Decrease in Susceptibility Toward Induction of Apoptosis and Alteration in G1 Checkpoint Function as Determinants of Resistance of Human Lung Cancer Cells against the Antisignaling Drug UCN-01 (7-Hydroxy staurosporine)

Kazuyo Sugiyama, Tadakazu Akiyama, Makiko Shimizu, Tatsuya Tamaoki, Carol Courage, Andreas Gescher, and Shiro Akinaga

ABSTRACT

7-Hydroxy staurosporine (UCN-01) is a protein kinase inhibitor that is under development as an anticancer agent in the United States and Japan. Long-term exposure of human A549 non-small cell lung cancer cells to UCN-01 furnished cells (A549/UCN) with acquired resistance against UCN-01. In this study, the sensitivity of these cells toward the growth-arresting properties of certain conventional cytotoxic agents was explored. Cells were not cross-resistant against Adriamycin, Taxol, staurosporine, and UCN-02, but they displayed 14- and 4.4-fold resistance against cisplatin and mitomycin C, respectively. Previous studies on the mechanism(s) of action of UCN-01 suggest that induction of apoptosis and G1 phase accumulation are important for its anticancer activity; therefore, we compared induction of apoptosis and cell cycle distribution caused by UCN-01 in wild-type A549 and A549/UCN cells using flow cytometry. UCN-01 (0.4 μM) induced apoptosis (62% terminal deoxynucleotidyl transferase-mediated nick end labeling-positive cells) in A549 cells, but not in A549/UCN cells. The percentages of cells that accumulated in G1, when exposed to UCN-01 (0.4 μM) were 22% in A549 cells and 67% in A549/UCN cells. These results suggest that acquired resistance of cancer cells against UCN-01 is characterized by attenuation of apoptosis induction associated with reinforcement of the G1 checkpoint and that apoptosis regulation is drastically altered in A549/UCN cells as compared with A549 cells. Cyclin-dependent kinase (CDK) inhibitor proteins p21 and p27 in A549/UCN cells against UCN-01 is characterized by attenuation of apoptosis induction associated with reinforcement of the G1 checkpoint and that apoptosis regulation is drastically altered in A549/UCN cells as compared with A549 cells. Cyclin-dependent kinase (CDK) inhibitor proteins p21 and p27 in A549/UCN cells were up-regulated, which was accompanied by overexpression of G1 cyclins D1 and E, but UCN-01 hardly affected levels of these proteins. In contrast, cyclin A, cyclin B1, retinoblastoma, and CDK2 proteins were apparently down-regulated, without changes in CDK4/6. UCN-01 hardly affected the expression level of cyclin B1 and induced dephosphorylation of retinoblastoma in both cell types. UCN-01 induced down-regulation of cyclin A level and CDK2 activity accompanied with its dephosphorylation in A549/UCN cells, but not in A549 cells. The antiapoptotic protein bel-2 was apparently up-regulated in A549/UCN cells, however, bel-xl, another antiapoptotic protein, was down-regulated, without changes in bak and bax. Taken together, these results are consistent with the notion that induction of apoptosis and block of cell cycle in G1 are important determinants of the sensitivity of cancer cells to UCN-01 and suggest that inhibition of CDK2 activity accompanied by its dephosphorylation and decrease of expression level of cyclin A might play an important role in the G1 phase accumulation induced by UCN-01.

INTRODUCTION

UCN-01 is originally isolated from the culture broth of Streptomyces sp. as a PKC-selective inhibitor (1). Several studies from our and other laboratories revealed that the drug possessed potent antitumor activity in several in vitro and in vivo preclinical models (2–6).

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2 The abbreviations used are: UCN-01, 7-hydroxy staurosporine; CDK, cyclin-dependent kinase; PKC, protein kinase C; RB, retinoblastoma; STP, staurosporine; PARP, poly(ADP-ribose) polymerase; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT-mediated nick end labeling; PI, propidium iodide.

