G1 Phase Accumulation Induced by UCN-01 Is Associated with Dephosphorylation of Rb and CDK2 Proteins as well as Induction of CDK Inhibitor p21/Cip1/WAF1/Sdi1 in p53-mutated Human Epidermoid Carcinoma A431 Cells

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ABSTRACT

UCN-01 (7-hydroxyl-staurosporine) was originally isolated as a Ca2+ and phospholipid-dependent protein kinase C selective inhibitor and now is being developed as an anticancer agent. Results from our and other laboratories have suggested that UCN-01 induces preferential G1-phase accumulation in several human tumor cell lines tested. To elucidate this mechanism, we examined the effects of UCN-01 on several cell cycle-regulatory proteins critical for G1-S-phase transition in p53-mutated human epidermoid carcinoma A431 cells. After 24 h exposure at around 50% growth-inhibitory concentrations (IC50), 260 and 520 nM, UCN-01 induced the accumulation of pRb (the dephosphorylated retinoblastoma protein form). The protein expression of cyclin A but not cyclin E was markedly reduced and that of cyclin D1 was partially reduced under the same condition. UCN-01 also showed the concentration-dependent inhibitions of the activity of cyclin-dependent kinase 2 (CDK2) using histone H1 and pRb as substrates in vitro (IC50 530 and 640 nM, respectively). In addition, CDK2 activities of the cells pretreated with UCN-01 for 24 h at 260 and 520 nM were markedly inhibited, giving IC50 of far less than 260 nM. When the same cell lysates were analyzed by Western blotting for CDK2, the lower band (e.g., active and phosphorylated CDK2) was remarkably reduced, in accordance with the reduced activity. Furthermore, UCN-01 induced the expression of the CDK inhibitor p21 protein and its complex formation with CDK2 after 24 h exposure at 260 and 520 nM, whereas the expression level was very low or undetectable in untreated or DNA-damaged cells. The increase of p21 mRNA levels was also induced under the same condition. UCN-01 further increased luciferase activities in A431 cells transiently transfected with p21 promoter-luciferase reporter plasmid after 24 h exposure at 260 and 520 nM. UCN-01 also increased the expression of the CDK inhibitor p27 protein after 24 h exposure at 260 and 520 nM. These results suggest that G1-phase accumulation induced by UCN-01 is associated with dephosphorylation of Rb and CDK2 proteins as well as induction of CDK inhibitors p21 and p27.

INTRODUCTION

UCN-01 (7-hydroxyl-staurosporine) was originally isolated from the culture broth of Streptomyces sp. as a protein kinase C-selective inhibitor (1). Previous studies from our laboratory and other laboratories have shown that the drug exhibits potent antitumor activity against several rodent and human cancer cell lines in vitro and in vivo (2–6). However, the precise mechanism of action for its antitumor activity is still not fully understood. Recent studies have revealed that UCN-01 inhibits cell cycle progression from G1 to S phase in various mammalian cell lines. In addition, UCN-01 was shown to enhance antitumor activities of several important cancer chemotherapeutic drugs, such as mitomycin C, cisplatin, and 5-fluorouracil in vitro and in vivo (4, 6, 10–12). Based on these unique preclinical data, UCN-01 has already entered into Phase I clinical trials in the United States and Japan.

Recent studies indicate that cell cycle progression in mammalian cells is regulated by a family of serine/threonine protein kinases termed CDKs (13). The activity of CDKs is regulated by binding to their partner cyclins (13) and phosphorylation by CAK (14). Progression through early to mid G1 phase of the cell cycle is dependent on CDK4 and/or CDK6, which are activated by D-type cyclins. Transition through mid G1 to S phase is regulated by activation of CDK2 by cyclin E. CDK2 and cyclin A is also required for late G1 to S-phase progression. A critical target of these cyclin/CDK complexes is pRb, the protein product of the retinoblastoma tumor suppressor gene (15). Phosphorylation of pRb occurs in mid G1 to late G1 phase and is required for entry into S phase (15). ppRb inactivates the function to suppress transcriptional activation driven by E2F (16).

