UCN-01-Induced Cell Cycle Arrest Requires the Transcriptional Induction of p21\textsuperscript{wafl/cip1} by Activation of Mitogen-Activated Protein/Extracellular Signal-Regulated Kinase Kinase/Extracellular Signal-Regulated Kinase Pathway

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Received 12/1/03; revised 2/17/04; accepted 3/11/04.

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ABSTRACT

The small molecule UCN-01 is a cyclin-dependent kinase (CDK) modulator shown to have antiproliferative effects against several in vitro and in vivo cancer models currently being tested in human clinical trials. Although UCN-01 may inhibit several serine-threonine kinases, the exact mechanism by which it promotes cell cycle arrest is still unclear. We have reported previously that UCN-01 promotes G1-S cell cycle arrest in a battery of head and neck squamous cancer cell lines. The arrest is accompanied by an increase in both p21\textsuperscript{wafl/cip1} and p27\textsuperscript{kip1} CDK inhibitors leading to loss of G1 CDK activity. In this report, we explore the role and the mechanism for the induction of these endogenous CDK inhibitors. We observed that p21 was required for the cell cycle effects of UCN-01, as HCT116 lacking p21 (HCT116 p21\textsuperscript{-/-}) was refractory to the cell cycle effects of UCN-01. Moreover, UCN-01 promoted the accumulation of p21 at the mRNA level in the p53-deficient HaCaT cells without increase in the p21 mRNA half-life, suggesting that UCN-01 induced p21 at the transcriptional level. To study UCN-01 transcriptional activation of p21, we used several p21\textsuperscript{wafl/cip1} promoter-driven luciferase reporter plasmids and observed that UCN-01 activated the full-length p21\textsuperscript{wafl/cip1} promoter and a construct lacking p53 binding sites. The minimal promoter region required for UCN-01 (from ~110 bp to the transcription start site) was the same minimal p21\textsuperscript{wafl/cip1} promoter region required for Ras enhancement of p21\textsuperscript{wafl/cip1} transcription. Neither protein kinase C nor PDK1/AKT pathways were relevant for the induction of p21 by UCN-01. In contrast, the activation of mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway was required for p21 induction as UCN-01 activated this pathway, and genetic or chemical MEK inhibitors blunted p21 accumulation. These results demonstrated for the first time that p21 is required for UCN-01 cell cycle arrest. Moreover, we showed that the mechanism for the induction of p21 is transcriptional via activation of the MEK pathway. This novel mechanism, by which UCN-01 exerts its antiproliferative effect, represents a promising strategy to be exploited in future clinical trials.

INTRODUCTION

Despite the substantial knowledge about mechanisms of carcinogenesis, the survival of patients with advanced solid tumors has not significantly improved over the past 30 years (1). Thus, it is crucial to identify the molecular mechanisms underlying the genesis of human cancer.

One of the most frequent events required for human cancer development is the deregulation of the cell cycle machinery (2, 3). Normal eukaryotic cells progress through the cell cycle in a regulated manner because of a cascade of biochemical events that coordinates the transition of cells from one phase to another. A series of events must take place before entry into S phase. These events include elevations in D- and E-type cyclin levels, activation of cyclin-dependent kinases (CDKs), phosphorylation of the retinoblastoma protein, and subsequent activation of the E2F transcription factor family (4, 5). Once activated, E2F stimulates the transcription of genes of which the protein products are required for S phase entry and transition (6–8). After the S phase, cells enter the G2 phase, in which additional cyclin/CDK complexes are activated, in particular, the cyclin B1/CDC2 complex that stimulates mitotic entry (4, 5). At least 90% of human neoplasms have abnormal cell cycle control because of inactivation of the tumor suppressor gene Rb responsible for preventing the transactivation of the transcriptional factor E2F (6). In a few cases, Rb is a mutant such as in retinoblastoma (9). However, in most cases, Rb function is lost because of phosphorylation by the CDKs, leading to release of the transcriptional factor. Thus, modulation of CDK activity may represent a very attractive target for the treatment and prevention of human neoplasms to release of the transcription factor E2F (2, 3, 10).

UCN-01 is a derivative of the serine/threonine kinase inhibitor staurosporine, currently being tested in human clinical trials (11–13). It was initially thought to have more specific inhibitory effects against protein kinase C (PKC; Refs. 12, 14). However, in some models, the cell cycle effects observed with this agent could not be explained by the effects on PKC function (15, 16). Despite the growing interest in UCN-01 as an antineoplastic agent, the mechanisms by which it induces cell cycle arrest remain incompletely understood. Several groups, including our laboratory, investigated the mechanism required to promote G1-S arrest (10, 12, 16–23). Although UCN-01 can inhibit CDKs in vitro, this activity occurs at concentrations ~2-fold higher than the ones necessary for cell cycle arrest (15, 16). In contrast, UCN-01 can up-regulate the endogenous CDK inhibitors p21wafl/cip1 (hereafter, p21) and p27kip1, and this up-regulation is associated with loss in G1 CDK activity and Rb dephosphorylation (16, 20). The exact relevance of the up-regulation of these endogenous inhibitors and the mechanism by which UCN-01 increases the expression of these inhibitors are still unknown.