These studies also showed a unique fingerprint pattern referred to as “compare negative” in the in vitro anticancer drugs screening panel of the National Cancer Institute, suggesting that the mechanism of action of the drug is unique (7). In addition to its anticancer activity as a single agent, UCN-01 was shown to enhance the cytotoxicity of some DNA-damaging agents and antimetabolites in vitro or in vivo preclinical models (8–13). On the basis of these preclinical data, clinical investigations have been initiated in the United States and Japan to explore the potential clinical usefulness of UCN-01.

Although the PKC enzyme family has been considered to be a major target of UCN-01, it is unclear how PKC inhibition can cause anticancer activity of the drug, and the most important target for UCN-01 is still obscure. Cell cycle analysis revealed that UCN-01 induces G1 phase accumulation in a wide spectrum of cancer cell lines (5, 9, 14, 15), suggesting that this event is one of the major consequences of treatment of human cancer cells with UCN-01. This feature is rarely observed with traditional cytotoxic anticancer agents. We have also shown that the UCN-01-induced G1 phase accumulation is mediated via direct and indirect inhibition of Rb kinase(s), such as CDK2 (16).

Furthermore, studies from the National Cancer Institute group (6, 17, 18) and our laboratories (14) suggest that induction of apoptosis is intrinsically linked to the cytotoxic effect of UCN-01. UCN-01 induced apoptosis both in leukemia and colon carcinoma cells, the latter being relativity resistant to apoptosis induction by conventional cytotoxic agents, such as camptothecin (17).

The aim of this study was to improve our understanding of the mechanism of antineoplastic action of UCN-01. Its special objective was to explore the intriguing association between apoptosis induction, G1 phase accumulation, and Rb kinase inhibition, which determines the cellular response toward this drug using a human A549 lung adenocarcinoma cell line with acquired resistance against UCN-01 (A549/UCN cells). When these cells were derived and compared with their wild-type counterparts, they were shown to possess down-regulated PKC-α, -ε, and -θ and to express the multidrug resistance-associated protein, but they lacked cross-resistance against STP and its PKC-specific analogue CGP 41251 (19). Specifically, we wished to compare A549/UCN and A549 cells in terms of: (a) their sensitivity to the growth-arresting properties of selected anticancer drugs with differing mechanisms of cytotoxicity; (b) their susceptibility toward induction of apoptosis and toward G1 phase accumulation elicited by UCN-01; and (c) their susceptibility toward UCN-01-induced changes in expression of proteins involved in apoptosis and G1 and S phase checkpoint functions.

UCN-01 is one of the first representatives of a relatively new generation of kinase-directed signal transduction modulators that are currently progressing into clinical trials. Therefore, the characterization of cellular resistance mechanisms operative against this drug was thought to help predict some determinants of clinical resistance that might eventually complicate therapy using such kinase-directed agents.
Table 1: Growth-inhibitory activity of UCN-01 or other anticancer drugs against A549 and A549/UCN cell lines

<table>
<thead>
<tr>
<th>Drug</th>
<th>A549 IC_{50} (μM)</th>
<th>A549/UCN</th>
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<tbody>
<tr>
<td>UCN-01</td>
<td>0.099</td>
<td>0.36</td>
</tr>
<tr>
<td>STP</td>
<td>0.020</td>
<td>0.012</td>
</tr>
<tr>
<td>UCN-02</td>
<td>0.72</td>
<td>0.65</td>
</tr>
<tr>
<td>CGP 41251</td>
<td>0.19</td>
<td>0.49</td>
</tr>
<tr>
<td>Flavopiridol</td>
<td>0.082</td>
<td>1.1</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>5.3</td>
<td>74</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>0.096</td>
<td>0.42</td>
</tr>
<tr>
<td>Adriamycin</td>
<td>0.45</td>
<td>0.59</td>
</tr>
<tr>
<td>Taxol</td>
<td>0.0096</td>
<td>0.0059</td>
</tr>
<tr>
<td>Navelbine</td>
<td>0.0081</td>
<td>0.0045</td>
</tr>
</tbody>
</table>

\* MT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.
\* Ratio of IC_{50} in A549/UCN cells and IC_{50} in A549 cells.