Recent studies also reveal that the activity of CDKs in G1 is negatively regulated by two families of CDK inhibitor proteins. One CDK inhibitor family includes p16INK4a, p15INK4b, p18INK4c, and p19INK4d, that target the CDK4 and CDK6 kinases and prevents their interaction with cyclin D (17). The other CDK inhibitor family includes p21 (also termed Cip1, WAF1, Sdi1, CAP20, and Pice1), p27Kip1, and p57Kip2 (17). p21 binds to cyclin/CDK complexes and either inhibits their kinase activities (17) or prevents their activation by cyclin D (17). p21 family proteins are also shown to have potent inhibitory activities against diverse CDKs in G1 (17). In addition, p21 binds proliferating cell nuclear antigen, a processivity factor for DNA polymerase-δ, and inhibits proliferating cell nuclear antigen-dependent DNA replication in vitro (19).

Several antiproliferative agents including rapamycin (20), staurosporine (21, 22), anti-estrogen ICI182780 (23), TGF-β (24), IFN-α (25), and tumor necrosis factor-α (26) were shown to induce G1-phase accumulation, dephosphorylation of pRb, and reduced expression of some G1 cyclins in mammalian cultured cells. In addition, recent reports have shown that the expression of p21 is induced by various cell growth-inhibitory signals that cause G1-phase arrest, including a DNA-damaging agent by a p53-dependent mechanism (27), and other antiproliferative agents such as TGF-β (28, 29), staurosporine (22), ICI182780 (23), tumor necrosis factor-α (26), retinoid derivatives (30), and differentiation inducers (31, 32) in either p53-dependent or independent pathways. In addition, p27Kip1 has also been shown to be increased in response to antiproliferative agents such as TGF-β (33), lovastatin (34), cyclic AMP (35), rapamycin (36), staurosporine (22), and vitamin D3 (37) when they induce G1-phase accumulation.

In this report, we have examined the effects of UCN-01 on expression of G1 cyclins, phosphorylation state of pRb, and kinase activity

Received 8/19/96; accepted 2/20/97.

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2 The abbreviations used are: CDK, cyclin-dependent kinase; CAK, cyclin-dependent kinase-activating kinase; pRb, retinoblastoma protein; ppRb, phosphorylated retinoblastoma protein; TGF, transforming growth factor; EGF, epidermal growth factor; RT-PCR, reverse transcription-PCR.
of CDK2 in vitro and in cell culture as well as induction of universal CDK inhibitor p21/Cip1/WAF1/Sdi1 in A43l epidermoid carcinoma cells to elucidate the mechanism of its preferential G1-phase accumulation.

MATERIALS AND METHODS

Drugs and Reagents. UCN-01 was produced by fermentation in our laboratories as described previously (1). The drug was dissolved in DMSO and freshly diluted with cell culture medium (DMEM (Life Technologies, Inc.). The cell cultures were then cultured for 24 h, followed by UCN-01 treatment for another 24 h. The cells were washed once with PBS, harvested, and lysed in lysis buffer [100 mM KH2PO4 (pH 7.8), 1% Triton X-100, and 1 mM EDTA] containing 100 mM NaCl, 0.1% Triton X-100, 50 mM sodium fluoride, 80 mM β-glycerophosphate, 0.1 mM sodium oxanate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, p300, p300, and aprotinin. The lysates were subjected to 2%-agarose gel electrophoresis, followed by Western blotting with anti-CDK2 antibody and subjected to SDS-PAGE. The protein was transferred to a nylon membrane and subjected to Western blotting with anti-CDK2 antibody and mixed gently for 2 h at 4°C. The immunoprecipitate was washed with lysis buffer twice and washed buffer [50 mM Hepes/NaOH (pH 7.4), 150 mM NaCl, 0.1% Triton X-100, 50 mM sodium fluoride, 80 mM β-glycerophosphate, 0.1 mM sodium oxanate, 1 mM EDTA] containing 10-15 tubes. Each CDK2-immunoprecipitate was mixed with 40 μl of kinase buffer [50 mM Hepes/NaOH (pH 7.4), 10 mM MgCl2, and 1 mM DTT] and separated into two times the IC50 of 520 flM (Fig. 1). The same magnitude of G1-phase accumulation was also induced at two concentrations, 50% growth-inhibitory concentration (IC50) of 260 μM (Fig. 1). The results of UCN-01 treatment were indicated using the following concentrations: 50 μM, 100 μM, and 200 μM. The cell lysate was centrifuged at 14,000 rpm for 10 min at 4°C, and its protein content was determined using the protein assay kit (Bio-Rad Laboratories, Hercules, CA).

