

Antitumor Activity of UCN-01 in Carcinomas of the Head and Neck Is Associated with Altered Expression of Cyclin D3 and p27^{KIP1}

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ABSTRACT

Altered and deregulated cyclin-dependent kinase (cdk) activity is now believed to play a major role in the pathogenesis of head and neck squamous cell carcinomas (HNSCC), thus providing a suitable cellular target for therapeutic intervention. UCN-01 (7-hydroxy-staurosporine), a known protein kinase C and cdk modulator, demonstrates antiproliferative and antitumor properties in many experimental tumor models and may represent a potential candidate to test in HNSCC. In this study, UCN-01 displayed potent antiproliferative properties (IC₅₀ of ~17–80 nM) in HNSCC cells. Cell cycle analysis revealed that UCN-01 treatment of HNSCC cells for 24 h leads to a G₁ block with a concomitant loss of cells in S and G₂-M and the emerging sub-G₁ cell population, confirmed to be apoptotic by terminal deoxynucleotidyl transferase-mediated nick end labeling analysis. Additional *in vitro* studies demonstrated a G₁ arrest that was preceded by depletion in cyclin D3, elevation of p21^{WAF1} and p27^{KIP1} leading to a loss in activity of G₁ cdks (cdk2, cdk4), and reduction in pRb phosphorylation. Antitumor properties of UCN-01 were also assessed *in vivo* by treating HN12 xenografts (7.5 mg/kg/i.p./daily) with UCN-01 for 5 consecutive days. Total sustained abolition of tumor growth ($P < 0.00001$) was obtained with only one cycle of UCN-01 treatment. Terminal deoxynucleotidyl

transferase-mediated nick end labeling staining of xenograft samples revealed a higher incidence of apoptosis in treated tissues when compared with control. Additional tissue analysis demonstrated that elevated p27^{KIP1} with minimal increase in p21^{WAF1} and reduced cyclin D3 levels were readily detected in those animals treated with UCN-01, similar to those observed in HNSCC cells. Thus, UCN-01 exhibits both *in vitro* and *in vivo* antitumor properties in HNSCC models, and these effects are associated with a decrease in cyclin D3 and an increase in p27^{KIP1} protein levels, thus providing appropriate surrogate markers to follow treatment efficacy *in vivo* and, therefore, a suitable drug candidate for treating HNSCC patients.

INTRODUCTION

Recent advances in our understanding of the molecular events that drive tumor progression has provided an opportunity to apply novel approaches for the identification of drug candidates for the treatment and management of malignant diseases (1, 2). For instance, using cell based assays intended to mimic different forms of human malignancies, it is now feasible to screen thousands of novel compounds, thus aiding in the identification of new drug candidates of therapeutic interest (3, 4). A paradigm for this approach is the 60 human cancer cell line drug-screening efforts of the Development Therapeutics Program, NCI³, where the expression of several relevant targets for cancer progression and therapy were examined, and the sensitivity to a large collection of cytotoxic/cytostatic agents has been determined in relationship to the expression of these drug targets (5). This primary screen has already identified several interesting compounds, and based on their novel antineoplastic properties, some of these agents have been selected for additional preclinical and clinical evaluation. Among them, UCN-01, also known as 7-hydroxy-staurosporine, presented a number of favorable properties, and this drug is currently undergoing Phase I clinical exploration (6–8).

UCN-01 was originally developed as an inhibitor of the PKC family of serine-threonine kinases (9, 10) and has been shown to affect the *in vitro* and *in vivo* growth of many types of tumor cells, including those from breast, lung, and colon (11–13). However, recent studies evaluating the antiproliferative properties of this compound demonstrated that many of its biological actions do not correlate with the ability of UCN-01 to block PKCs (14). For example, UCN-01, but not other potent PKC antagonists such as GF 109203X, induces

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³ The abbreviations used are: NCI, National Cancer Institute; PKC, protein kinase C; cdk, cyclin dependent kinase; HNSCC, head and neck squamous cell carcinoma; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

a G₁ cell cycle block, which correlates with the loss of cdk2 activity and the elevated expression levels of the endogenous cdk inhibitors p21^{WAF1} and p27^{KIP1} (11, 15, 16). In addition, recent studies have documented that UCN-01 can inhibit other kinases important in cell cycle regulation, including chk1 (17–19). Cell cycle blockade by UCN-01 is therefore likely because of action at more than one target also related to the mutational status of key regulatory proteins such as pRb (20).