MATERIALS AND METHODS

Drugs, Reagents, and Antibodies. UCN-01 was produced by fermentation in our institute, as described previously (1). STP, CGP 41251, UCN-02, flavopiridol, navelbine, mitomycin C, and adriamycin were purchased by Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). Taxol and cisplatin were purchased from Sigma Chemical Co. (St. Louis, MO). Drugs were dissolved in DMSO and freshly diluted with culture medium. Tris and Tween 20 were purchased from Bio-Rad Laboratories (Hercules, CA); NaCl, EDTA, and sodium fluoride from Kanto Chemical Co., Inc. (Tokyo, Japan); Triton X-100 from Yoneyama Yakuhin Kogyo Co., Ltd. (Osaka, Japan); and HEPES, 
\( \beta \)-glycerophosphate, sodium o-vanadate, phenylmethylsulfonyl fluoride, aprotinin, and leupeptin from Sigma Chemical Co.

Antibodies were purchased from the following suppliers: anti-p21, p27, CDK4, CDK6, and bax polyclonal antibodies and anti-cyclin B1 monoclonal antibody from Santa Cruz Biotechnology (Santa Cruz, CA); anti-Rb and cyclin E monoclonal antibodies from Pharmingen (San Diego, CA); anticyclin D1 monoclonal antibody from IBL (Gunma, Japan); anti-PARP monoclonal antibody from Enzyme Systems Products (Dublin, CA); anti-bcl-2 monoclonal antibody from Genosys (Cambridgeshire, United Kingdom); anti-bcl-xL polyclonal antibody from Transduction Laboratories (Lexington, KY); anti-bak monoclonal antibody from Calbiochem (Cambridge, MA); anticyclin A and CDK2 monoclonal antibodies were prepared as described previously (16).

Cell Cycle and Assessment of Inhibition of Cell Growth. A549/UCN cells were derived from their parent A549 cells, as described previously (19). Cells were maintained in Ham's F-12 medium supplemented with 10% fetal bovine serum, 100 IU of penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine (Life Technologies Inc., Grand Island, NY) in an atmosphere of 5% CO₂. A549/UCN cells were maintained in the presence of 200 nM UCN-01.

In cell growth inhibition experiments, cells were incubated in culture medium for 24 h in 96-well microwell plates (Nunc, Roskilde, Denmark) before incubation with drug for 72 h. Cell viability was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co.) assay (20).

**Cell Cycle Analysis.** Cells were incubated in the culture medium for 24 h in Falcon 3003 plastic dishes (Becton Dickinson, Lincoln Park, NJ) before incubation with UCN-01 for 24 h. The cells were harvested by trypsin-EDTA treatment, fixed with ice-cold ethanol (70%), and stored at 4°C. For flow cytometric analysis, the RNA was hydrolyzed using 250 μg/ml RNase A (type 1-A; Sigma Chemical Co.) at 37°C for 30 min, and the cells were stained with propidium iodide (PI; Sigma Chemical Co.) for 20 min. DNA content of cells was analyzed using an EPICS ELITE flow cytometer (Coulter Electronics, Hialeah, FL). Cell cycle distribution was calculated by a Multicycle program (Phoenix Flow Systems, Inc., San Diego, CA).

**Assessment of Apoptosis.** For the TUNEL assay, cells were processed according to the manufacturer's instructions using ApopTag Direct Kit (Oncor, Inc., Gaithersburg, MD, Ref. 21). Briefly, cells were fixed in 1% formaldehyde solution for 15 min on ice, washed in PBS, suspended in 70% ethanol, and stored at -20°C. After washing in PBS, the cells were resuspended first in equilibration buffer for 15 min at room temperature, then in TdT reaction buffer (50 μl) at 37°C for 30 min. After stopping the TdT reaction, the cells were incubated in PBS (1 ml) containing 10 μg/ml PI and 10 μg/ml RNase A for 15 min at room temperature in the dark. Bivariate analysis of apoptosis (green fluorescence) and DNA content (PI) was performed flow cytometrically. The resulting bivariate plots allow detection of apoptotic events initiated within the cell cycle. Using the control specimen to define normal levels of green fluorescence (i.e., basal levels of apoptosis), the R1 region was set. Cells with fluorescence within the R1 region were considered apoptotic. Data from 20,000 cells were collected and analyzed using the Multi2D program (Phoenix Flow Systems).