In this study, we demonstrated that the induction of p21 (and not p27kip1) is crucial for the cell cycle arrest induced by UCN-01. The p21 accumulation does not require p53 function, occurs at the transcriptional level, and the minimal p21 promoter region required is similar to the one required for the proto-oncogene Ras. Finally, we demonstrated that the transcriptional activation of p21 is not dependent on PKC or PDK1/AKT activity but requires the activation of the mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway.

MATERIALS AND METHODS

Cell Culture. Exponentially growing HaCaT human keratinocyte cell lines were grown in DMEM containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA) in a 5% CO2 humidified atmosphere at 37°C. HCT116 cells of various genetic backgrounds (wild-type, p53\textsuperscript{-/-}, and p21\textsuperscript{-/-}) were a kind gift from Dr. Bert Vogelstein (Johns Hopkins, Baltimore, MD) and maintained as described above and in the presence of 350 μg/ml geneticin (Sigma Chemical Co., St. Louis, MO).

Drugs and Chemicals. UCN-01 was provided by Kyowa Hakko Kogyo Co. Ltd. (Tokyo, Japan) to the Developmental Therapeutics Program, National
Cancer Institute (Bethesda, MD). UCN-01 was reconstituted in DMSO at a stock concentration of 10 mM. Phosphatidylinositol 3'-kinase (PI3K)-specific inhibitor LY294002 and actinomycin D were purchased from Sigma. Wortmannin and the MEK inhibitors U0126 and PD98059 were from Calbiochem (La Jolla, CA). Stock solutions for all of the pharmacological inhibitors were prepared in DMSO at a concentration of ≥1000-fold. The final concentration of DMSO in the culture medium was always ≤0.1%.

Protein Extraction and Immunoblot Analysis. HaCaT and HCT116 cells were plated and grown overnight to ∼70% confluence and exposed to chemical inhibitors as described in the figure legends. To study the role of MEK/ERK pathway, HaCaT cells were cultured under serum-free conditions for 24 h as well as under fetal bovine serum-containing conditions and exposed to 100 nM UCN-01 for either 4 h or 12 h. Cell lysates for immunoblotting were prepared as described previously (24, 25). Briefly, 25 μg of protein was electrophoretically resolved by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane Immobilon-P (Millipore, Bedford, MA). Appropriate primary antibodies were used, and reactions were detected by horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence following the manufacturer’s directions (Pierce, Rockford, IL). Antibodies to ERK2 and phosphorylated (phospho-)ERK1/2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies to p21 and p27 were from Transduction Laboratories (San Diego, CA); and monoclonal antibody to β-actin was from Chemicon (Temecula, CA). Polyclonal antibodies to AKT1 and phospho-AKT (T308 and S473) were purchased from BD PharMingen (San Diego, CA); phospho-MEK and MEK were obtained from Cell Signaling (Beverly, MA); and phospho-adducin was from Upstate Biotechnology (Lake Placid, NY). Horseradish peroxidase-conjugated antirabbit and antimouse antibodies (1:5000) were purchased from Amersham Biosciences (Arlington Heights, IL).

Northern Blot Analysis. Total RNA was extracted using TRIzol (Invitrogen) following the manufacturer’s recommendations. Twenty-five μg of total RNA was separated on 1% formaldehyde-agarose gels and transferred to nylon membranes (Hybond-XL; Amersham Pharmacia Biotech) by capillary action. Probes for p21 promoter were generated by digestion of pCMV p21 (obtained from Michele Pagano, New York University, New York, NY) with XhoI and HindIII and then gel purified using GENECLEAN spin kit (Bio 101, Carlsbad, CA). Glyceraldehyde-3-phosphate dehydrogenase was obtained from Ambion Inc. (Austin, TX). Probes were labeled with [α-32P]dATP by random priming using Ready-To-Go DNA Labeling kit (Amersham Biosciences). Northern blot hybridization to 32P-labeled probes was carried out using Hybrisol I solution (Intergen Co., Purchase, NY) following the manufacturer’s protocol. Signals were detected by phosphorimager, and quantitation of signals was performed by densitometry.

Cell Cycle Analysis. Analysis of cellular DNA content by flow cytometry was performed as described previously (25). Briefly, UCN-01 (100 nm) and vehicle-treated HCT116 and isogenic variants were harvested after 12 h, washed briefly in ice-cold PBS, and fixed in 70% ethanol. DNA content was obtained by incubating cells in PBS containing propidium iodide (50 μg/ml) and RNase A (1 mg/ml) for 30 min at 37°C. Fluorescence was measured and analyzed using FACScalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) and ModFit (Verity Software, Topsham, ME), respectively.