In Vitro CDK2 Kinase Assays. Exponentially growing A43l cells were harvested with 0.25% trypsin, washed with PBS, and stored at −80°C. The cells 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 β-glycerophosphate, 0.1 mM sodium oxanate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, p300, and aprotinin], p300, and to stop the reaction, heated for 5 min at 95°C, and subjected to SDS-PAGE. The gel was dried, stained with Coomassie Brilliant Blue, and analyzed by BAS2000 image analyzer (Fuji Photo Film Co., Tokyo, Japan).

Cell Culture CDK2 Kinase Assay and Western Blotting. A43l cells (3 × 10^6/dish) were precultured in Falcon 3003 plastic dishes (Becton Dickinson, Lincoln Park, NJ) overnight and treated with the indicated concentrations of UCN-01 for 24 h. The cells were harvested by treatment with 0.25% trypsin, fixed with ice-cold 70% ethanol solution, hydrolyzed with 25 μg/ml of RNase A (type 1-A; Sigma Chemical Co.) at 37°C for 30 min, and stained with propidium iodide (Sigma) for 20 min. The DNA content of the cells was analyzed by a FACScan flow cytometer (Becton, C例行, FL). The cell cycle distribution was calculated by a MULTICYCLE program (Coulter).

In Vitro CDK2 Kinase Assays. Exponentially growing A43l cells were harvested with 0.25% trypsin, washed with PBS, and stored at −80°C. The cells 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 β-glycerophosphate, 0.1 mM sodium oxanate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, p300, and aprotinin], p300, and to stop the reaction, heated for 5 min at 95°C, and subjected to SDS-PAGE. The gel was dried, stained with Coomassie Brilliant Blue, and analyzed by BAS2000 image analyzer (Fuji Photo Film Co., Tokyo, Japan).
MECHANISM OF G1 PHASE ACCUMULATION BY UCN-01

Fig. 1. Effect of UCN-01 on cell cycle distribution of A431 cells. The cells were harvested after 24 h treatment with UCN-01 (260 and 520 nM) or without (Control). Cell fixation, RNA hydrolysis, and DNA staining with propidium iodide were performed as described in “Materials and Methods.” DNA histograms (top) and cell cycle phase proportions (bottom) were analyzed by a flow cytometer.

<table>
<thead>
<tr>
<th>Cell-cycle phase (%)</th>
<th>G1</th>
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<tr>
<td>Control</td>
<td>37.7</td>
<td>45.4</td>
<td>17.0</td>
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<tr>
<td>UCN-01 260 nM</td>
<td>61.6</td>
<td>32.4</td>
<td>6.4</td>
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<tr>
<td>UCN-01 520 nM</td>
<td>66.8</td>
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Effect of UCN-01 on Protein Expression of CDK Inhibitor p21/Cip1/WAF1/Sdi1 in A431 Cells. Recent reports show that CDK2 activity is negatively regulated by a CDK inhibitor protein such as the p21 family of proteins (13, 15, 17). In addition, several antiproliferative agents are reported to induce p21 protein in either a p53-dependent or -independent manner when they induce G1-phase accumulation in target cells (22, 23, 26, 28, 29, 31, 32). To see whether UCN-01 also affects the p21 protein expression, we have investigated the effect of UCN-01 on p21 protein level in A431 cells by a Western blotting technique. In untreated control A431 cells, the p21 protein level was very low or undetectable (Fig. 4A), and the complex formation between p21 and CDK2 was also undetectable (Fig. 4B).

