependent on the molecular status of key cell cycle regulatory genes and the ability to induce Cdk inhibitors, Rb hypophosphorylation, and G1 arrest. To address this, we investigated the effects of UCN-01 on growth and modulation of gene expression in human NSCLC cell lines with differential abnormalities of p16CDKN2, RB, and p53.

### MATERIALS AND METHODS

#### Cell Cultures. The NSCLC cell lines A549, Calu1, and H596 were acquired from the American Type Culture Collection (Rockville, MD). The normal human fetal lung fibroblast strain T3891 was initiated in our laboratory (25). Human iso- genic models of Rb expression, the bladder cancer cell line 5637 (Rb-null), and the prostate cancer cell line DU145 (Rb mutant) have been reported previously (26, 27). Cells were cultured in either RPMI 1640 or DMEM (BioWhittaker, Walkersville, MD) with 10% heat-inactivated fetal bovine serum, supplemented with penicillin and streptomycin. Cells were maintained at 37°C in 5% CO2 in air.

#### Drug Treatment. Stock solutions were prepared by dissolving UCN-01 in DMSO at a concentration of 1 mg/mL and were stored at −20°C. Immediately prior to treatment, the UCN-01 stock solution was further diluted in serum-free media. UCN-01 was provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute.

The protein extracts for the Western blots and the cells for flow cytometry from untreated controls were both obtained from log phase cells at ~60% confluence. Similarly, the cultures exposed to drugs were treated at ~40%-60% confluence and harvested at the respective time points.

#### Western Blotting. Soluble protein extracts were prepared from cell pellets in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 0.1% NP40, 1 mM phenylmethylsulfonyl fluoride, 5 mM NaF, and 1 mM sodium o-vanadate. DNase (20 units/mL) was added, and the mixture was placed on ice for 30 min with occasional vortexing. The lysate was cleared by centrifugation at 12,000 rpm in a microfuge for 15 min at 4°C. Protein concentrations were quantitated from duplicate readings using a modified Bradford assay (Bio-Rad Laboratories). Protein samples were diluted with lysis buffer to either 20 or 30 µg/µl to facilitate equal loading of samples. SDS-PAGE gels were cast in a discontinuous fashion using a 4% stacking gel with slight modification of the methods of Laemmli, as reported previously (25, 28). Rb was analyzed on a 7.5% resolving gel (Hoefer Scientific Instruments, San Francisco, CA). p16CDKN2 and p21CDKN1 were separated on a 15% gel using a mini-gel system (Protein II; Bio-Rad Laboratories). Proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories) at a constant 125V for 70 min.

Membranes were blocked in a solution consisting of 5% blocking grade nonfat dry milk (Bio-Rad Laboratories) in TBST [25 mM Tris-HCl (pH 8.0), 31.25 mM NaCl, and 0.025% Tween 20]. Blots were incubated either overnight at 4°C or at room temperature for 1 h with one of the following primary antibodies: anti-Rb mouse monoclonal (G-245), anti-p16CDKN2 rabbit polyclonal, or anti-p21CDKN1 mouse monoclonal (6B6; all from PharMingen, San Diego, CA). Blots were washed three times in the blocking solution (15 min each) and incubated in a 1:1250 dilution of the appropriate biotinylated (p16CDKN2 and p21CDKN1) or horseradish peroxidase-conjugated (Rb) secondary antibody (Vector Laboratories, Burlingame, CA or Promega Corp., Madison, WI) for 1 h. Blots were washed once in blocking solution and three times in TBST (10 min each). Blots using

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### Table 1 NSCLC mutational status and UCN-01 IC50

<table>
<thead>
<tr>
<th>Cells</th>
<th>IC50 (nM)</th>
<th>p16</th>
<th>p53</th>
<th>RB</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3891</td>
<td>103</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>A549</td>
<td>187</td>
<td>Null</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>Calu1</td>
<td>484</td>
<td>Null</td>
<td>Null</td>
<td>wt</td>
</tr>
<tr>
<td>H596c</td>
<td>3025d</td>
<td>wt</td>
<td>mt</td>
<td>Null</td>
</tr>
</tbody>
</table>

* wt, wild type.

* Undetectable protein expression (31–33).

* Statistically different IC50 from other cell lines (P < 0.001).

* Extrapolated IC50.

* Codon 245, GGC→TGC, Gly→Cys, and loss of heterozygosity (13).
biotinylated secondary antibodies were incubated with streptavidin-horseradish peroxidase (Boehringer Mannheim, Indianapolis, IN) diluted to 1:1000 in TBST for 30 min, followed by two washes in TBST. All blots were washed in TBS (TBST lacking Tween) for 5 min and incubated with chemiluminescent detection reagents (ECL; Amersham, Arlington Heights, IL) as per instructions of the manufacturer. Finally, membranes were exposed to Kodak XAR films. After development, blots were stained with Ponceau S total protein stain (Sigma Chemical Co., St. Louis, MO) to confirm equivalent transfer of proteins. The protein bands of interest were compared with expression of the endogenous control β-actin detected with antibody clone AC15 (Sigma).