**Western Blotting.** Western blotting was performed as described previously (16). Cells exposed to drug were harvested by trypsin-EDTA treatment, washed with PBS, and stored at -80°C. They were lysed in lysis buffer (50 mM HEPES/NaOH [pH 7.4], 150 mM NaCl, 0.1% Triton X-100, 50 mM sodium fluoride, 80 mM glycerophosphate, 0.1 mM sodium o-vanadate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) for 20 min at 4°C. The cell lysate was centrifuged (14,000 rpm) for 10 min at 4°C, and its protein content was determined using a protein assay kit (Bio-Rad Laboratories). Equal amounts of protein were heated in SDS-sample buffer for 5 min at 95°C and subjected to SDS-PAGE. Protein was transferred to a nitrocellulose membrane (Amersham Life Sciences, Buckinghamshire, United Kingdom), and detected by enhanced chemiluminescence (Amersham Life Sciences).

**CDK2 Kinase Assay.** CDK2 kinase assay was performed, as described previously (16). Cell lystate was prepared as described above. Equal amounts of protein (300 μg) of the cell lysate were added to protein A-Sepharose CL-4B (20%, v/v; Pharmacia, Uppsala, Sweden) pre-associated with anti-CDK2 antibody and mixed gently for 2 h at 4°C. CDK2-immunoprecipitate was washed with lysis buffer twice and washed buffer [50 mM HEPES/NaOH (pH 7.4), 10 mM MgCl₂, and 1 mM DTT] two times, and incubated with kinase buffer (40 μl) [50 mM HEPES/NaOH (pH 7.4), 10 mM MgCl₂, 1 mM DTT, 16 μM of histone H1 (Boehringer Mannheim, Mannheim, Germany), 50 μM ATP, and 2.5 μC [γ-32P]ATP (5000 Ci/mmol; Amersham Life Sciences)] for 10 min at 30°C. Each sample was mixed with 3 × SDS-sample buffer (30 μl) to stop the reaction, heated for 5 min at 95°C, and subjected to SDS-PAGE. Gel

![Fig. 1. Effect of UCN-01 on cell cycle distribution in A549 and A549/UCN cells. Cells were harvested after 24 h of treatment with UCN-01 (0.08, 0.24, and 0.4 μM). Cell fixation, RNA hydrolysis, and DNA staining with PI were performed as described in “Materials and Methods.” Data shown are representative of three independent experiments.](image-url)
was dried, stained with Coomassie Brilliant Blue, and analyzed by a BAS2000 image analyzer (Fuji photo film, Tokyo, Japan).

RESULTS

Sensitivity of A549/UCN Cells to STP Analogues and Cytotoxic Agents. A549/UCN cells have been previously characterized by 4-fold resistance against UCN-01 and hardly any cross-resistance against STP and its analogues CGP 41251 and Ro 31–8220 (19). Because the A549/UCN cells used in the work described here were routinely cultured in the presence of UCN-01, we reevaluated their sensitivity to the growth-arresting effects of STP and its congeners UCN-01, UCN-02, and CGP 41251. As shown in Table 1, in comparison with wild-type A549 cells, A549/UCN cells showed 3.6- and 2.6-fold resistance against UCN-01 and CGP 41251, respectively, but lacked cross-resistance against STP and UCN-02. Dose-response curves of UCN-01 showed that acquired resistance to UCN-01 was more pronounced at higher concentrations [i.e., at 300 nM UCN-01 the percentage decrease in cell number was 90% for A549, but only 55% for A549/UCN cells (data not shown)]. Dose-response curves of CGP 41251 also showed that resistance against CGP 41251 was weak because at higher concentrations both cell lines were sensitive to the drug, A549/UCN cells even more so than A549 cells at concentrations of above 3 μM CGP 41251 (data not shown). To characterize A549/UCN cells further, their potential sensitivity to the following clinically used anticancer drugs was investigated: Adriamycin representing DNA-intercalating topoisomerase II poisons; cisplatin and mitomycin C representing DNA intrastrand cross-linking agents; and Taxol and navelbine representing mitotic poisons. A549/UCN cells were not cross-resistant against Adriamycin, Taxol, or navelbine. However, they showed 4.4- and 14-fold cross-resistance against mitomycin C and cisplatin at IC₅₀ levels, respectively (Table 1). A549/UCN cells were also investigated in terms of their sensitivity to flavopiridol, a CDK inhibitor currently under clinical scrutiny (22), because UCN-01 has also been shown to inhibit CDK2 in a direct and indirect manner (15, 16). A549/UCN cells were not resistant against flavopiridol (Table 1).