Luciferase Reporter Studies. HaCaT cells were plated overnight into six-well plates at a density of ∼70,000 cells/well. Cells were then transfected with 1 μg of plasmid reporter construct using Fugene Transfection (Roche, Indianapolis, IN) reagent according to the manufacturer’s protocol. Twelve h after transfection, cells were exposed to 100 nM UCN-01 for 18 h. Cells were then lysed, and luciferase activity was assayed using Dual Luciferase Reporter Assay System (Promega, Madison, WI). Luciferase activities were normalized based on total protein concentrations and Renilla luciferase activity. All of the p21 promoter constructs used in this work were reported previously (26). Briefly, the 2.4-kb genomic fragment containing the p21 start site was cloned into pGL2-basic (Promega) to create p21 basic (full length). To create p21P53, p21P was digested with SacI and religated to remove ∼250 bases from the S’ end of the p21 promoter, including a p53 consensus site. p21P53a1 was created by digesting p21P with SmaI and religation. p21P53mu-lac (minimal promoter region for Ras activity) was created by cloning the 50-bp SmaI fragment of the p21 promoter into pGL-2 basic (26). Plasmids encoding vectors for activating mutants for Ras (Ras V12) and dominant-negative MEK (MEKK4A) were kindly provided by Silvio Gutkind (National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD) and described previously (27).

ERK in Vivo Kinase Assays. Exponentially growing HaCaT cells were seeded starved for 24 h and then exposed to increasing concentrations of UCN-01 for 1 h or with 100 nM UCN-01 for different time periods. After treatment, cells were lysed as described previously (16), and 200 μg of total cellular lysate was immunoprecipitated with ERK-2 antibody (Santa Cruz Biotechnology, Inc.) for 1 h at 4°C. Then, Gammabind G Sepharose was used to capture the immune complexes. Kinase reactions were carried out in kinase assay buffer [12.5 mM 4-morpholinopropanesulfonic acid or 3-(N-morpholino)-propanesulfonic acid (pH 7.5), 12.5 mM β-glycerophosphate, 7.5 mM MgCl2, 0.5 mM EGTA (pH 8), 0.5 mM NaF, and 0.5 mM NaV] containing γ[32P]ATP [ (3000 Ci/mmol; NEN, Boston, MA) and 50 μg of Myelin Basic Protein as substrate. Reactions were incubated at 37°C for 30 min and terminated by the addition of SDS-gel loading buffer. The resolved and dried gels were subjected to autoradiography and quantified by phosphorimager.

Infection of HaCaT Cells with Akt Adenoviruses (AdVs). AdVs for constitutively active (Myr-Akt) and kinase inactive (T308A and S473A) Akt mutants were kindly donated by Marcelo Kazanietz (University of Pennsylvania, Philadelphia, PA) and described elsewhere (28). Subconfluent HaCaT cells were plated overnight in six-well plates and infected with AdVs for 14 h at multiplicity of infection ranging from 1 to 100 plaque-forming units/cell in DMEM supplemented with 2% fetal bovine serum. After removal of the virus, the cells were incubated for an additional 24 h in medium supplemented with 10% fetal bovine serum. Maximum expression after adenoviral infection was achieved using this protocol. Expression of the recombinant protein remained stable throughout the duration of the experiment. For these experiments, we used titers of viral stocks >1 × 10^9 plaque-forming units/cell.

Immunofluorescence Studies. Exponentially growing HaCaT cells were exposed to UCN-01 for 4 h in the presence or absence of the MEK inhibitor PD98059, and then fixed in 4% paraformaldehyde for 15 min at room temperature. After washing with PBS, fixed cells were permeabilized in PBS containing 0.2% Triton X-100 and 5% BSA for 30 min at room temperature. The cells were exposed to primary antibodies to p21 (as described in the immunoblot section) for 1 h, washed in PBS, and exposed to secondary antibodies linked to Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA) in 4% BSA along with 4’,6-diamidino-2-phenylindole (2 μg/ml; nuclear stain) for 1 h at room temperature. Cells were then washed in PBS, sealed with mounting medium, and examined using a Leica TCS-SP2 confocal system (Heidelberg, Germany).

RESULTS

G-S Arrest Induced by UCN-01 Requires the Up-regulation of p21 in HCT116 Cell Lines. Several groups, including ours, have shown previously that UCN-01 arrests a wide variety of human tumor cell lines at the G1-S transition (10, 12, 16–23). In several in vitro models, including head and neck squamous cancer cell lines, the block in cell cycle progression is associated with the accumulation of the endogenous CDK inhibitors p21 and p27 (16, 21). To better understand the role of p21 and p27 in the G1-S arrest induced by UCN-01, we took advantage of the availability of a panel of colon carcinoma isogenic cell lines, HCT116 wild-type, and clones that lack p21 (p21/s). To test the cell cycle effects of UCN-01 in these genetic-defined models, we treated these isogenic cell lines (wild-type and p21−/−) with UCN-01 and analyzed the cell cycle effects after 12 h (Fig. 1). Exposure of the wild-type cells to UCN-01 (≥30 nm) caused, as expected, a significant decrease in cells at the G1 phase (Fig. 1A). Interestingly, the effects of UCN-01 in p21−/− clones were lost even at higher (≥300 nm) concentrations, suggesting that the presence of p21 is required for the cell cycle effects of UCN-01. To examine the role of p35 in p21 up-regulation by UCN-01, we determined the expression of p21 in isogenic HCT116 cell lines upon UCN-01 treatment (Fig. 2). The induction of p21 occurred both in the wild-type and p35−/− clones, suggesting strongly that the induction of p21 is independent of p35. However, the expression of p27 on
UCN-01 treatment was unaltered in all of the clones (wild-type, p53\(^{-/-}\), and p21\(^{-/-}\)), suggesting that the G\(_1\)-S arrest induced by UCN-01 is associated with p53-independent p21 accumulation only and not by accumulation of both p21 and p27, as suggested elsewhere (16, 20, 29).