**Growth Curves.** Cell growth was assessed using the MTT assay (Sigma Chemical Co.; Ref. 29). Each experiment consisted of five or six replicate wells at each treatment dose, and each experiment was performed at least four times. Cells were plated on 96-well plates at a density of 5000 cells/well. One day after plating, cells were treated with UCN-01 at concentrations ranging from 10 to 1000 nM. After drug exposure for 24 h, the UCN-01 containing medium was removed, and the wells were washed and replaced with fresh medium. When control cells reached 90% confluence, 25 μl of MTT (2 mg/ml in PBS, pH 7.4) was added to each well and incubated for 3 h at 37°C. The medium was then removed, and 175 μl of DMSO were added and incubated for 15 min while agitated on an orbital rotator. Signal from the MTT dye was read by absorbance using the E-max microplate reader with Softmax software (Molecular Devices, Sunnyvale, CA). Absorbance readings were converted to cell numbers by comparison with the standard curves (four-parameter fittings) for each cell line and then divided by the cell number in the untreated control wells to obtain the fraction of cells present after treatment with each dose of UCN-01. The fraction affected was equal to 1.0 minus the fraction present in the treated wells. These data were then analyzed using CalcuSyn software (Biosoft, Cambridge, United Kingdom) to determine the concentration at which cell growth is inhibited by 50% (IC$_{50}$).

**Flow Cytometry.** Cells were pelleted and resuspended in fixative (95% methanol, 5% glacial acetic acid) for 3 or more
RESULTS

UCN-01 Inhibits Growth of NSCLC Cell Lines with Wild-Type RB. To correlate the growth response after UCN-01 treatment with the molecular status of selected cell cycle regulatory genes, human NSCLC cell lines with differential abnormalities of p16CDKN2, p53 and RB were evaluated in comparison to the normal human fetal lung fibroblast strain, T3891 (Table 1). A549 cells are p16CDKN2-null, Calu1 cells are p53-null, and H596 cells are RB-null and additionally harbor a p53 point mutation (codon 245, GGC→TGC, Gly→Cys, and loss of heterozygosity; Refs. 12, 13, 31–33). We have shown previously that in Calu1 cells, p16CDKN2 protein is undetectable, despite the presence of mRNA by reverse transcription-PCR (34). Further characterization by Western blotting of A549 and H596 cells verified the absence of protein expression of p16CDKN2 and Rb, respectively (data not shown).

To examine the involvement of p16CDKN2 in the response to UCN-01, A549 and T3891 cells were compared. Treatment resulted in a dose-dependent inhibition of cell growth with no significant difference between these p16CDKN2-null and the normal cells (P > 0.05; Fig. 1). Thus, in this model, p16CDKN2 is not required for UCN-01-induced growth inhibition. Because of the established roles of p53 in cell cycle regulation and p21CDKN1 induction, we examined the consequence of mutant p53 in the response to UCN-01 by comparing Calu1 with A549 and T3891. Calu1 cells (p53-null) were also responsive to UCN-01 treatment (Fig. 1). Although Calu1 cells were somewhat less inhibited, there were no significant differences when the responses of T3891 or A549 versus Calu1 were compared (P > 0.05 in both cases). Thus, p53 is not required for UCN-01-induced growth inhibition. In contrast, H596 cells (RB-null) were more refractory to UCN-01 treatment (Fig. 1). Using these data, the extrapolated IC50 of H596 was 3025 nM. The difference in growth response between this cell line and each of the others was statistically significant (P < 0.001 in all cases), suggesting the possibility that RB status influences growth response to UCN-01. IC50s from these experiments are correlated with the molecular status of p16CDKN2, p53, and RB of these cell lines in Table 1.

UCN-01 Treatment Results in Rb Hypophosphorylation. To investigate changes in the phosphorylation state of Rb, T3891, and A549 cells were cultured with 100, 300, and 600 nM concentrations of UCN-01 for both 24 and 72 h. These treatments resulted in a shift of Rb to the hypophosphorylated state (Fig. 2A). The Rb in T3891 cells was completely hypophosphorylated by 100 nM UCN-01 at 24 h. In A549 cells, RB phosphorylation was both time and dose dependent, showing complete hypophosphorylation at 72 h of 600 nM UCN-01. Changes in RB phosphorylation in Calu1 cells were investigated
at a dose of 500 nM UCN-01 for 24 h. Hypophosphorylation was observed at this dose (Fig. 2B). To determine the time dependence of Rb hypophosphorylation, T3891 cells were treated with 100 nM of UCN-01 for 12 h. Hypophosphorylation of Rb was nearly complete at this time point (Fig. 2C).