Susceptibility of A549/UCN Cells to UCN-01-induced Changes in Cell Cycle Distribution and to Drug-induced Apoptosis. To assess whether A549/UCN cells are susceptible toward two key biochemical effects of UCN-01, induction of G₁ phase accumulation (5, 16), and induction of apoptosis (17, 18), cells were incubated for 24 h with UCN-01 at concentrations near the IC₅₀ values, 0.08 μM in the case of A549 cells and 0.24 and 0.4 μM for A549/UCN cells. Analyses of cell cycle distribution and apoptosis were performed by PI single staining and TUNEL assay using flow cytometry, respectively. Another marker of apoptotic cell death, cleavage of PARP protein, which is executed by caspase 3, was tested by Western blotting. Fig. 1 shows that UCN-01 at 0.08 μM induced only a slight accumulation of A549 cells in G₁ phase, whereas the drug at higher concentrations blocked cells in the S and G₂M phases, but not in G₁ phase. The percentages of cells in G₁ phase were 43%, 47%, 34%, and 22% at 0, 0.08, 0.24, and 0.4 μM of UCN-01, respectively (Fig. 1). In contrast, in A549/UCN cells, UCN-01 induced a clearly concentration-dependent increase in the G₁ phase population (Fig. 1).
two independent experiments.

cleavage assay (Fig. 5), and assessment of morphological indicators as to induction of apoptosis (data not shown). In sharp contrast, the shrinkage, chromatin condensation, and nuclear fragmentation in appearance of an 85-kDa protein was also observed after UCN-01 concentration-dependent cleavage of PARP protein assessed by the positive in the TUNEL assay (Fig. 2). Furthermore, an apparent positive cells and in the sub-G_1 cell population, which was also cells, UCN-01 elicited a concentration-dependent increase in TUNEL-

Lane 1

C

untreated

staurosporine 0.3 μM

taxol 0.1 μM

Lane 2

A549

A549/UCN

DNA strand breaks

DNA content

Lane 3

B

A

Fig. 3. Induction of apoptosis by STP and Taxol in A549 and A549/UCN cells. Cells were harvested after 24 h of treatment with STP (0.3 μM) and Taxol (0.1 μM) or left untreated. Apoptotic cells were detected by flow cytometry using the TUNEL technique and by Western blotting, as described in “Materials and Methods.” Apoptotic cells were identified as those contained in the R1 region of a contour plot (A). B, degree of apoptosis is expressed as percentage of cells. C, the effects of STP and Taxol on expression levels of intact PARP (116 kDa) and its proteolytic cleavage product (85 kDa): Lane 1, untreated; Lane 2, 0.3 μM STP; Lane 3, 0.1 μM Taxol. Data shown are representative of two independent experiments.

percentages of cells in G_1 phase were 43%, 58%, 66%, and 67% at 0, 0.08, 0.24, and 0.4 μM of UCN-01, respectively (Fig. 1). In A549 cells, UCN-01 elicited a concentration-dependent increase in TUNEL-positive cells and in the sub-G_1 cell population, which was also positive in the TUNEL assay (Fig. 2). Furthermore, an apparent concentration-dependent cleavage of PARP protein assessed by the appearance of an 85-kDa protein was also observed after UCN-01 treatment in these cells (Fig. 5). Morphological changes such as cell shrinkage, chromatin condensation, and nuclear fragmentation in A549 cells after treatment with UCN-01 supported the inference made as to induction of apoptosis (data not shown). In sharp contrast, the drug induced hardly any apoptosis in A549/UCN cells at concentrations as high as 0.4 μM, as adjudged by TUNEL assay (Fig. 2), PARP cleavage assay (Fig. 5), and assessment of morphological indicators (data not shown). These results suggest that attenuation of apoptosis induction and augmentation of G_1 phase checkpoint function might be pivotal determinants of the acquired resistance of A549/UCN cells against UCN-01.

