UCN-01 (7-Hydroxystaurosporine) Inhibits in vivo Growth of Human Cancer Cells through Selective Perturbation of G1 Phase Checkpoint Machinery

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Mechanisms underlying tumor sensitivity to the antitumor agent UCN-01 (7-hydroxystaurosporine) were examined in the nude mouse model using three human tumor xenografts, two pancreatic cancers (PAN-3-JCK and CRL 1420) and a breast cancer (MX-1). UCN-01 antitumor activity was evaluated in terms of relative tumor weights in treated and untreated mice bearing the tumor xenografts. The activity of cyclin-dependent kinase 2 (CDK2), levels of p21 and p27 proteins, pRb status and cell cycle were evaluated. Induction of p21 and apoptosis were also assessed immunohistochemically in CRL 1420. UCN-01 was administered intraperitoneally at a dose of either 5 or 10 mg/kg daily for 5 days followed by a further 5 injections after an interval of 2 days. UCN-01 significantly suppressed the growth of both pancreatic cancers, but was ineffective against MX-1. p21 protein expression was markedly induced in the UCN-01-sensitive pancreatic carcinoma xenografts at both doses, but p21 induction was only evident in the UCN-01-resistant MX-1 at 10 mg/kg. MX-1 exhibited CDK2 activity that was 6-fold higher than that of pancreatic cancer strains, which may explain the resistance of MX-1 to UCN-01 despite the induction of p21 at the dose of 10 mg/kg. The UCN-01-sensitive tumors exhibited G1 arrest and increased levels of apoptosis, changes not observed in resistant MX-1. In conclusion, it appears that a determining factor of in vivo UCN-01 sensitivity involves the balance of CDK2 kinase activity and p21 protein induction, resulting in augmented pRb phosphorylation, G1 cell cycle arrest and apoptosis.

Key words: UCN-01 — Human xenografts — In vivo sensitivity

Protein phosphorylation is a major mediator of signal transduction during progression through the cell cycle. Members of the cyclin-dependent kinase (CDK) family and cyclins have important roles at different points in the cell cycle. For instance, cell cycle progression from early to mid-G1 phase is dependent on CDK4 and/or CDK6 activation by D-type cyclins whilst binding of CDK2 to cyclins A and E is necessary for the coordinated progression from mid-G1 to S phase.1) In addition to binding to the appropriate partner cyclin, CDKs also require the proper sequence of stimulation and phosphorylation by cyclin-activating kinases.2–4) Transition through G1 to S phase is regulated by activation of CDK2 by cyclin E, and the resultant cyclin/CDK complexes phosphorylate pRb to give ppRb,5) which then activates E2F-driven transcriptional activation.6) E2F promotes expression of the thymidine kinase, thymidylate synthetase (TS), dihydrofolate reductase, cyclin E, and DNA polymerase-α genes, among others.

Recent studies have revealed that CDK activity during G1 is negatively regulated by two families of CDK inhibitor proteins; the INK4 family including p16INK4a and the CIP/KIP family which includes p21WAF1/CIP1 and p27Kip1.7–10) p21 induction during irradiation-induced p53-mediated G1 arrest and apoptosis indicates the involvement of p21 in G1/G2 arrest and subsequent inhibition of CDK function.11) Therefore, p21 appears to be an important effector of p53-mediated cell cycle arrest.

The p27 protein was originally recognized as a cyclin/CDK complex binding protein that inhibited cyclin E/CDK2 activity in growth arrest induced by contact inhibition and transforming growth factor-β.10) Recent studies have indicated a potentially key role for p27 in the progression of several human cancers, and decreased p27 protein levels have been associated with aggressive tumors.12–14)