**p16<sup>CDKN2</sup> Protein Expression Is Not Induced by UCN-01 Treatment.** Induction of p16<sup>CDKN2</sup> expression after treatment with UCN-01 was examined by Western blotting. In T3891 cells, p16<sup>CDKN2</sup> showed no substantial change after 24 h of 100 nM UCN-01 (Fig. 3).

**UCN-01 Induction of p21<sup>CDKN1</sup> Is p53 Independent.** Protein expression of the CDK inhibitor p21<sup>CDKN1</sup> was assessed after 24 h treatment with UCN-01 at 100 and 500 nM concentrations. Induction of p21<sup>CDKN1</sup> was evident in all cell lines examined (Fig. 4). In A549 and H596 cells, basal expression of p21<sup>CDKN1</sup> was observed, which was further induced after treatment. In the p53-null Calu1 line, basal expression was not observed; however, after UCN-01 treatment, p21<sup>CDKN1</sup> was readily detectable, indicating that UCN-01-induced expression of p21<sup>CDKN1</sup> is p53 independent. Induction of p21<sup>CDKN1</sup> was
Fig. 6  A, UCN-01-induced Rb hypophosphorylation in RB-transfected 5637 cells. The RB-expressing RB5 subline was treated with UCN-01 at 100 or 500 nM for 48 h. Treatment resulted in Rb hypophosphorylation at both doses. B, UCN-01-induced G1 arrest in RB5 cells. Treatment with UCN-01 at 100 or 500 nM for 24 h resulted in accumulation of cells in the G1 phase of the cell cycle in the RB-expressing 5637 subline B5. In parent 5637 cells, modest G1 arrest is seen when treated with 500 nM UCN-01.
also observed in the normal fibroblast strain T3891 (data not shown).

**NSCLC Cells with Wild-Type RB Arrest in G₁ after UCN-01 Treatment, Independent of p53 Status.** The DNA content of individual cells was analyzed to examine cell cycle modulation after exposure to UCN-01. After UCN-01 treatment, T3891 and A549 cells showed a near complete absence of cells in S-phase and an accumulation of cells in G₀-G₁ (Fig. 5, A and B). T3891 cells treated with UCN-01 showed a percentage of cells in G₂ (10% or less) as did A549 cells, whereas cells arrested by serum starvation accumulated almost exclusively in the G₀-G₁ phase. UCN-01-treated Calu1 cells (p53-null) showed a decrease of ~30% of cells in S-phase and an equivalent increase of cells in the G₀-G₁ phase and a small G₂ peak (data not shown). The RB-null NSCLC line H596 was also investigated for changes in cell cycle response. At both the 100 and 500 nm dose, the percentage of cells in S-phase was largely unchanged, indicating continuing exit from G₁. In addition, a substantial decrease in cells in G-M was noted (Fig. 5C).

**RB Status Influences UCN-01-induced G₁ Arrest.** To further test the role of RB in UCN-01 response, we investigated the effects using two established human isogenic models of RB expression, the bladder cancer cell line 5637 (RB-null) and the prostate cancer cell line DU-145 (RB-mutant). These cell lines were each stably transfected with wild-type RB, yielding the 5637 subline RB5 and the DU-145 subline DU1.1 and B5 (26, 27). The expressed RB protein in RB5 was hypophosphorylated after treatment with both 100 and 500 nm UCN-01 (Fig. 6A). At 100 nm UCN-01, RB5 cells showed marked accumulation in G₁ phase in contrast to the RB-null parent line 5637, demonstrating the role of RB in G₁ arrest in response to UCN-01 (Fig. 6B). G₁ accumulation in the parent line became apparent only at 500 nm.

The prostate carcinoma model of RB expression revealed similar results at 500 nm UCN-01 for 30 h (Table 2; Fig. 7). Increases in G₁ accumulation were greater in the sublines (DU1.1, +32%; B5, +27%; average, 29.5%) than in the parent (DU-145, +20%). Similar results were obtained from cells treated with 100 nm UCN-01 for 72 h. Decreases in percentages of cells in S-phase from the two sublines expressing wild-type RB (DU1.1, −24%; B5, −18%; average, −21%) were also greater than the RB-mutant parent line (DU-145, −14%). These data further support a role for wild-type RB in G₁ arrest in response to UCN-01.