To further characterize the role of these endogenous CDK inhibitors, we used HaCaT cells, a p53 nonfunctional immortalized keratinocyte cell line that accumulates in G\(_1\) upon UCN-01 treatment (16). Dose response (Fig. 3A) and time course (Fig. 3B) studies demonstrated that concentrations >30 nM UCN-01 for 12 h promoted the accumulation of p21 protein. This accumulation occurs as early as 3 h after exposure to 100 nM UCN-01 concentrations. Again, in parallel samples, the expression of p27\(^{kip1}\) did not increase. This result confirms our previous observation using the isogenic HCT116 model (Fig. 2), suggesting that accumulation of p27\(^{kip1}\) is not required for UCN-01-induced cell cycle arrest.

The Increase in p21 Expression by UCN-01 Is Due to an Increase in p21 mRNA Steady-State Levels. On the basis of the biological relevance of p21 in UCN-01-induced cell cycle arrest, we focused on the mechanism by which UCN-01 induces this endogenous CDK inhibitor. To this end, we asked whether p21 protein accumulation by UCN-01 is the result of an increase in p21 mRNA. Dose-response analyses (Fig. 3C) and time course studies (Fig. 3D) demonstrated that p21 mRNA increases by 3 h, at concentrations ≥100 nM, as measured by Northern blot analysis, similar to the concentrations necessary for the increase in p21 protein levels as measured by Western blot analysis (see Fig. 3A). Thus, the increase in p21 mRNA by UCN-01 occurs at similar times and concentrations required for the increase in p21 protein expression. To determine whether UCN-01-induced accumulation of p21\(^{waf1/cip1}\) was due to post-transcriptional control, we conducted pulse-chase analysis in the presence of actinomycin D and measured p21 mRNA by Northern blot studies. We observed that p21 mRNA expression decreased to a similar extent in both vehicle and UCN-01-treated cells up to 6 h after actinomycin D (85% and 82%, respectively). These experiments demonstrate that p21 accumulation promoted by UCN-01 is not explained by increased in p21 mRNA half-life. Instead, it appears to be due to transcriptional effects.

Induction of p21 mRNA by UCN-01 Is at the Transcriptional Level, and the Minimal Promoter Region Is the Same One Required for Ras to Activate the p21 Promoter. To confirm that the increase in p21\(^{waf1/cip1}\) mRNA induced by UCN-01 was due to transcriptional activation of the p21\(^{waf1/cip1}\) promoter, we transiently transfected HaCaT cells with several p21\(^{waf1/cip1}\) promoter-driven luciferase reporter plasmids (26, 30). Twelve h after transfection, HaCaT cells were exposed to 100 nM UCN-01 for 18 h, and luciferase
activity was measured as described in “Materials and Methods.” As a positive control, we transiently transfected HaCaT cells with activated Ras (Ras V12), a known transcriptional activator of the p21waf1/cip1 promoter (26). As shown in Fig. 4, UCN-01 significantly enhanced the transcriptional activity of the p21 full-length 2.4 kb promoter (p21P-luc) by ~5-fold. Similar activation (~6-fold) was observed with Ras V12 (positive control). Moreover, we transfected HaCaT cell lines (a keratinocyte cell line with deficient p53 function; Ref. 31) with the p21PΔp53-luc reporter, a p21waf1/cip1 construct lacking 250 bases from the 5’ end corresponding to the p53 consensus DNA binding site. Again, UCN-01 activates this construct (~6-fold) despite the lack of the p53 consensus site, indicating that neither the presence of p53 binding sites in the p21waf1/cip1 promoter nor the presence of functional p53 protein was required for UCN-01 to activate the p21waf1/cip1 promoter.

To determine the minimal promoter region required for the transcriptional activation of p21waf1/cip1 by UCN-01, additional promoter deletion constructs were tested. We initially used the p21PSma-luc construct (26, 30), which contains the p21waf1/cip1 promoter sequences from base −111 through the transcriptional initiation site. Of note, this construct represents the minimal promoter region activated by Ras (26, 30). As shown in Fig. 4, UCN-01 significantly activates this minimal promoter region (~7-fold) similar to Ras V12. Moreover, when HaCaT cells were transfected with full-length p21waf1/cip1 construct lacking the Ras minimal promoter region p21PSmaΔ1-luc the induction was lost for both Ras V12 and UCN-01. Thus, the minimal p21waf1/cip1 promoter region for UCN-01 is similar to that of Ras, and it seems to be represented by a region in the p21waf1/cip1 promoter proximal to the Smal site at −111 (p21PSma-luc). To test whether the p21waf1/cip1 transcriptional activation by UCN-01 occurs in other cell types, we transfected pSMA-luc into HCT116 isogenic cell lines (wild-type or p53 null cells). Again, UCN-01 activated pSMA-luc in both cell lines (data not shown), demonstrating that the induction of the p21 minimal promoter by UCN-01 occurs in other cell types and is also independent of p53 function.