Recently, CDK modulators have been developed as chemotherapeutic agents, including purines and purine analogs,15–17) butyrolactone,18) flavopiridol,19) and 7-hydroxystaurosporine (UCN-01).20) UCN-01 is a new antitumor agent originally isolated as a selective inhibitor of Ca2+ and phospholipid-dependent protein kinase C21–24) UCN-01 treatment induces preferential G1 phase accumulation in several types of human cell lines, although the mechanism of this has not been completely defined.20, 25–27) UCN-01 was assessed by disease-orientated screening at the National Cancer Institute,28) where it was found to inhibit renal, pancreatic and lung cancers, exerting antitu-
mor activity via a novel mode of action compared to conventional agents. Mack et al. demonstrated that UCN-01 induced G1 arrest in cells null for p53 and p16, with Rb status influencing the ability of UCN-01 to induce this arrest.29)

Normal cells have low p53 protein levels, with p53 up-regulation being the result of “stress,” such as DNA damage by genotoxic agents.30) The rapidly increased cellular p53 levels allow p53 to act as a checkpoint as to whether to initiate G1 arrest or apoptosis. Several reports have demonstrated that p53 is involved in triggering apoptosis following various stimuli.31, 32) It is becoming clear that p53-dependent apoptosis can modulate the toxic effects of anticancer agents, and that resistance to therapy correlates well with the acquisition of p53 mutations. Several studies have indicated that p53 mutations are relatively common in adenocarcinoma of the pancreas, occurring in up to 70% of patients.33–36) Treatment of p53-mutated cancers requires an antitumor agent that suppresses tumor growth through a p53-independent pathway. Knowledge of how the signal transduction pathway controls tumor growth would more clearly identify patients who would receive benefit from such treatment approaches.

In the present study, we assessed the effects of UCN-01 on different human cancer xenografts in the nude mouse model to elucidate the mode of action of this agent.

MATERIALS AND METHODS

Human tumor xenografts The human pancreatic carcinoma xenograft PAN-3-JCK was established as a serially transplantable human pancreatic carcinoma xenograft in the nude mouse at the Central Institute for Experimental Animals, Kawasaki. CRL 1420 (MIA PaCa-2) was supplied from the American Tissue Culture Collection. These tumors are histologically defined as poorly differentiated adenocarcinomas and are resistant to conventional antitumor agents.37, 38) The human breast cancer xenograft MX-1 was derived from the breast cancer of a 29-year-old female patient,39) and was kindly supplied to our institute by Dr. K. Inoue (Cancer Chemotherapy Center, Tokyo) in 1979. MX-1 is defined histologically as an invasive ductal carcinoma and is sensitive to conventional antitumor agents.40, 41) All xenografts were maintained in our laboratory as serially transplantable tumors in nude mice.

Tumor inoculation, measurement of tumor size, and evaluation of agent activity Male nude mice with a BALB/c genetic background (CLEA Japan Co., Ltd., Tokyo) were maintained under specific pathogen-free conditions using an Isorack in our experimental animal center. Mice were fed sterile food and water ad libitum. Five-week-old mice weighing 20–22 g were used for our studies. Two fragments of tumor tissue, each measuring approximately 3×3×3 mm in size, were inoculated bilaterally into the subcutaneous tissue of the dorsum of ether-anesthetized mice using a trocar needle. The tumors were measured (length and width) with sliding calipers three times weekly by the same observer. Tumor weights were calculated from linear measurements using the formula: tumor weight (mg) = length (mm) × [width (mm)]² / 2.42) When tumors reached 100 to 300 mg, the mice were randomly allocated to test groups (n=5 per group) and the treatment was initiated.

UCN-01 was supplied by Kyowa Hakko Kogyo Co., Ltd. (Tokyo). UCN-01 was dissolved at 0.2 or 0.4 mg per ml of solvent containing 20 mM citrate buffer pH 6.0 and stored at 4°C until use. Aliquots (0.5 ml) of UCN-01 solution were administered intraperitoneally at doses of either 5 or 10 mg/kg daily for 5 days, with the treatment repeated once after a 2-day interval. Control mice were given vehicle alone simultaneously.