After 24 h exposure to UCN-01 at 260 or 520 nM, a significant increase of p21 protein in A431 cells was induced (Fig. 4A), and the apparent complex formation between p21 and CDK2 was also observed (Fig. 4B), suggesting that UCN-01-induced p21 would inhibit CDK2 in vivo. To examine the function of p53 in A431 cells, the cells were treated with DNA-damaging agents such as X-ray as well as Adriamycin, both of which were reported to induce p21/WAF1 in p53 wild-type cell lines (27, 48). As shown in Fig. 5, the levels of p21 was used as a substrate instead of histone H1 in vitro, giving an IC50 of 640 nM (Fig. 3B). To see if UCN-01 would inhibit the cellular CDK2 activity in vivo, A431 cells were pretreated with UCN-01 for 24 h, and then the CDK2 activity was determined by the immunoprecipitation method described above using histone H1 as a substrate. As shown in Fig. 3C, CDK2 activities of the cells pretreated with UCN-01 for 24 h at 260 and 520 nM were inhibited by more than 90% of the activity of untreated control cells. CDK2 has been shown to be active when the protein is phosphorylated by CAK at threonine 160 (47). We then analyzed the phosphorylation state of the CDK2 protein by gel mobility shift assay using a Western blotting method (21) after the treatment of A431 cells with UCN-01 at 260 and 520 nM, as described above. In the drug-treated cells, the amount of faster-mobilizing protein band (e.g., active and threonine 160-phosphorylated CDK2; Ref. 21) was remarkably reduced (Fig. 3D) in accordance with the reduced CDK2 activity shown in Fig. 3C. These results suggest that the decrease of phosphorylated and active form of CDK2 protein also might be an additional mechanism(s) for inhibition of CDK2 activity in UCN-01-treated A431 cells other than direct inhibition of CDK2 enzyme by the drug.

Effects of UCN-01 on CDK2 Kinase Activity in Cell-free Systems and in Cultured Cells. To see if UCN-01 would directly inhibit CDKs in A431 cells, CDK2 kinase activity of the cells was determined after immunoprecipitation with anti-CDK2 monoclonal antibody using histone H1 as a substrate. As shown in Fig. 3C, exponentially growing A431 cells exhibited apparent CDK2 (H1 kinase) activity, which was inhibited by UCN-01 in a concentration-dependent manner in vitro, giving an IC50 of 530 nM (Fig. 3A). The drug also exhibited apparent inhibitory activity against CDK2 when pRb phosphorylation of ppRb in A431 cells.
MECHANISM OF G1 PHASE ACCUMULATION BY UCN-01

we studied the effect of UCN-01 on p21 mRNA by RT-PCR. As shown in Fig. 6, p21 mRNA was constitutively expressed in untreated A431 cells, and the level was clearly increased after 24 h exposure to UCN-01 at 260 or 520 nM. In contrast to this induction of p21 mRNA,

Fig. 4. Effect of UCN-01 on protein expression of p21 and its complex formation with CDK2 in A431 cells. A431 cells were harvested after 24 h treatment with UCN-01 (260 and 520 nM) or without (0 nM). p21 protein in the crude cell lysates (A) and CDK2 and p21 proteins in the CDK2-immunoprecipitates (B) were detected by Western blotting as described in "Materials and Methods."

Fig. 5. Effect of a growth factor and DNA-damaging agents on p21 protein expression in A431 cells. A431 cells were harvested after 24 h treatment with UCN-01 (260 and 520 nM), EGF (20 nM, Lane 4), Adriamycin (400 nM, Lane 5), or without (Control, Lane 1). X-ray treatments (20 Gy, Lane 6) were performed 4 h before cell harvest by using an X-irradiator. Cell lysis and Western blotting with anti-p21 antibody were performed as described in "Materials and Methods."

protein induced by Adriamycin and the high dose of X-ray were very low and undetectable in A431 cells, respectively, suggesting that p53 protein in A431 cells is not functional. As reported previously (49), 20 nM of EGF induced the expression of p21 in A431 cells, possibly in a p53-independent manner (Fig. 5, Lane 4), when the growth factor inhibited the growth of A431 cells and induced apparent G1-phase accumulation.

Effects of UCN-01 on mRNA Level and Transcriptional Activation of p21/Cip1/WAF1/Sdi1. To determine whether this induction of p21 protein by UCN-01 was due to an increase of the mRNA,
UCN-01 exhibited little, if any, effect on the steady-state mRNA level of β-actin in the cells, even at higher concentration (Fig. 6). To elucidate the mechanism by which UCN-01 induces the accumulation of p21 mRNA, a plasmid construct harboring a luciferase reporter gene under the transcriptional control of p21 promoter, WWP-Luc, was transiently transfected into A431 cells, and UCN-01-induced luciferase activity was measured. UCN-01 treatment (24 h) of the transfected cells resulted in a 2–3-fold increase in luciferase activities at both concentrations equally (Fig. 7). Wild-type but not His273 mutant-type human p53 increased the luciferase activity when the expression plasmids coding these types of p53 were cotransfected with WWP-Luc into A431 cells in this system (data not shown). These results suggest that UCN-01-induced accumulation of p21 mRNA is in part a consequence of transcriptional activation of the gene in A431 cells.