Of interest, alterations in the regulatory pathways that contribute to the proper function of the cell cycle machinery have been frequently observed in HNSCCs, the sixth most common cancer among men in the developed world, which results in ~13,000 deaths each year in the United States alone (21). For example, overexpression of cyclin D1 and loss of p16^{INK4A} protein have been reported in HNSCC (22, 23) and found to be predictive for early relapse and reduced survival (24, 25). Similarly, high and low levels of cdk4 and p27^{KIP1} proteins, respectively, may be associated with the progression of this disease (26, 27). Furthermore, overexpression of cyclin A and cyclin B1 proteins in HNSCC suggests that there may be aberrant cell cycle progression at the G₂-M checkpoint as well (28). Unfortunately, advanced refractory head and neck neoplasms are often poor responders to available chemotherapy, which contributes to a poor 5-year survival rate (21, 29). It follows that there is a clear need to identify and develop new strategies for the treatment and management of HNSCC patients.

We have recently characterized a panel of HNSCC cell lines derived from primary and secondary cancer lesions of contrasting clinical staging (T₂ to T₄; Ref. 30). These cells have been extensively assessed for alterations of major tumor suppressor genes (31, 32). For example, aberrant forms of p53 and p16^{INK4A} were present in all of the cell lines tested with the exception of one, HN30, which was found to express wild-type p53, thus displaying normal p53 function (32). Components affecting the cell cycle machinery were also assessed in these cell lines, and major alterations included overexpression of G₁-S cyclins (cyclin A, cyclin E) and cdk4 and cdk6, leading to increased cdk activity (33). Furthermore, we have previously documented the insensitivity of one of these cell lines, HN30, to some of the currently available therapeutics, including γ -irradiation and bleomycin (34, 35).

In this study, we have used this panel of HNSCC cell lines and tumor xenograft models to evaluate the suitability of UCN-01 as a treatment modality for squamous carcinomas, particularly for those lesions that are well advanced and refractory to currently available treatments. We report here that UCN-01 is growth inhibitory *in vitro* against this representative panel of HNSCC cells and that this effect is irrespective of p53 function, resulting from the blockade of cells in the G₁ phase of the cell cycle. Additionally, UCN-01 in these cells reduced cdk2 and cdk4 activity, concomitant with increased expression of p21^{WAF1} and p27^{KIP1}, and induction of apoptosis. Furthermore, we observed that UCN-01 exhibited significant evidence of antitumor activity in the HN12 xenograft model of HNSCC, also associated with induction of apoptosis, increased p27^{KIP1}, and decreased cyclin D3 expression.

MATERIALS AND METHODS

Cell Culture. Culture conditions of cell lines established from carcinomas of the head and neck are described elsewhere (35). Briefly, cells were maintained on a layer of lethally irradiated Swiss 3T3 fibroblasts in DMEM supplemented with 10% fetal bovine serum and 0.4 μ g/ml hydrocortisone at 37°C in 95% air/5% CO₂, and before subculturing or experimental procedures, feeder cells were removed as described previously (35). HaCaT cells (immortal epidermal keratinocytes) were used as a control phenotype and were maintained without a feeder layer support with culture conditions as described previously (35).

Drug. UCN-01 was provided by Kyowa Hakko Kogyo Co. Ltd., Japan, to the Development Therapeutics Program, NCI. For *in vitro* studies, this compound was reconstituted in DMSO as a 10 mM stock solution, which was further diluted to the working concentration (0–1000 nM) in culture media. The final concentration of DMSO in the culture medium was $\leq$ 0.1%. The diluent for the *in vivo* studies was 2% sodium citrate (pH 3.5).

Assessment of Thymidine Incorporation. Assessment of cell proliferation by uptake of [³H]thymidine (ICN Pharmaceuticals, Inc., Costa Mesa, CA) was essentially as described previously (35). Briefly, HNSCC and HaCaT cells (1–2 $\times$ 10⁴/well) were grown overnight in 24-well plates and treated with UCN-01 (1–1000 nM) and DMSO (0.1%, final concentration) for the vehicle control wells. After treatment (24–48 h), cells were pulsed with [³H]thymidine (1 μ ci/well) for 4–6 h, fixed (5% trichloroacetic acid), and solubilized (0.5 M NaOH) before scintillation counting. Experiments were performed in triplicate.