Susceptibility of A549/UCN Cells Toward Induction of Apoptosis by STP and Taxol. Because A549/UCN cells exhibited decreased susceptibility toward induction of apoptosis by UCN-01 as compared with their wild-type counterparts, the hypothesis was tested that these cells might have also acquired resistance against apoptosis induction by known apoptosis inducers represented by cytoxic agents, such as Taxol and STP. A549/UCN or A549 cells were treated with 0.3 μM STP or 0.1 μM Taxol for 24 h, and induction of apoptosis was determined by TUNEL and PARP cleavage assays. Both STP and Taxol induced apoptosis in either cell line; their apoptogenicity was slightly more pronounced in A549/UCN cells than in wild-type A549 cells (Fig. 3). This result suggests that A549/UCN cells display selective resistance against UCN-01-induced apoptosis, which is consistent with the sensitivity pattern of these cells to the growth-arresting properties of UCN-01, Taxol, and STP (Table 1).

Difference in between A549 and A549/UCN Cells Expression of Proteins Involved in Cell Cycle Regulation and Apoptosis Induction. To elucidate the mechanism(s) of resistance to UCN-01 in A549/UCN cells, we compared expression levels of proteins germane to the regulation of the cell cycle and apoptosis in A549/UCN cells with those in A549 cells. With regard to G_s and S phase cell cycle regulatory proteins, CDK inhibitor proteins p21 and p27 and G_1 cyclins D1 and E were up-regulated in A549/UCN cells as compared with A549 cells (Fig. 4A). In contrast, expression of Rb and S and G_s cyclins A and B1 and CDK2 was down-regulated, whereas levels of CDK4 and CDK6 proteins were only slightly decreased (Fig. 4A). The apoptosis-regulatory proteins, the levels of which were measured, were the antiapoptotic molecules bcl-2 and bcl-xL and the proapoptotic species bak, bax, and PARP. Whereas wild-type A549 cells expressed bcl-xL but not bcl-2, A549/UCN cells expressed bcl-2 with only traces of bcl-xL (Fig. 4B). A549/UCN cells displayed slight up-regulation of expression of bak and bax proteins (Fig. 4B). PARP, an important substrate for the death-inducing enzyme caspase 3, was apparently down-regulated in A549/UCN cells (Fig. 5). Because PKC family proteins were reported to play a role as an upstream regulator of Raf-1 protein (23), we have examined the expression level of Raf-1 protein in A549/UCN and A549 cells. There was no difference in Raf-1 expression level between both cell lines (data not shown).

Susceptibility of A549/UCN Cells Toward UCN-01-induced Changes in Expression Levels of Proteins Involved in Cell Cycle Regulation and Apoptosis Induction. We wished to elucidate further the role of cell cycle- and apoptosis-regulatory proteins as arbiters of the resistance of A549/UCN cells against UCN-01. To that end, we studied the effect of UCN-01 on the expression level of the proteins forward to be differentially expressed between A549 and A549/UCN cells (see Fig. 4). Cells were incubated with UCN-01 (0.08, 0.24, and 0.4 μM) for 24 h and analyzed by Western blotting. Although UCN-01 did not affect the level of p21 in A549 cells, it induced its expression in A549/UCN cells (Fig. 5), a phenomenon that has previously been reported in other cell types (16). UCN-01 did not affect the expression level of p27 in either A549 or A549/UCN cells (Fig. 5), which differs from its effects reported previously (16). UCN-01 hardly affected the levels of cyclins D1, E, and B1 in either cell line (data not shown). The expression level of cyclin A was decreased slightly by UCN-01 in A549 cells; in contrast, in A549/UCN cells, it was markedly reduced to undetectable levels (Fig. 5). In both cell types, UCN-01 induced a decrease in Rb protein levels and the emergence of a Rb species with faster mobility, indicating dephosphorylation of Rb (Fig. 5). In A549/UCN cells, UCN-01 induced the disappearance of a faster migrating band of CDK2, which indicates dephosphorylation of the CDK2 protein (Fig. 5). This result suggests that the dephosphorylation of CDK2 might play an important role in the G_s phase accumulation induced by UCN-01. Although the expression levels of bcl-2 were not
affected by UCN-01, bcl-xL protein levels were slightly decreased by UCN-01 in both cell lines, although its basal expression level was much higher in A549 cells than in A549/UCN cells. UCN-01 also did not cause a shift in mobility of the bcl-2 and bcl-xL proteins in these cells, an event that has been reported for mitotic poisons and interpreted as a marker of M phase arrest and/or apoptosis induction (24, 25).