Transcriptional Up-Regulation of p21 by UCN-01 Does Not Require PKC nor PDK1/AKT Pathways. UCN-01 is a known inhibitor of several serine-threonine kinases, including PKC and PDK1 (14, 32, 33). To test whether the p21 transcriptional effects of UCN-01 might be related to PKC modulation, we assessed whether the putative effects of UCN-01 on PKC function occur at times and concentrations relevant for the p21 induction induced by this agent. To this end, we assessed the phosphorylation of adducin, a known substrate of PKC (16, 34). As demonstrated in Fig. 5A, the phosphorylation of adducin was unaltered at the highest UCN-01 concentrations tested. Thus, the putative effects of UCN-01 in PKC activity appear not to be relevant for p21 transcription, as demonstrated previously with respect to apoptosis and cell cycle arrest (15, 17). Sato et al. (33) demonstrated recently that UCN-01 inhibits PDK1, a serine-threonine kinase responsible for the activation of Akt (35, 36). Of note, fully active Akt requires the phosphorylation of 2 residues, threonine 308 (site phosphorylated by PDK1) and serine 473 (site phosphorylated by an undefined kinase; Refs. 35, 36). To assess whether UCN-01 activates p21 transcription by modulation of Akt signaling, we tested whether UCN-01 promotes the loss in Akt phosphorylation in HaCaT cell lines. As shown in Fig. 5A, although UCN-01 induced p21 at concentrations >30 nm (as shown before), the effects of UCN-01 on dephosphorylation of Akt on site S473 (PDK-independent site) occurred only at concentrations >300 nm. Moreover, the PDK1-dependent site, threonine 308, was unchanged at the times and concentrations required for p21 induction by UCN-01. In contrast, the small molecule PI3k inhibitor LY294002 dephosphorylated Akt at both residues (threonine 308 and serine 473) while having minimal effects on p21 expression (Fig. 5A). Similar results were observed with another chemically unrelated PI3k inhibitor, wortmannin (data not shown). Furthermore, coexposure of HaCaT cells to UCN-01 in combination with either LY294002 or wortmannin showed unaltered induction of p21 (data not shown). These results suggest that induction of p21 by UCN-01 is not related to the capacity of UCN-01 to inhibit PI3k/Akt pathway.

To additionally exclude the possibility that the effects of UCN-01 in the PDK1/AKT pathway might be responsible for p21 up-regulation, we expressed a constitutively active form of Akt (Myr-Akt) and a kinase-inactive form of Akt (T308A and S473A) in HaCaT cells using adenoviral delivery. Upon infection with increasing multiplicity of infection of the Akt AdVs, a dose-dependent increase in the
expression of the corresponding proteins was detected, as verified by Western blot using an antibody against total AKT (Fig. 5B). Then, HaCaT cells infected with myr-AKT adenovirus were exposed to UCN-01. Consistent with Fig. 3, p21 was significantly increased in HaCaT cells exposed to UCN-01 (Fig. 5B, Lane 6). Increasing multiplicity of infection of activated AKT failed to prevent this up-regulation (Fig. 5B, Lanes 7–10). Moreover, increasing multiplicity of infection with kinase-deficient AKT failed to increase p21, strongly suggesting that the effects of UCN-01 in p21 expression appear not to be related to the effects on the PDK1/AKT pathway reported previously.

Transcriptional Up-Regulation of p21 by UCN-01 Is Due to Activation of the MEK/ERK Pathway. Because the transcriptional up-regulation of p21 by UCN-01 is p53 independent and the minimal promoter region is similar to the one required for the proto-oncogene Ras to activate the p21 promoter, we tested whether UCN-01 could modulate signal pathways downstream to Ras. Ras modulates transcriptional events by activating the mitogen-activated protein kinase (MAPK) kinase kinase, Raf (37–40). In turn, Raf activates MEK and this, in turn, phosphorylates and activates the ERK1 and ERK2, leading to the phosphorylation and activation of several transcriptional factors. To test whether UCN-01 activates the MEK/ERK pathway, we exposed serum-starved HaCaT cells to increasing concentrations of UCN-01 for 60 min or for increasing time periods followed by in vitro kinase reactions. UCN-01 at concentrations ≥100 nM significantly activated ERK kinase activity (Fig. 6A). Similar results were obtained when protein lysates were immunoblotted with a phospho-specific ERK1/2 antibody (data not shown). Time course analysis revealed that 30 min of 100 nM UCN-01 exposure were sufficient to activate the MEK/ERK pathway (Fig. 6A, right). Thus, MAPK activation occurs at similar concentrations (≥100 nM) required for p21 up-regulation. Moreover, as demonstrated in Fig. 6A, the activation of MAPK temporally precedes the increase in p21 mRNA (3–6 h; Fig. 3C). To confirm the involvement of the MEK/ERK pathway in UCN-01-induced p21 transcriptional up-regulation, we used two structurally unrelated chemical inhibitors of the MAPK kinase, MEK (PD98059 and U0126), at concentrations that were specific for MEK inhibition (41, 42). HaCaT cells either exposed to UCN-01 (Fig. 6B, Lane 2) or transfected with Ras (Fig. 6B, Lane 5) demonstrated an increase in p21 promoter activity, as previously shown in Fig. 4. However, cotransfection with dominant-negative MEK (MEKAA) or preincubation with the MEK inhibitor PD98059 blunted the activation of the p21 promoter by either UCN-01 or Ras. Similar results were obtained when p21 expression was determined by Northern blot studies (Fig. 6C).