Relative mean tumor weights (RW) were calculated as: 

$$\text{RW} = \frac{W_i}{W_o} \times 100\%$$

where $W_i$ is the mean tumor weight at any given time and $W_o$ is the mean tumor weight just prior to the commencement of treatment. Antitumor effects were assessed using the lowest $T/C$ ratio (%) during the experiment, where $T$ is the relative mean tumor weight of the treated group and $C$ is the relative mean tumor weight of the control group at any given time. Antitumor activity was considered present when the lowest $T/C$ ratio was less than or equal to 42%.40

Western blotting for p21, p27, CDK2, and Rb activity assay Reagents were obtained from Sigma Chemical, St. Louis, MO unless otherwise stated. PAN-3-JCK, CRL 1420 and MX-1 xenografts were harvested 24 h after the last UCN-01 treatment. Tumors were washed with phosphate-buffered saline (PBS) (–), cut and homogenized in trypsin-EDTA solution on ice. The cells were lysed in lysis buffer containing 50 mM HEPES, NaOH (pH 7.4), 150 mM NaCl, 0.1% Triton X-100 (Wako Pure Chemical Industries, Ltd., Tokyo), 50 mM sodium fluoride, 80 mM α-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. Cell lysates were cleared by centrifugation at 15,000 rpm for 30 min at 4°C and protein content determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were heated in sodium dodecyl sulfate (SDS) sample buffer for 5 min at 95°C, separated by SDS-polyacrylamide (Nacalai Inc., Kyoto) gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore Co., Bedford, MA).43) p21, p27 and CDK2 were separated using 15% resolving gels, and Rb was separated using 7.5% resolving gels, and Rb was separated using 7.5% resolving gels. Membranes were incubated in blocking buffer (5% skim milk [DIFCO Laboratories, Detroit, MI] and 1% bovine serum albumin (BSA) in PBS (–)). Blots were incubated overnight at 4°C with the following primary antibodies;
mouse monoclonal anti-human p21 (sc-817), polyclonal rabbit anti-human p27 (sc-528) (Santa Cruz Biotechnology Inc., Santa Cruz, CA), mouse monoclonal anti-human Rb (14001A) (Pharmingen Co., San Diego, CA) or mouse monoclonal anti-CDK2 antibody (Kyowa Hakko Kogyo Tokyo Research Laboratories, Tokyo). Blots were washed with washing buffer containing Tween 20 (Bio-Rad Laboratories), and incubated with the either horseradish peroxidase-conjugated (Amersham Life Sciences, Buckinghamshire, UK) rabbit anti-mouse IgG (H+L) or rabbit anti-rabbit IgG (H+L) (Zymed Laboratories, Inc., San Francisco, CA) as the secondary antibody and detected with the enhanced chemiluminescence system (Amersham Pharmacia Biotech). Membranes were then exposed to Hyperfilm ECL (Amersham) and resultant band densities were measured with an Image Densitometer (Molecular Analyst, Bio-Rad Laboratories) to evaluate p21 induction and accumulation of phosphorylated Rb protein.

CDK2 kinase assay The CDK2 kinase assay was performed as previously described using histone H1 as a substrate. Following UCN-01 treatment, the PAN-3-JCK, CRL 1420 and MX-1 xenografts were removed from the nude mice and homogenized in trypsin-EDTA solution on ice. Cells were washed with PBS and lysed in lysis buffer as described above at 4°C for 20 min. Lysates were clarified by centrifugation at 14,000 rpm for 10 min at 4°C and protein content was determined using a protein assay kit (Bio-Rad Laboratories). One milligram aliquots of protein were added to protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) preassociated with anti-CDK2 antibody and mixed gently for 2 h at 4°C. Immunoprecipitates were washed twice with lysis buffer and twice with washing buffer (50 mM HEPES/NaOH pH 7.4, 10 mM MgCl2, 1 mM DTT). Pellets of 20 µl of CDK2 immunoprecipitate were mixed with 40 µl of kinase buffer (50 mM HEPES/NaOH, pH 7.4, 10 mM MgCl2, 1 mM DTT, 16 µl histone H1 (Boehringer Mannheim, Mannheim, Germany), 50 µM ATP, 2.5 µCi [γ-32P]ATP (5000 Ci/mmol; Amersham Life Science) and protein content was determined using a protein assay kit (Bio-Rad Laboratories) for 10 min at 30°C. Samples were then mixed with 30 µl of 3x SDS-sample buffer to stop the reactions, heated for 5 min at 95°C, and subjected to SDS-PAGE. Gels were dried, stained with Coomassie Brilliant Blue and analyzed using a BAS2000 image analyzer (Fuji Photo Film Co., Tokyo).