Cell Cycle Analysis. Analysis of DNA content of cells by flow cytometry was performed as described previously (36). Briefly, cells (HN12, HN30, HaCaT) grown exponentially to 40–50% confluency were exposed to UCN-01 (0–1000 nM) or DMSO (0.1%), harvested at the indicated time (0–24 h), washed briefly in ice-cold PBS, and fixed in 70% ethanol. DNA was stained by incubating the cells in PBS containing propidium iodide (50 μ g/ml) and RNase A (1 mg/ml) for 30 min at 37°C, and fluorescence was measured and analyzed using a Becton Dickinson FACScan and the Cell Quest software (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Analysis of Apoptosis by TUNEL. *In situ* labeling of apoptosis-induced DNA strand breaks was carried out using the *In Situ* Cell Death Detection Kit (Boehringer Mannheim Biochemicals, Indianapolis, IN) according to the manufacturer's recommendations. Briefly, cells (HN12, HN30, HaCaT) were grown to 50–60% confluency on glass coverslips and treated for 24 h with 300 nM UCN-01 or 0.1% DMSO. After washing with PBS, cells were fixed and permeabilized with paraformaldehyde (4% in PBS containing 0.05% Tween 20), and DNA strand breaks were end-labeled with fluorescein-conjugated nucleotides by terminal deoxynucleotidyl transferase. After several rinses in PBS, the coverslips were mounted on glass slides with media containing 4',6-diamidino-2-phenylindole (Vectashield; Vector Laboratories, Burlingame, CA) and analyzed using confocal microscopy. TUNEL labeling of frozen tissue sections was carried out according to the manufacturer's recommendation.

Analysis of Cdk Activity and Immunoblotting. Assessment of cdk2 and cdk4 activity and protein levels in cells was essentially determined as previously reported (35–37). Briefly, exponentially growing cells (HN12, HN30, HaCaT) were exposed to UCN-01 (0–300 nM) or DMSO (0.1%) for 24 h, lysed in NP40 lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 20 mM EDTA, 0.5% NP40, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml aprotinin, 5 µg/ml leupeptin]. Five hundred µg of total cellular protein was initially precleared by incubating with control IgG and Gammabind G Sepharose (Pharmacia Biotech, Piscataway, NJ) for 30 min at 4°C, and after centrifugation, the supernatant was subsequently used to immunoprecipitate cdk2 and cdk4 for 1 h at 4°C, with appropriate antibodies (sc-163 and sc-260-G, respectively; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Cdk complexes were captured with Gammabind G Sepharose and after several washes, kinase reactions were carried out in kinase assay buffer [50 mM HEPES (pH 7.5), 10 mM MgCl₂, 1 mM DTT] containing γ-[³²P]ATP (3000 Ci/mmol; NEN, Boston, MA), and for cdk2 assays, 25 µM ATP and histone H1 (0.2 mg/ml; Boehringer Mannheim Biochemical) or 2.5 µM ATP and glutathione S-transferase (1 µg) from the COOH-terminal portion of the Rb protein (amino acids 773–928) expressed in bacteria as a glutathione S-transferase fusion protein for cdk4 assessment. Reactions were incubated at 37°C for 30 min and terminated by the addition of SDS-gel loading buffer, resolved, and the dried gels were subjected to autoradiography and quantification by phosphorimaging (Molecular Dynamics, Sunnyvale, CA). IgG immunoprecipitates were used as background controls for the immunoprecipitated kinase. Western blot analysis of the immunoprecipitates and total cell lysates were carried out using appropriate antibodies to the indicated proteins (cdk2, cdk4: as above; cdc25C, cyclin D1, and cyclin D3, 1:500; sc-327, sc-246, and sc-182, respectively, Santa Cruz Biotechnology Inc.; p21^{WAF1} and p27^{KIP1}, 1:750; 6B6 and G173-534, respectively, Transduction Laboratories, San Diego, CA; phospho-α adducing, 1:500; Upstate Biotechnology, Lake Placid, NY; β-actin, 1:500, Chemicon International, Inc., Temecula, CA) and reactions detected by horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Amersham, Arlington Heights, IL). Polyclonal antisera to pRb recognizing phosphorylated threonine and serine residues 356 and 780, respectively, have been described previously (37).