**Effect of UCN-01 on Protein Expression of CDK Inhibitor p27Kip1 in A431 Cells.** To see if UCN-01 would induce other member(s) of the CDK inhibitor protein family, we have examined the effect of UCN-01 on expression of p27 protein, a member of the p21 family (13, 17, 33), by a Western blotting technique. p27 protein was constitutively expressed in untreated A431 cells, and the p27 protein expression was significantly increased after 24 h exposure to UCN-01 at 260 or 520 nM (Fig. 8). In our experimental conditions, we could not detect p16INK4A and p15INK4B proteins by Western blotting in A431 cells.

**DISCUSSION**

Results from our laboratory and other laboratories have suggested that UCN-01 induces preferential G1-phase accumulation in several types of mammalian cell lines examined (3, 7–9). These studies did not clearly define the mechanism for G1-phase accumulation induced by UCN-01. However, we have demonstrated here that both direct inhibition of kinase activity of CDK2 by UCN-01 as a protein kinase inhibitor and indirect inhibition of CDK2 through induction of CDK inhibitor protein p21 by UCN-01 are important for cell cycle arrest in G1.

At the IC50 and two times the IC50 for growth inhibition, in which condition apparent G1-phase accumulation was evident (Fig. 1), UCN-01 decreased the expression of cyclin A and cyclin D1 but not cyclin E (Fig. 2, B–D), suggesting that the drug-treated cells could not enter into S phase. In addition, concentration-dependent dephosphorylation of ppRb (Fig. 2A) induced by UCN-01 in the cells also suggests that the UCN-01-treated cells are arrested before entry into S phase. We have also examined the effects of UCN-01 using other cell lines, indicating that UCN-01 induces G1-phase accumulation as well as dephosphorylation of ppRb in Rb-intact cell lines including MCF-7, HCT116, and WiDr cells. On the other hand, although we have extensively examined the effect of UCN-01 on cell cycle distribution of Rb-null Saos-2 cells by flow cytometric analysis, we have not yet obtained definitive results in this cell line. Further studies are needed to clarify the effect of UCN-01 on cell cycle distribution of Rb-null cells. Previous studies showed that a protein kinase inhibitor, staurosporine, which has a similar indolocarbazole structure to UCN-01, induced G1-phase accumulation and the dephosphorylation of ppRb at a lower concentration of 5 nM in mammalian fibroblasts and lymphocyte cells (50–53). Staurosporine was shown to block cell cycle progression through G1 between cyclin D and cyclin E restriction points because the drug reduced the expression of cyclin E in normal lymphocyte cells activated with phytohemagglutinin (50). These results suggest that staurosporine and UCN-01 might block cell cycle progression of G1 phase at separate points, although more studies are needed to define the accurate arrest points.

Previous studies revealed that both UCN-01 and staurosporine exerted inhibitory activity against CDK2 in cell-free systems (5, 9, 54). In our experimental conditions, UCN-01 and staurosporine also exhibited a concentration-dependent inhibitory effect on CDK2 obtained from cycling A431 cells, giving IC50 of 530 nM (Fig. 3A) and 32 nM, respectively, when histone H1 was used as a substrate. To see if UCN-01 inhibits the CDK2 activity of A431 cells at the cellular level, the CDK2 activities of UCN-01-treated A431 cells were determined. UCN-01 inhibited more than 90% of CDK2 activity in cell culture after 24 h exposure at a 50% growth-inhibitory concentration of 260 nM (Fig. 3C). At 260 nM, UCN-01 could only inhibit the CDK2 activity isolated from A431 cells by 30 to 40% in the cell-free systems (Fig. 3, A and B). This discrepancy suggests that there might be additional mechanism(s) for inhibition of CDK2 activity by UCN-01 in the cells. In addition, if the inhibitory activity of UCN-01 against CDK2 is completely reversible after the immunoprecipitation process, there should be other mechanism(s) for inhibition of CDK2 in cell culture. The CDK2 inhibition by these direct and indirect actions might be one of the mechanisms of dephosphorylation of ppRb by UCN-01.
UCN-01. Another possibility is that UCN-01 might inhibit CDK4 and/or CDK6 (9), which are other kinases for pRb in the cells. In our experimental conditions, CDK4 kinase activity in A431 cells was too low to examine the inhibitory activity of UCN-01. Additional studies are needed to determine whether CDK4 and/or CDK6 inhibition contribute to the induction of dephosphorylation of pRb and G1-phase accumulation by UCN-01 in A431 cells.