Cell cycle analysis Following UCN-01 treatment, PAN-3-JCK, CRL 1420, and MX-1 xenografts were removed from the nude mice and cut into pieces with scissors in PBS without calcium. Single cell suspensions were prepared enzymatically by treatment with 0.5 mg/ml pronase, 0.2 mg/ml collagenase type 1 and 0.2 mg/ml DNase for 30 min at 37°C. After centrifugation at 3000 rpm, tumor cells were suspended in PBS (−) and diluted to 5×10^5 cells/ml. Cellular DNA content was measured by flow cytometry. Cells were fixed in ice-cold 70% ethanol solution, and stored at 4°C. After having been washed with PBS (−), cells were incubated with 250 µg/ml ribonuclease A (type 1-A) containing 0.1% Triton X-100 (Wako Pure Chemical Industries) in PBS (−) for 20 min at 37°C. Cells were then stained with propidium iodide at a final concentration of 50 µg/ml for 20 min on ice. Fluorescence of individual cells was measured with a flow cytometer (Epics Elite, Coulter, Hialeah, FL). Cell cycle distribution was calculated using the Multicycle program (Coulter).

Immunohistochemical staining UCN-01-treated (10 mg/kg) or untreated CRL 1420 specimens were formalin-fixed and paraffin-embedded. Serial deparaffinized 4 µm sections were subjected to methanol 0.03% hydrogen peroxide treatment for 30 min to block endogenous peroxidase activity. Sections were then heated in citrate buffer (0.01 M, pH 6.5) for 3 cycles of 5 min for antigen retrieval, hydrated with Tris-buffer saline (TBS, pH 7.4) for 15 min, and incubated with blocking solution of 5% skim milk (DIFCO Laboratories) and 1% BSA in PBS (−) to block non-specific binding sites. Sections were then incubated with monoclonal mouse anti-human p21 antibody (sc-817) (diluted 1:500, Santa Cruz Biotechnology Inc.) at 4°C overnight, followed by incubation with biotinylated horse anti-mouse serum at room temperature for 20 min, and incubation with horseradish peroxidase-conjugated avidin-biotin complex (ABC) (Vector Laboratories, Inc., Burlingame, CA) at room temperature for 15 min. Staining was developed by incubating sections in 3,3'-diaminobenzidine tetrahydrochloride substrate at room temperature for 8 min, and sections were weakly counter-stained with 0.1% hematoxylin. Finally, sections were dehydrated, mounted and positive reaction detected as a brown color. Triplicate samples of five areas of 200 cells were selected randomly and p21-positive cells in each area were counted. The percentage of p21-positive cells in the specimens was determined as the positive cells per 1000 tumor cells in five areas. Immunohistochemical examination was conducted blindly by two independent investigators.