Animal Studies. All animal studies were carried out using the appropriate NIH animal care and user protocol. Female athymic (*nu/nu*) nude mice (Harlan Sprague Dawley, Frederick, MD), 5–6 weeks of age and weighing 18–20 g, used in the study were housed in appropriate sterile filter-capped cages and fed and watered *ad libitum*. All handling and transplantation procedures were conducted in a laminar-flow biosafety hood.

Establishment and Treatment of Tumor Xenografts in Athymic *nu/nu* Mice. HN12 cells were used to induce xenografts in athymic mice as described previously (35). Briefly, exponentially growing cells were harvested, washed, resuspended in DMEM, and 1×10^7 viable cells were transplanted s.c. in the neck region of the athymic mice. The animals were monitored twice weekly for tumor formation, and drug treatment was commenced when tumor volume reached ~ 0.9 cm³ (38, 39). Drug treatment of tumor-bearing animals was essen-

tially as previously described (35). Briefly, animals were randomly grouped (control: $n = 10$; test: $n = 10$) and treated with UCN-01 (7.5 mg/kg/day) or an equal volume of diluent [2% sodium citrate (pH 3.5)]. Treatment schedule was a single injection per animal, given i.p. consecutively for 5 days. At the end of the treatment period, one animal from each group was euthanized for tissue retrieval, which were fixed (4% paraformaldehyde over night before processing for paraffin embedding) or snap frozen for apoptosis and immunohistochemical analysis. On completion of the 5-day treatment, animals were monitored (twice weekly) for tumor growth and body weight. For analysis, tumor weight was determined as described previously (39), whereby tumor volume [$(L \times W^2)/2$; where L and W represent length and the width of the tumor] was converted to weight. Results of animal experiments were expressed as mean $\pm$ SE, and paired Student's t test was used to determine the difference between treated and control groups. $P < 0.05$ was considered to be statistically significant.

Immunohistochemical Analysis of Tumor Xenografts. Serial sections of paraffin-embedded tumor xenografts from control and treated animals (day 5) were analyzed for the expression of p21^{WAF1} and p27^{KIP1} (as above) and cyclin D1 and cyclin D3 (1:40; P2D11F11 and DCS-22, respectively; Novocastra Laboratories, Burlingame, CA) by immunohistochemistry using an automated immunostainer following manufacture's paraffin slide protocol (Ventana Medical Systems, Inc., Tucson, AZ).

RESULTS

UCN-01 Inhibits the Growth of HNSCC Cells. With no available data regarding the sensitivity of HNSCC to UCN-01, we initially assessed if cell lines derived from this tumor type responded with decreased proliferation to increasing concentrations of the drug by assaying the incorporation of [³H]thymidine into cellular DNA. As demonstrated in Fig. 1, all cells exposed to UCN-01 (0–1000 nM) for 24 h resulted in a dose-dependent inhibition of [³H]thymidine uptake. The sensitivity, as assessed by the IC₅₀, was similar among all cell lines (HaCaT, 17 nM; HN4, 21 nM; HN8, 68 nM; HN12, 80 nM; HN30, 59 nM), achieving IC₈₀s with ~ 400 nM of UCN-01. No significant differences in IC₅₀s were noted with cells exposed to UCN-01 for 48 h (data not shown). The results suggest that, *in vitro*, all HNSCC cells respond similarly to the antiproliferative properties of UCN-01, and based on this, we chose for subsequent experiments the immortalized (HaCaT) or malignant (HN12 and HN30) cell lines. Of note, HN30 is resistant to certain anticancer agents (34, 35), and HN12 harbors a mutant p53 and is highly tumorigenic *in vivo* (35). Thus, each cell line exhibits distinct biological features, together representing useful models for this cancer type.

UCN-01 Affects Cell Cycle Progression in HNSCC Cells. As our previous results indicated that HNSCC cells are sensitive to the antiproliferative properties of UCN-01, we performed a more detailed analysis of the effect of the drug (0–1000 nM) on the cell cycle. Interestingly, when cells (HN12, HN30, HaCaT) were exposed for 24 h to increasing amounts of UCN-01 (0–300 nM), a distinct G₁ cell cycle block with a concomitant loss of those cells in S and G₂-M phase is demon-