**DISCUSSION**

Lung cancer is now the most common cause of cancer-related death in both men and women. Because most patients present with advanced stage disease, improved systemic therapy is essential. UCN-01 represents a new class of anticancer agents with a novel therapeutic target: the cell cycle. Disruption of cell cycle regulation is a common motif in lung cancer. In NSCLC cell lines, abnormal RB is found at a frequency of 15%, whereas p16CDKN2 alterations are more common, estimated at 45% (11, 12). In contrast, RB abnormalities are common in SCLC, reported as high as 88%, whereas p16CDKN2 abnormalities occur rarely (11, 12). In both types of cancer, p53 alterations are relatively common, with frequencies of ~45% for NSCLC and as high as 80–100% for SCLC (35). Reported here are investigations of the molecular mechanisms of UCN-01 response using...
an experimental model of human NSCLC lines with differential abnormalities in these cell cycle regulatory genes.

This report demonstrates that response to UCN-01 is associated with wild-type RB status in NSCLC cells and is characterized by G1 checkpoint arrest. Evidence supporting the role of wild-type RB status as a determinant of UCN-01 response includes the induction of p21CDKN1 expression and the modulation of the phosphorylation state of Rb, a possible mechanism of G1 arrest.

To further test the role of RB in the response to UCN-01, we used two established human isogenic models of RB expression, one a bladder carcinoma line and the other a prostate carcinoma, because of the absence of a NSCLC model. In the bladder cancer model, the RB-expressing subline showed Rb hypophosphorylation and G1 arrest at 100 nM treatment, in contrast to the parent line. In the prostate cancer model, the wild-type RB-expressing sublines showed greater G1 accumulation and a greater diminishment of S-phase cells when treated with UCN-01. Thus, these data also support the hypothesis that RB status is a determinant of response to UCN-01. However, the limited G1 arrest seen in both parent lines of these isogenic models indicates that RB is not the sole determinant of G1 arrest in response to UCN-01.

Previously, Seynaeve et al. (15) reported differential sensitivity to UCN-01 treatment in a series of breast cancer cell lines, with some lines exhibiting near complete, irreversible growth arrest, whereas others showed a more limited response. On the basis of our findings, these results may be clarified by the observation that the MDA-MB-468 cell line, which was the least responsive, is RB-mutant (36). The association of UCN-01 response with wild-type RB is further supported by similar results reported by our group with staurospermine, a closely related compound (26, 37, 38). RB was required for staurospermine-induced G1 arrest in human bladder carcinoma 5637 cells as demonstrated by the RB5 subline.

Additional conclusions from this study are as follows: (a) because p16CDKN2-null cells (A549) were strongly inhibited by UCN-01 and no increase in expression of the Cdk inhibitor was observed, we conclude that p16CDKN2 is not required for UCN-01-induced growth arrest; and (b) the finding that UCN-01 induced p21CDKN1 expression and growth arrest in p53-null cells (Calu1) suggests that UCN-01 functions, at least in part, through the p53-independent induction of p21CDKN1. These observations do not rule out the possibility that UCN-01 may operate additionally through a p53-dependent pathway; p53-null Calu1 cells were growth inhibited less than the wild-type p53 cell lines T3891 and A549. However, this difference was not statistically significant. Recently, Husain et al. (39) showed that the activity of UCN-01 as a single agent was independent of p53 status in a human ovarian cancer model. However, their p53 transfection experiments suggested that G1 arrest in response to UCN-01 was p53-dependent. In contrast, our data in a p53-null NSCLC cell line implicate the p21CDKN1 and RB genes as playing key roles in the G1 arrest.

UCN-01 is also of therapeutic interest due to its ability to potentiate the anticancer activity of chemotherapy and radiation. Others have reported that potentiation of DNA-damaging agents, such as radiation and cisplatin, by UCN-01 is greater in cells with disrupted p53 (18, 19). In our studies, UCN-01 as a single agent demonstrated activity in cell lines both wild-type and null for p53. The ability of UCN-01 to sensitize cells to cisplatin or radiation appears to result from an abrogation of the DNA damage-induced G1 checkpoint arrest (18, 19). In contrast, the mechanism of anticancer action of UCN-01 as a single agent is clearly different, where G1 arrest is a predominant feature.

In conclusion, our findings suggest that the molecular profile of key cell cycle regulatory genes in human cancers may predict responsiveness to new antineoplastic agents. Because UCN-01 is now entering early clinical trials in cancer patients, additional investigations designed to clarify further the molecular mechanisms of action of this novel therapeutic agent are warranted.

ACKNOWLEDGMENTS

We thank Drs. Arline Deitch and Kayoko Nishi for advice on these studies. We also thank Dr. Edward A. Sausville for providing the UCN-01 and Dr. David W. Goodrich for providing the 5637 cells and the RB-expressing subline RB5. Additionally, our thanks go to Jared T. Muenzer, Jeff Yun, Mahesh Patel, and Andrew Hufton for assistance with cell culturing and Western blotting.

REFERENCES

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