Detection of apoptosis in tissue cryosections UCN-01-treated (10 mg/kg) or untreated CRL 1420 specimens were stained using “ApopTag” Plus (In situ Apoptosis Detection Kit) (ONCOR, Gaithersburg, MD). Cryosections were fixed in 1% paraformaldehyde in PBS (pH 7.4) in a Coplin jar for 10 min at room temperature and washed in two changes of PBS for 5 min. Specimens were quenched in 3% hydrogen peroxide in PBS for 5 min at room temperature and rinsed twice with dH2O for 5 min. Immediately, 75 µl aliquots of equilibration buffer were applied directly to the specimens, which were incubated for 10 s at room temperature, after which 55 µl/5 cm² working strength TdT enzyme was immediately pipetted onto the sections. Incubation was continued in a humidified chamber at 37°C for 1 h. Anti-digoxigenin peroxidase was then applied to the slides at approximately 65 µl/5
cm² at room temperature. Excess peroxidase substrate was then applied to completely cover the specimens (75 µl/5 cm²), which were stained for 6 min at room temperature and then counter-stained with 0.5% (w/v) methyl green in a Coplin jar for 10 min at room temperature. Washing was performed by dipping the specimens 10 times in 100% N-butanol for the first and second washes, followed by 30 s without agitation for the third wash. Specimens were then dehydrated, mounted with Permount and examined by fluorescence microscopy. The incidence of apoptotic chromatin condensation was counted as fluorescent spots using an Olympus BH-2 fluorescence microscope equipped with a BH2-DM2U2UV Dichetomic mirror cube filter. The apoptotic index was determined by the same method as used for immunohistochemical examination.

Statistical analysis The statistical significance (P) of the experimental results was determined by Student’s t test using Macintosh Microsoft Excel Version 2.01. A value of P<0.05 was considered to be statistically significant.

RESULTS

In vivo antitumor activity The antitumor activity of UCN-01 against three human tumor xenografts is shown in Fig. 1. The intraperitoneal administration of UCN-01 in a schedule of either 5 or 10 mg/kg daily for 5 days followed by a further 5-day course after an interval of 2 days resulted in significant dose-dependent suppression of the growth of PAN-3-JCK and CRL 1420. Compared to controls, the lowest relative mean tumor weight T/C ratios for the 5 and 10 mg/kg doses were 36.3% and 16.8% respectively for PAN-3-JCK (Fig. 1a) and 32.3% and 16.2% respectively for CRL 1420 (Fig. 1b). The actual tumor weights of the two pancreatic cancer xenografts were also significantly suppressed by UCN-01 (P<0.001) compared to controls. In contrast, UCN-01 did not significantly suppress the growth of MX-1 xenografts, with the lowest T/C ratios being 89.9% and 66.1% at the 5 and 10 mg/kg doses, respectively (Fig. 1c).

Changes in p21 and p27 protein expression, and CDK2 and pRb phosphorylation as detected by western blotting Western blotting revealed strong up-regulation of p21 expression in the two pancreatic tumor xenografts following UCN-01 treatment (Fig. 2). The relative increases

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**Fig. 1.** Relative tumor weights of the three tumor xenografts following UCN-01 treatment. UCN-01 solution (0.5 ml) was administered intraperitoneally at doses of 5 or 10 mg/kg daily for 5 days, and the treatment repeated once following a 2-day interval. The statistically significant decline in relative tumor weights of two pancreatic tumors (a) PAN-3-JCK and (b) CRL 1420 compared with the untreated control group indicates an antitumor effect. MX-1 tumor weights (c) were not significantly different from controls. ■ control, ○ UCN-01 5 mg/kg i.p., ▲ UCN-01 10 mg/kg i.p.

**Fig. 2.** Effect of UCN-01 upon expression of four cell cycle regulatory proteins. PAN-3-JCK, CRL 1420, and MX-1 tumor xenografts were harvested after UCN-01 treatment and analyzed by western blotting using antibodies against p21, CDK2, p27 and pRb.
in band intensities for the 5 and 10 mg/kg doses were 72.9% and 75.9%, respectively, for PAN-3-JCK, and 49.8% and 55.1%, respectively, for CRL 1420. In contrast, p21 protein expression in MX-1 was unchanged in the 5 mg/kg group, although p21 protein accumulation was observed at the 10 mg/kg dose (65.6%). PAN-3-JCK and MX-1 p27 protein levels remained stable following UCN-01 treatment, whilst p27 levels in CRL 1420 were reduced by UCN-01 treatment (Fig. 2).

CDK2 protein was expressed in all three tumor xenografts. The amount of the faster-mobilizing protein band of phosphorylated active CDK2 was markedly reduced in CRL 1420 following UCN-01 treatment, indicating reduced CDK2 activity (Figs. 2 and 3). However, CDK2 protein levels in PAN-3-JCK and MX-1 were not significantly altered following UCN-01 treatment.