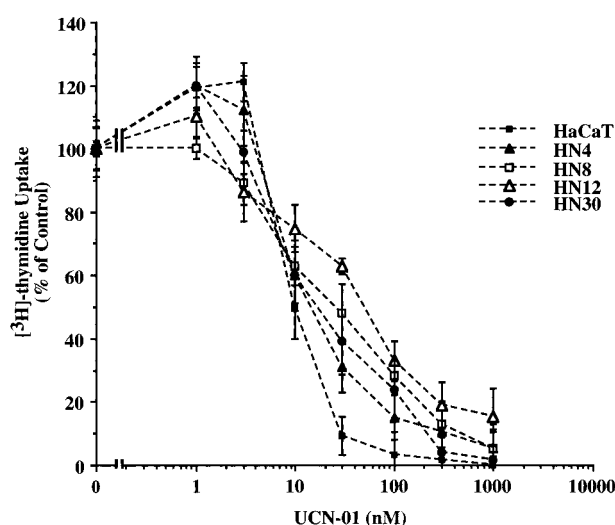


Fig. 1 UCN-01 inhibits DNA synthesis in HNSCC cell. HaCaT and HNSCC cells (HN4, HN8, HN12, HN30) were seeded ($1-2 \times 10^4$ /well) into 24-well plates and grown for 12–18 h. Cells were further grown for 24 h in the presence of UCN-01 (1–1000 nM) or DMSO (0.1% final concentration). Subsequently, the cells were pulsed with [3 H]thymidine, fixed, and solubilized as described in “Materials and Methods,” and DNA synthesis was assessed by the cell’s ability to incorporate [3 H]thymidine. The antiproliferative effect of UCN-01 in HNSCC cells is illustrated as percentage of inhibition of [3 H]thymidine incorporation relative to control. The results are the mean ($\pm$ SE) of three independent experiments and used to calculate the IC_{50} s of each cell line tested.

strated. Furthermore, with exposure levels of the drug in excess of 300 nM, an emerging sub- G_1 cell population was noted in these cells (Fig. 2). The data indicate that UCN-01 can induce the blockade of HNSCC cells in G_1 with loss of cells in S and G_2 -M and with a subsequent increase in the cell population with a sub- G_1 content of DNA.

UCN-01-induced G_1 Arrest of HNSCC Cells Correlates with Loss in Cdk2 and Cdk4 Activity. To determine the mechanisms by which UCN-01 arrest cells in G_1 , we next investigated the status of cdk2 and cdk4 activity. For this approach, exponentially growing HN12 cells were exposed to increasing concentrations of UCN-01 (0–300 nM) or 0.1% DMSO for 24 h and harvested for assessment of cdk activity as described above. As observed in Fig. 3A, a notable reduction in cdk2 (22–74%) and cdk4 (58–86%) activity was achieved with 30–100 nM UCN-01. Additionally, further increases in drug concentration (100–300 nM) correlated with a near complete reduction (81–91%) in kinase activity. Values for kinase activity were corrected for IgG background (percentage of control) after quantification by phosphorimaging (Molecular Dynamics). In contrast, similar levels of cdk2 and cdk4 mass were recovered after immunoprecipitation, indicating that the drug treatment had little or no effect on protein levels for these kinases (Fig. 3A). Similar results were observed in HaCaT and HN30 cells (data not shown). To determine whether this effect of UCN-01 occurs as a consequence of direct inhibition of enzymatic activity, *in vitro* kinase reactions were performed on cdk complexes isolated from exponentially growing cells. Cdk complexes demonstrated no loss in activity when exposed to drug