UCN-01 Promotes the Increase in Nuclear p21 Protein by Activation of the MEK/ERK Pathway. To test whether MAPK was also required for the induction of p21 at the protein level, serum-starved HaCaT cells treated with UCN-01 were preincubated with the chemical MEK inhibitor PD98059. Again, the loss in MEK activity induced by PD98059, as measured by loss in ERK phosphorylation, blunted the p21 protein induction induced by UCN-01 (Fig. 7A). Moreover, we assessed whether the activation of MEK occurs also in serum containing exponentially growing conditions. To this end, subconfluent HaCaT cells treated with UCN-01 were preincubated with another structurally unrelated MEK inhibitor, U0126. As clearly demonstrated in Fig. 7B, the induction of p21 by UCN-01 was associated with activation of MEK, as measured by antiserum that only recognizes phosphorylated MEK. Moreover, preincubation with the MEK inhibitor blunted the induction of p21 by UCN-01. Together, UCN-01 induces the activation of MEK/ERK pathways in both serum-free and serum-containing conditions, and this activation is required for the up-regulation of p21.

Next, we performed immunofluorescence studies to assess whether the p21 accumulation induced by UCN-01 was nuclear or cytoplasmic and to test whether MEK blockade prevented not only the induction but also the redistribution of p21. As clearly demonstrated in Fig. 7B, UCN-01 significantly increased p21 expression, similarly to what was observed previously at the Western blot level (Fig. 3). Moreover, the increase in p21 was exclusively nuclear as the immunostaining colocalized with 4′,6-diamidino-2-phenylindole, a known nuclear fluorescence stain, without evidence of redistribution to the cytoplasmic compartment (see Fig. 7C, inset). Furthermore, preincubation with the MEK inhibitor PD98059 significantly diminished the total expression of nuclear p21. Thus, induction of p21 by UCN-01 appears to be
almost exclusively nuclear, and its expression requires the activation of MEK/ERK pathway.

In summary, UCN-01 promotes cell cycle arrest through an increase in p21 protein expression. This increase is p53 independent and is due to transcriptional activation of the p21\textsuperscript{waf1/cip1} promoter. The minimal promoter region of p21 is similar to the one activated by Ras, and p21 transcriptional activation by UCN-01 requires the activation of the MEK/MAPK pathway. This finding contributes to the understanding of the mechanism of cell cycle arrest by this compound and may help also in designing more adequate clinical trials with this agent.

DISCUSSION

In this report, we demonstrate, for the first time, that UCN-01, a novel CDK modulator, induces G\textsubscript{1}-S cell cycle arrest by up-regulation of the endogenous CDK inhibitor p21\textsuperscript{waf1/cip1}. Up-regulation of p21\textsuperscript{waf1/cip1} by UCN-01 is transcriptional, and the minimal promoter region required for UCN-01 is similar to the one required for p21\textsuperscript{waf1/cip1} induction by the proto-oncogene Ras. Moreover, the transcriptional activation of p21 by UCN-01 requires MEK/ERK activity as UCN-01 activates this pathway, and the use of genetic and/or chemical MEK inhibitors blunted p21 up-regulation.

Initial studies demonstrated that UCN-01 promotes cell cycle arrest and apoptosis in different in vitro models (12, 14, 15, 17). Although UCN-01 can inhibit CDKs in vitro, the concentrations required for this effect (\( \geq 600 \text{ nM} \)) are 10 times the concentration required for cell cycle arrest (15, 17). Additional studies demonstrated that PKC, a known in vitro target of UCN-01, was not required for this effect (15). Instead, several groups have shown that UCN-01, at concentrations relevant for cell cycle arrest (50–300 nM), increases the expression for the endogenous CDK inhibitors p21\textsuperscript{waf1/cip1} and p27\textsuperscript{kip1}, thereby leading to cellular CDK inhibition and Rb dephosphorylation (16, 20, 29). Although the increase in p21 and p27 occurs at concentrations relevant for cell cycle arrest and precedes the onset of the arrest induced by UCN-01 (16), the exact requirement of these CDK endogenous inhibitors has not yet been fully explored. In this report, we explored the role of p21 and p27 in the cell cycle arrest induced by UCN-01. To this end, we exposed a battery of isogenic cells, HCT116 wild-type and HCT116\textsuperscript{p53\textminus}, to UCN-01 and observed that in the wild type, G\textsubscript{1}-S arrest induced by UCN-01 was associated with the up-regulation of p21 without changes in p27 expression. However, UCN-01 failed to block cells lacking p21, suggesting that p21 (and not p27) is required for the cell cycle effects of UCN-01.