Susceptibility of A549/UCN Cells Toward CDK2 Kinase Activity. Because the results of Fig. 5 have suggested that dephosphorylation of CDK2 might play an important role in G1 phase accumulation induced by UCN-01, we further compared the effect of UCN-01 on CDK2 kinase activity in A549 and A549/UCN cells. Cells were incubated with UCN-01 (0.08, 0.24, and 0.4 μM) for 24 h, and CDK2 activity was determined after immunoprecipitation with anti-CDK2 monoclonal antibody using histone H1 as a substrate. Although there was little change on CDK2 activities in A549 cells treated with UCN-01, CDK2 activity of A549/UCN cells was inhibited by UCN-01 in a concentration-dependent manner (Fig. 6). This result is consistent with the mobility shift of CDK2 and cyclin A protein level (Fig. 5), suggesting that inhibition of CDK2 activity might play an important role in the G1 phase accumulation induced by UCN-01.

DISCUSSION

UCN-01 is an anticancer drug candidate with a novel chemical structure and a unique mode of action. Initial clinical studies at the National Cancer Institute suggest that the drug can be administrated safely to humans at dosages that, on the basis of preclinical efficacy studies in mice, might elicit anticancer activity (26). Because the ultimate molecular target of UCN-01 remains elusive, it is very important to elucidate components that might contribute to its mechanism(s) of action. Preclinical studies from our and other laboratories using cultured human cell lines suggest that UCN-01 exhibits two key biochemical effects, accumulation of cells in the G1 phase of the cell cycle and induction of apoptosis, both of which might be important for...
its anticancer activity (5, 6, 15–18). The results described here demonstrate, for the first time, that human non-small cell lung cancer cells with acquired resistance against UCN-01, unlike their wild-type counterparts, are refractory against the ability of UCN-01 to induce apoptosis. This conclusion suggests that in some cancer cell types the induction of apoptosis is a pivotal arbiter of UCN-01 sensitivity, which is consistent with results presented previously (6, 17, 18). Intriguingly UCN-01 induced G1 phase accumulation more intensely in A549/UCN cells than in A549 cells, and this cell cycle arrest was inversely correlated with susceptibility toward induction of apoptosis, suggesting that G1 checkpoint function might be another critical determinant of UCN-01-sensitivity in cancer cells.

The determination of expression levels of G1 checkpoint proteins (i.e., of negative regulators such as the CDK inhibitors p21, p27, and Rb, as well as of positive regulators such as cyclins and CDKs) allowed further inferences to be made as to the mechanism of resistance against UCN-01 operative in A549/UCN cells. In the resistant cells, p21 and p27, as well as G1 cyclins D1 and E, were unambiguously clearly up-regulated, suggesting that these cells are characterized by a compromised balance of cell cycle-inhibitory and -accelerating molecules. Because p21 and p27 proteins are thought to play an important role as G1 checkpoint proteins (27), it is conceivable that the reinforcement of the G1 checkpoint function renders cells resistant against UCN-01. It has previously been suggested that dephosphorylation of both Rb and CDK2 proteins is important for the G1-phase accumulation induced by UCN-01 (16). However, in the study described here, UCN-01 induced the dephosphorylation of Rb protein in both A549 cells and their UCN-01 resistant counterparts, suggesting that a step downstream of Rb protein dephosphorylation might be responsible for G1 phase accumulation. The fact that the phosphorylated form of CDK2 was down-regulated by UCN-01 only in A549/UCN cells but not in wild-type A549 cells suggests that CDK2 dephosphorylation plays a more important role than Rb dephosphorylation in the G1 phase accumulation induced by UCN-01. Because UCN-01 has been shown to inhibit CDK2 activity in several cell lines (15, 16), the results presented here suggest that a CDK2 substrate other than Rb might be crucial for cell cycle blockage and/or apoptosis induced by UCN-01. Alternatively, the effects of UCN-01 on CDK2 in A549/UCN cells may be the consequence rather than the cause of the altered cell cycle arrest phenotype in the resistant cells. It is also conceivable that a protein upstream of CDK2, such as CDK-activating kinase, might be inhibited by UCN-01, thus engineering the down-regulation of CDK2 activity.