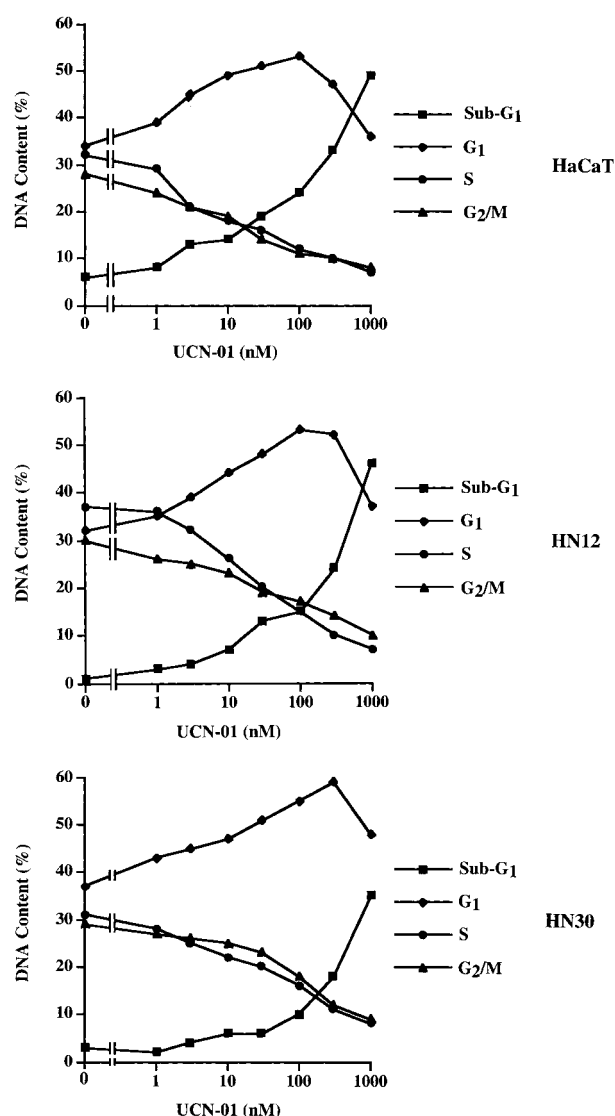


Fig. 2 G_1 cell cycle arrest in UCN-01-treated HNSCC cells. Asynchronous HaCaT, HN12, and HN30 cells, grown exponentially to 40–50% confluency, were exposed to different concentrations of UCN-01 (1–1000 nM) or 0.1% DMSO for 24 h. Cells were harvested, fixed, and DNA stained with propidium iodide. DNA content (%) of cells was obtained by flow cytometry, and values for each phase of the cell cycle (sub- G_1 , G_1 , S, G_2 -M) for each concentration of UCN-01 are depicted graphically. This figure is representative of three similar independent experiments.

levels < 300 nM but those in excess of > 300 nM may directly inhibit cdk (data not shown) as observed in our previous study (14). Thus, loss of cdk activity observed in HNSCC cells treated with UCN-01 (30–300 nM) could not be explained by direct inhibition of cdk activity.

Effect of UCN-01 on the Expression of G_1 -S Transition Proteins in HNSCC Cells. Active cdk phosphorylates several endogenous substrates, including the gene product of the retinoblastoma tumor suppressor gene (pRb). Phosphorylation of pRb occurs at several sites and phospho-specific antibodies