On the basis of the relevance of p21 in the cell cycle arrest induced by this agent, we focused on the mechanism by which UCN-01 induces p21. The endogenous CDK inhibitor p21\textsuperscript{waf1/cip1} is a known
universal CDK inhibitor of which the induction may lead to several phenotypes, including cell cycle arrest, cellular senescence, apoptosis, and differentiation (43–54). In this report, we demonstrated that UCN-01 promoted the accumulation of p21 mRNA at similar times and concentrations required for the induction of p21 protein. Moreover, using pulse-chase actinomycin D studies, we demonstrated that p21 mRNA stability was not significantly altered by UCN-01. To assess the effects of UCN-01 on p21 transcription, we used a battery of p21 promoter luciferase constructs. When HaCaT cells were transfected with the full-length promoter or with a construct lacking p53 consensus sites, a significant activation was observed, suggesting that UCN-01 transactivates the p21 promoter, and this transactivation does not require p53. Of note, the induction of the p21PΔp53 construct by UCN-01 (~3.5-fold), a construct lacking ~300 bp from the 5′ end of the full-length p21 promoter (p21P), appears to be lower than the full-length promoter (~5-fold). The lower induction observed with the p21PΔp53-luc could be explained not only by the loss in p53 binding sites but also by the loss in several known recognition sites such as p73, Stats, CAAT/enhancer-binding protein β, and other unrecognized positive (and/or negative) sites (55). Currently, we are investigating the nature for this event. Finally, we determined that the minimal promoter region required for UCN-01, from −114 bp to the transcription start site (p53a-luc), is the same minimal promoter region necessary for the proto-oncogene Ras to induce p21 (26). Thus, these results suggest that UCN-01 promotes the transcriptional up-regulation of p21 in a way similar to Ras. We believe that the induction of p21 by UCN-01 does not require p53 for the following reasons: first, induction of p21 by UCN-01 occurs in cells with wild-type (HCT116 wild-type; Fig. 2), mutant p53 cells (HaCaT; Fig. 3), or p53 null cells (HCT116 p53−/−; Fig. 2); and second, UCN-01 transactivates the luciferase reporter Δ p53 (construct lacking p53 binding sites) or pSma (minimal promoter region for both UCN-01 and Ras; −100 bp from transcriptional site) when transfected in a cell line with mutant p53 (HaCaT; Fig. 4) and p53 null (HCT116 p53−/−; data not shown).

To establish the exact mechanism by which UCN-01 activates p21 transcription, we tested whether known targets of UCN-01, such as PKC, chk1, and the PDK1/akt pathways, are involved in the transcriptional induction of p21\textsuperscript{waf1/cip1} (12, 14, 17, 33). First, we assessed the phosphorylation status of adducin, a known PKC substrate, and we demonstrated that at concentrations and times when p21 was maximally induced, PKC activity was not altered. These results are consistent with our previous results, where the cell cycle effects of UCN-01 in leukemia and/or squamous malignant tumors were not due to loss in PKC activity (15, 16). Moreover, we demonstrated recently that the cell cycle effects of UCN-01 in head and neck squamous cell cancer were not related to loss in chk1 activity (16). Also recently, Sato et al. (33) demonstrated that UCN-01 may also have the capacity to inhibit PDK1, an important kinase in the P38/akt pathway. Of note, AKT, also known as protein kinase B, is activated by phosphorylation in two specific sites, threonine 308 (PDK1-dependent site) and serine 473 (PDK1-independent site; Refs. 35, 36). To determine whether UCN-01-induced p21 transcriptional up-regulation is due to effects on the PDK1/akt pathway, we set out several experiments.

Fig. 7. Activation of the mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway is required for UCN-01-induced p21 nuclear protein expression. A, serum-starved HaCaT cells were exposed to UCN-01 or vehicle for 4 h in the presence or absence of PD98059 (50 μM), which was added 30 min before UCN-01. Cells were harvested, and protein lysates were immunoblotted against p21, phosphorylated-ERK, and ERK2 as discussed in “Materials and Methods.” As a control, expression levels of ERK1/2 were analyzed by Western blot in the same lysates. Two additional experiments yielded comparable results. B, exponentially growing HaCaT cells were exposed to UCN-01 or vehicle for 4 h in the presence or absence of UO126 (10 μM), which was added 30 min before UCN-01. Cells were harvested, and protein lysates were immunoblotted against p21, phosphorylated MEK, and MEK as discussed in “Materials and Methods.” As a control, expression levels of actin were analyzed by Western blot in the same lysates. Two additional experiments yielded comparable results. Arrow denotes phosphorylated MEK species. C, HaCaT cells grown in coverslips were exposed to UCN-01 or vehicle for 4 h in the presence or absence of PD98059 (50 μM), which was added 30 min before UCN-01. Cells were permeabilized and immunostained as described in “Materials and Methods.” For nuclear staining, coverslips were mounted with 4,6-diamidino-2-phenylindole-containing mounting media. Top panels, Cy3 immunofluorescence depicts p21 expression. Bottom panels, Merge represents the co-staining of Cy3 (p21 expression) and 4',6-diamidino-2-phenylindole (chromatin). Images are ×100. Inset, control and UCN-01 exposed cells shown at ×400. This panel corresponds to a representative experiment. Nearly identical results were obtained in two additional experiments.
First, we asked whether UCN-01 promotes the loss in AKT phosphorylation. Indeed, at the concentrations and times tested, a minimal decrease in phosphorylation of Ser 473 appeared only at the highest UCN-01 concentration tested (300 nM by 4 h). In contrast, there was no decrease in the phosphorylation of the PDK1-dependent site Thr 308, even at the highest UCN-01 concentrations tested. Thus, at times and concentrations where p21 is maximally induced, it appears that there was no significant loss in AKT phosphorylation. Second, we tested whether the exposure of HaCaT cells to small molecule PI3k inhibitors could lead to p21 up-regulation. Indeed, exposure of HaCaT cells to LY294002 failed to show a significant increase in p21. Similar effects were observed with an unrelated small molecule PI3k inhibitor, wortmannin (data not shown). Third, to additionally challenge this hypothesis, we infected HaCaT cells with adenovirus containing kinase-dead AKT for 24 h demonstrating no increase in p21. Fourth, we asked whether infection with an adenovirus containing constitutively-active AKT (myr-AKT) could rescue the effects of UCN-01. AKT activation did not rescue the induction of p21 by UCN-01, suggesting strongly that UCN-01 modulates p21 transcription through an AKT-independent mechanism.