As to apoptosis-regulatory proteins, although A549 cells expressed bcl-xL but not bcl-2, in accordance with a previous study (28, 29), A549/UCN cells expressed bcl-2 but not bcl-xL. This difference suggests that bcl-2 might be an important determinant of UCN-01 sensitivity. It has been proposed that overexpression of bcl-2 can be responsible for resistance against apoptosis inducers (30, 31), a notion that prompted us to examine the susceptibility of UCN-01-resistant A549 cells toward apoptosis induction by Taxol and STP. A549/UCN cells were even more sensitive than A549 cells to apoptosis induction by both drugs, which is consistent with the pattern of susceptibility toward growth inhibitory potency, suggesting that the acquired resistance against UCN-01 in A549/UCN cells is a feature selective to UCN-01, at least among the drugs tested here.

Recently, p21 was reported to regulate negatively the induction of apoptosis, and the protein was shown to be a substrate of caspase 3, an important executor of apoptosis (32–34). p21 protein was clearly up-regulated by UCN-01 in A549/UCN cells but not in A549 cells, which harbor the wild-type p53 protein. These data are consistent with the notion that UCN-01 induces p21 via a p53-independent mechanism (16). p27, which has been shown to be associated with cytotoxic drug resistance in cells under anchorage-independent growth conditions (35, 36), was also up-regulated in A549/UCN cells. Taken together, the overexpression of p21 and p27 in A549/UCN cells might be an important determinant of acquired resistance against UCN-01, however, the mechanism of their overexpression remains to be elucidated.

The pattern of cross-resistance against a variety of anticancer agents observed in A549/UCN cells is in accordance with alterations in the checkpoint governing G1 rather than that regulating G2/M. The cells were not cross-resistant against Taxol and navelbine, which are M phase-acting drugs, or adriamycin, which arrests cells selectively at the G2 phase (37). Unexpectedly, A549/UCN cells exhibited cross-resistance against DNA cross-linking agents such as mitomycin C and cisplatin, both of which are thought to act on the G1-S phase, as well as the G2 phase, in p53 wild-type cells, such as A549. This cross-resistance can be interpreted to indicate that reinforcement of the G2 checkpoint also protects cells from the lethal consequences of DNA-damaging agents. Alternatively, UCN-01, cisplatin and mitomycin C could contribute directly to DNA damage leading to an increase in availability of DNA present in replication forks. Thus, susceptibility to apoptosis could arise from S phase arrest.

In conclusion, the results described above suggest that acquired resistance of A549/UCN cells against UCN-01 is mediated through the attenuation of apoptosis induction, which in turn might be the corollary of the enforcement of G1 checkpoint proteins, as well as antiapoptotic proteins. The results also suggest that the susceptibility of cells toward G1 phase accumulation elicited by UCN-01 can be inversely correlated to susceptibility to apoptosis induction and cellular sensitivity toward the drug. UCN-01 is one of several new kinase-directed antisignaling drugs that are currently entering clinical evaluation as anticancer drugs. Should any of these agents be eventually included into the oncologist’s chemotherapeutic armamentarium, it is prudent to be aware of the possibility that they may elicit clinical resistance as a consequence of the ability to alter processes that regulate the cell cycle machinery and apoptosis in a complicated fashion analogous to that described here for UCN-01.

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ATTENUATION OF APOPTOSIS INDUCTION BY UCN-01


Decrease in Susceptibility Toward Induction of Apoptosis and Alteration in G1 Checkpoint Function as Determinants of Resistance of Human Lung Cancer Cells against the Antisignaling Drug UCN-01 (7-Hydroxystaurosporine)

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