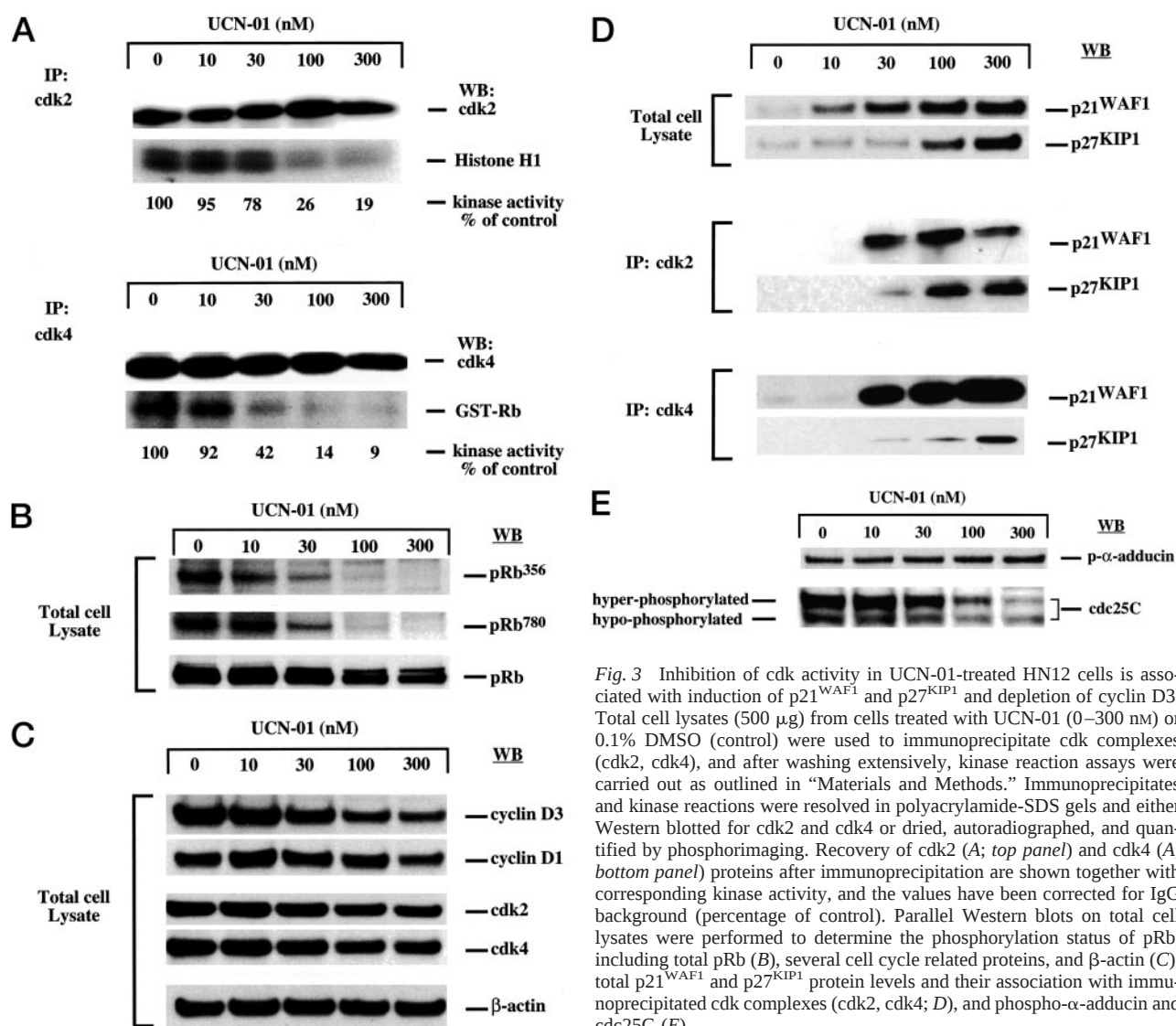


Fig. 3 Inhibition of cdk activity in UCN-01-treated HN12 cells is associated with induction of p21^{WAF1} and p27^{KIP1} and depletion of cyclin D3. Total cell lysates (500 µg) from cells treated with UCN-01 (0–300 nM) or 0.1% DMSO (control) were used to immunoprecipitate cdk complexes (cdk2, cdk4), and after washing extensively, kinase reaction assays were carried out as outlined in “Materials and Methods.” Immunoprecipitates and kinase reactions were resolved in polyacrylamide-SDS gels and either Western blotted for cdk2 and cdk4 or dried, autoradiographed, and quantified by phosphorimaging. Recovery of cdk2 (*A*; *top panel*) and cdk4 (*A*; *bottom panel*) proteins after immunoprecipitation are shown together with corresponding kinase activity, and the values have been corrected for IgG background (percentage of control). Parallel Western blots on total cell lysates were performed to determine the phosphorylation status of pRb, including total pRb (*B*), several cell cycle related proteins, and β-actin (*C*), total p21^{WAF1} and p27^{KIP1} protein levels and their association with immunoprecipitated cdk complexes (cdk2, cdk4; *D*), and phospho-α-adducin and cdc25C (*E*).

were created to recognize either cdk2 sites (pRb threonine 356) or cdk4/cdk6 (pRb serine 780; Ref. 36). To assess the endogenous pRb phosphorylation status of HN12 cells exposed to UCN-01 (0–300 nM) or vehicle, lysates prepared as described above, were resolved and immunoblotted with pRb phospho-specific antibodies. As shown in Fig. 3B, loss in phosphorylation of pRb at threonine 356 and serine 780 residues was obtained with concentrations ≥ 30 nM of UCN-01 while having a minimal effect on the amount of total pRb. The data indicate that UCN-01 at low nM levels reduces pRb phosphorylation and prevents G₁-S cell cycle progression. Thus, loss in cdk activity (Fig. 3A) can be corroborated with loss in endogenous Rb phosphorylation in intact cells exposed to UCN-01.

The loss of observed cdk activity could be the result of loss in cdk mass, loss in cofactors (*i.e.*, cyclins), overexpression of endogenous inhibitors (*i.e.*, p21^{WAF1}/p27^{KIP1}), or posttranslational modifications in specific residues in the catalytic subunit required for cdk function (13). As shown in Fig. 3C, parallel

lysates from HN12 treated with UCN-01 were resolved and immunoblotted for the indicated antibodies. The treatment with UCN-01 (≥ 100 nM) resulted in a partial loss of cyclin D3 and cyclin D1, the latter at concentrations > 300 nM. Thus, the decreased levels of these cyclins may account partially for the decreased activity of cdk in response to this agent but only at the highest concentrations. As a loading control, the steady state levels of β-actin remained unaltered. Similar results were obtained for HaCaT and HN30 cells (data not shown).