On the basis of our p21 promoter results, the minimal p21 promoter region is similar to the one required for Ras to transactivate p21; therefore, we asked whether UCN-01 activates p21 transcription due to modulation of signal transduction pathways downstream of Ras, namely MEK and ERK MAPK pathways (56, 57). We demonstrated first that UCN-01 activated MEK/ERK pathways at concentrations and times that preceded the accumulation of p21 in serum-free (Fig. 6a and Fig. 7A) or in serum-containing conditions (Fig. 7B). We then determined the exact role of MEK activation in p21 transcription. Induction by UCN-01 using either dominant-negative alleles and/or chemical inhibitors of MEK (PD 98059 and U0126) at concentrations that only block MEK activity (41, 42). Blockade of MEK by genetic or chemical inhibitors clearly blunted the induction of p21 transcription, promoter, mRNA, and protein levels. Of note, the activation of MAPK was reported previously in hematological malignant models (58). The authors showed that concentrations of 200 nM UCN-01 in combination with MEK inhibitors for ≥24 h promoted apoptosis (58). In our report, we did not observe an increase in apoptosis by the combination of UCN-01 and MEK inhibitors. Several different experimental conditions can explain the difference in phenotype: (a) we used a lower UCN-01 concentration (100 nM); (b) we incubated both drugs for no longer than 12 h; and (c) leukemic cells may be more sensitive to UCN-01 than human keratinocyte cell lines. To address whether UCN-01 promoted the redistribution of p21 from nucleus to cytoplasm, we performed immunofluorescence studies with p21 antibodies demonstrating that the induction observed was mostly nuclear and, again, this up-regulation was blocked by preincubation with MEK inhibitors. Taken together, these results demonstrated that UCN-01 activated the MEK/ERK pathway, and this activation was required for the induction of p21.

It is counterintuitive that a small molecule inhibitor with clear antiproliferative properties could activate a proliferative pathway such as the MEK/ERK MAPK pathway. However, Ras (a known activator of MEK/ERK) can, in some models, lead to antiproliferative phenotypes such as cell death or senescence due to activation of the MEK/ERK pathways (59, 60). Moreover, other antiproliferative agents including paclitaxel, perifosine, and cisplatin do indeed activate MEK/ERK MAPK pathways (61–63). Thus, activation of the MEK/ERK pathways by different stimuli, including proto-oncogenes or drugs, can, in some circumstances, mediate antiproliferative pathways. How UCN-01 activates the MEK/ERK pathway is still unknown. It is possible that UCN-01 may activate a stress response leading to the activation of MAPKs (64). Additional experiments are warranted to investigate the exact nature for this activation.

In summary, the G1/S arrest induced by UCN-01 is mediated by up-regulation of p21. This up-regulation is transcriptional, and the minimal p21 promoter region is similar to the one required for Ras to transactivate p21. Moreover, transactivation of p21 requires the activation of the MEK/ERK pathways, as UCN-01 promotes MEK/ERK activation, and loss in MEK activity blunted the effects of UCN-01 on p21 expression. Thus, this novel pathway whereby UCN-01 regulates p21 transcription and expression appears to be highly dependent on MAPK activation. Additional work will be necessary to identify the nature of the molecules required for UCN-01 to activate MAPK as well as to investigate the role of this novel signaling pathway in normal and aberrant cell growth.

ACKNOWLEDGMENTS

We thank Silvio Gutkind for reagents and Silvio Gutkind, Lourdes Villalba, and Alfredo Molinolo for critically reading the manuscript. We also acknowledge William Swaim and Neil Hardegen for technical support in confocal imaging and cell cycle fluorescence-activated cell sorter analysis, respectively.

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*Cancer Res* 2004;64:3629-3637.

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