In untreated PAN-3-JCK xenografts, pRb was constitutively hyper-phosphorylated and growth-permissive, with dephosphorylated pRb accumulating only after treatment with 5 mg/kg UCN-01. In CRL 1420 tumors treated with UCN-01, a dose-dependent accumulation of dephosphorylated pRb was observed. pRb levels were increased by 29.9% and 43.1% after treatment with 5 and 10 mg/kg UCN-01, respectively. In contrast, pRb levels in MX-1 were stable following UCN-01 treatment (Fig. 2). These results suggest that UCN-01 may mediate tumor growth inhibition by accumulating tumor cells in the G1 phase of the cell cycle through p21 protein induction and accumulation of dephosphorylated pRb.

CDK2 kinase activity As shown in Fig. 3, untreated MX-1 tumor exhibited an almost 6-fold higher level of CDK2 (H1 kinase) activity compared to the pancreatic cancer strains. This marked CDK2 activity was slightly inhibited by UCN-01 at doses of 5 and 10 mg/kg. In contrast, CDK2 activity in UCN-01-treated CRL 1420 tumors was less than 50% of that of untreated controls. Although CDK2 activity in MX-1 was also inhibited by UCN-01, the remaining activity was still higher than in untreated PAN-3-JCK and CRL 1420 tumors, and this may be related to the low sensitivity of MX-1 to UCN-01 treatment.

DNA content analysis by propidium iodide (PI) staining Results of the staining analysis of control and UCN-01-treated xenografts are shown in Fig. 4. In the two pancreatic cancer xenografts, the 5 mg/kg dose led to an accumulation of tumor cells in the G1 phase compared with untreated controls. The cell cycle distribution was then analyzed by flow cytometry using the FL-2 channel. The proportions of PAN-3-JCK cells that had entered the G1 phase in the control, 5 and 10 mg/kg groups were 37.7%, 61.6%, and 66.9% respectively. On the other hand, the proportions of CRL 1420 cells that had entered the G1 phase in the control, 5 and 10 mg/kg groups were 43.1%, 61.5%, and 52.3% respectively. Thus, the 10 mg/kg dose resulted in fewer CRL 1420 tumor cells entering the G0/G1 or apoptotic phase as a hypo-diploid peak in PI stained cells by flow cytometry, suggesting that UCN-01 induces apoptosis in a dose-dependent manner. In contrast, the cell cycle progression of MX-1 tumor cells was not altered after UCN-01 treatment, reflecting the low sensitivity of
MX-1 to UCN-01 in spite of the suppression of CDK2 activity.

**Immunohistochemistry** p21-positive cells were identified in tissue sections by brown nuclear staining. In untreated CRL 1420 tumor specimens, less than 2% of cells were positive for p21 (Fig. 5a). This was increased to 12.4±5% of total cells following UCN-01 treatment at a dose of 10 mg/kg (Fig. 5b). Immunohistochemical and apoptotic staining of PAN-3-JCK was difficult to interpret because of cyst formation.

**Apoptosis** No apoptotic changes were observed in untreated CRL 1420 tumor specimens (Fig. 5c). However, apoptosis was induced by 10 mg/kg UCN-01 treatment, resulting in a marked increase in the apoptotic index to 24.1±4% (Fig. 5d).