In accordance with the reduced activity, CDK2 protein was shown to be dephosphorylated after UCN-01 exposure, which was apparent from disappearance of the faster-migrating, threonine 160-phosphorylated active form of the CDK2 protein (Fig. 3D). Such type of inactivation of CDK2 was also reported for staurosporine-treated cultured cells (21). These results suggest that UCN-01 and/or staurosporine inhibit CAK activity in a direct and/or indirect manner. We do not have data for direct inhibitory activity against CAK, which also forms a cyclin/CDK complex, by UCN-01 and/or staurosporine. These possibilities should be examined in future studies. Another possibility for inhibition of CAK activity is induction of p21 (18) by UCN-01. Recently, several antiproliferative agents have been shown to induce p21 protein in target cells (22, 23, 26, 28, 31, 32) when they exhibit apparent G1-phase accumulation. As shown in Fig. 4, this was the case for UCN-01. In untreated A431 cells, the expression level of p21 protein was very low or undetectable because p53 in A431 cells was shown to be mutated at codon 273 (55). In accordance with this, X-ray irradiation and Adriamycin treatment did not fully induce p21 protein (Fig. 5, Lanes 5 and 6). These results suggest that UCN-01 induces p21 in a p53-independent manner. In our experimental conditions, EGF induced the expression of p21 protein in A431 cells, as reported previously (49). Studies with an RT-PCR method revealed that the steady-state level of p21 mRNA was up-regulated by UCN-01 (Fig. 6), possibly through transcriptional activation of the gene (Fig. 7) in A431 cells. This type of transcriptional activation of the p21 gene was also reported for the other antiproliferative agents such as TGFB and the retinoid derivatives (29, 30), which induce G1-phase accumulation in p53-independent pathways. Very recently, staurosporine at a lower concentration was shown to induce p21 protein as well as p27 protein when the drug showed G1-phase block in the cells. However, the authors did not describe the induction mechanism of p21 in this report (22). Other studies of staurosporine revealed that the drug as well as another protein kinase inhibitor, H7 induced the expression of p21 in MCF-7 cells, which have wild-type p53 (56). UCN-01 was shown to induce the expression of p21 protein in MCF-7 cells when the drug showed apparent G1-phase accumulation in the cells.3 Additional studies are needed to clarify precise mechanism(s) of action for transcriptional activation of the p21 gene by UCN-01 and related indolocarbazole compounds.

In this study, UCN-01 also increased the p27 protein level in A431 cells (Fig. 8), suggesting that p27 is also important for antiproliferative activity of UCN-01. Overproduction of p21 and p27 proteins in tumor cell lines have been shown to inhibit the growth of the cells in vitro as well as in vivo animal models and to induce apparent G1-phase accumulation (57–60). Taken together, p21 and/or p27 induction by UCN-01 might play an important role in its antiproliferative activity in cultured cell systems as well as in vivo animal models. These possibilities should be examined in experimental mice tumor models and/or clinical specimens before and after UCN-01 administrations.

ACKNOWLEDGMENTS

We thank Dr. Akira Mihara and Toshimitsu Takiguchi for providing highly purified UCN-01, Dr. Wafik S. El-Deiry for the WWPC-luc plasmid, and Dr. Nobuo Hanai and Akiko Furuya for providing excellent anti-cyclin A and anti-CDK2 antibodies. We also thank Yuka Watanabe and Hiroaki Narumi for excellent technical assistance. We are also grateful to Dr. Tatsuya Tamaoki for continuous encouragement and critical comments on the manuscript.

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