**DISCUSSION**

In this study, we assessed the antitumor activity of UCN-01 on three human tumor xenografts serially transplanted into nude mice. While two pancreatic cancer xenografts insensitive to conventional agents\(^{37,38}\) were sensitive to UCN-01, the MX-1 xenograft, sensitive to conventional agents\(^{40,41}\) was resistant to UCN-01. This indicated that UCN-01 has a different antitumor spectrum compared to other agents. In order to clarify the mode of action of UCN-01, we analyzed proteins involved in cell cycle signaling by western blotting. Expression of the CDK inhibitor p21 was strongly induced by UCN-01 (5 and 10 mg/kg) in the two UCN-01-sensitive pancreatic carcinoma xenografts. This was confirmed by immunohistochemical examination, in which increased numbers of p21-positive cells were observed in CRL 1420 tumor specimens treated with 10 mg/kg UCN-01. However, the active phosphorylated form of CDK2 together with the CDK inhibitor p27 were dose-dependently reduced by UCN-01 treatment only in CRL 1420. Nonetheless, an accumulation of dephosphorylated pRb was observed in response to 5 or 10 mg/kg UCN-01 treatment in both human pancreatic tumor xenografts. The tumor cells...
accumulated in the G1 phase in a dose-dependent manner, resulting in growth inhibition. Thus, it is possible that the inhibition of ppRb led to the perturbation of cell cycle progression at the G1 checkpoint in these pancreatic tumors.

In contrast, p21 expression was up-regulated in MX-1 xenografts treated with 10 mg/kg UCN-01, while no significant changes were observed in pRb phosphorylation status. However, CDK2 activity of MX-1 was about 6-fold higher than that of PAN-3-JCK, which may explain why increased p21 protein levels in MX-1 did not result in the accumulation of dephosphorylated pRb. Our results suggest that CDK2 activity may be directly related to UCN-01 sensitivity, because high CDK2 kinase activity in MX-1 was not significantly suppressed by UCN-01, as described by Akiyama et al.20

In addition, Akiyama et al. demonstrated that A431 cells (p53-mutated human epidermoid carcinoma cells) expressed p27 protein in vitro in response to 24 h exposure to 260 and 520 nM UCN-01.20 The CDK inhibitor protein p27 plays an important role in contact inhibition, and p27 protein status in tumor xenografts may differ between tumors cultured in vitro and in vivo.40 However, p27 levels were not affected by UCN-01 either in UCN-01-sensitive PAN-3-JCK or in UCN-01-resistant MX-1, while it was reduced in CRL 1420. This might account for the stable p27 expression observed in the present study using human tumor xenografts.

In cell cycle analysis, we observed that negative regulation was dependent on the balance of CDK2 kinase activity and p21 expression, which inhibited pRb phosphorylation independently of p53 status. Thus, the induction of p21 protein in the p53 mutant CRL 1420 xenograft suppressed tumor growth despite only modest changes in CDK2 activity. It has been reported that the accumulation of dephosphorylated pRb is involved in G1 arrest and apoptosis. Mack et al. demonstrated that UCN-01-induced G1 arrest occurred in cells null for p53 and p16INK4a, suggesting that Rb status may influence the ability of UCN-01 to induce G1 arrest.25 This result is consistent with our present study, in which the accumulation of dephosphorylated Rb was closely related to G1 arrest and apoptosis. In addition, our results indicate that upstream events of this signal transduction are regulated by the balance of CDK2 kinase activity and p21 protein expression, suggesting that UCN-01 modulates signal transduction mainly through affecting this balance. In addition, these changes in the upstream signal transduction resulted in the accumulation of dephosphorylated pRb, G1 block and increased levels of apoptosis in two sensitive pancreatic cancer strains. This result is similar to that of Sugiyama et al., who reported that induction of apoptosis and G1 cell cycle arrest are important determinants of the in vitro sensitivity of cancer cells to UCN-01, suggesting that inhibition of CDK2 activity together with CDK2 dephosphorylation and reduced cyclin A expression may be important in the G1 phase accumulation induced by UCN-01.45 Akiyama et al. have also suggested that G1 checkpoint function, including a CDK2 regulatory pathway, may be a significant determinant of the sensitivity of tumor cells to UCN-01 in vitro.27

Since the accumulation of dephosphorylated pRb is involved in the inactivation of the essential transcription factor E2F, the G1 block caused by UCN-01 may result in the downregulation of mRNA for enzymes critical for DNA synthesis such as thymidylate synthetase,46 dihydrofolate reductase, cyclin E and DNA polymerase-α. Thus, UCN-01 pretreatment combined with antitumor agents that target these enzymes may lead to synergistic antitumor activity.

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