We next assessed if an increased complex formation between cdk and the cdk inhibitors p21^{WAF1} and p27^{KIP1} could account for the observed reduction in intrinsic kinase activity in response to UCN-01. Indeed, a dramatic increase in p21^{WAF1} and p27^{KIP1} levels was observed at effective concentrations of UCN-01 (≥ 30 nM; Fig. 3D). Furthermore, when cdk2 and cdk4 complexes were immunoprecipitated from HN12 cells, we observed a remarkable increase in the levels of their associated p21^{WAF1} and p27^{KIP1} proteins (Fig. 3D), without affecting the

total levels of cdk2 and cdk4 or their recovery after immunoprecipitation (see above, Fig. 3, A and C). Thus, the elevated levels of p21^{WAF1} and p27^{KIP1} and their increased association with cdk2 may contribute to the inhibition of cdk activity by UCN-01.

To determine whether the effect in cell cycle progression and modulation of cell cycle regulatory proteins is because of the capacity of UCN-01 to target PKC, HN12 lysates prepared as described were resolved and immunoblotted with specific antisera to phospho- α -adducin, a known substrate of PKC (40). As shown in Fig. 3E, no evidence of modulation in phospho- α -adducin was observed at low concentrations of UCN-01 (<300 nM) where a clear loss in cdk activity and pRb phosphorylation (threonine 356 and serine 780) is observed (Fig. 3B), reinforcing the notion that PKC loss induced by UCN-01 may be dispensable for the cell cycle effects of UCN-01, as observed in certain leukemia models (14). Another recently documented target of UCN-01 is the human chk1, a protein kinase that upon DNA damage phosphorylates cdc25C at several residues, including serine 216 (41). Thus, we assessed whether UCN-01 affects the phosphorylation status of cdc25C in exponentially HN12 cells using specific antisera to this molecule (19). As observed in Fig. 3E, cdc25C migrates as two distinct electrophoretic forms, a slower migrating serine 216 hyperphosphorylated form (*top panel*) and a faster migrating hypophosphorylated form (*bottom panel*; Ref. 19). In this figure, the intensity of the top panel (hyperphosphorylated) in control cells is elevated (5-fold) compared with the bottom panel (hypophosphorylated) cdc25C band. However, with increased concentrations of UCN-01 (100–300 nM), a loss in cdc25C mass and particularly in the top panel was observed, which is similar to those previously reported (19) and consistent with modulation of chk1 in these cells at concentrations ≥ 100 nM. Furthermore, ratio of the two (control and 300 nM) phosphorylated forms of cdc25C demonstrated a substantial decrease (85%). Collectively, these studies identify a prominent effect of UCN-01 on cell cycle regulatory molecules, including p21^{WAF1} and/or p27^{KIP1} along with depletion in cyclin D3 as correlating with the capacity to cause arrest of cell cycle progression in G₁ in squamous carcinoma cells.

Cell Cycle Kinetics of UCN-01-treated HN12 Cells.

Time course analysis of HN12 cells exposed to UCN-01 (100 nM) demonstrated a notable loss in G₂-M DNA content by 6 h (Fig. 4A). However, longer exposure resulted in not only to loss in G₂-M but also an increase in G₁ and loss in S-phase DNA content, compatible with G₁ blockade as demonstrated in other models (15, 20, 42, 43). Western blot analysis obtained from parallel samples revealed a very prominent induction of p21^{WAF1} by 3 h and subsequently followed by induction of p27^{KIP1} and depletion of cyclin D3 by 6 h. Loss of Rb phosphorylation as measured by phospho-specific antibodies, reflecting loss in cdk2 and cdk4 activity, was demonstrated by 9 h (Fig. 4B). Partial loss of cyclin D1 was also observed by 12 h, but cyclin E levels were not affected (15), which served as a protein loading control. Thus, the most prominent biochemical effects of UCN-01 in HN12 cells, namely induction of p21^{WAF1}, p27^{KIP1}, and depletion in cyclin D3, precede cell cycle arrest observed in these cells. Similar results were obtained in HaCaT and HN30 cells (data not shown).

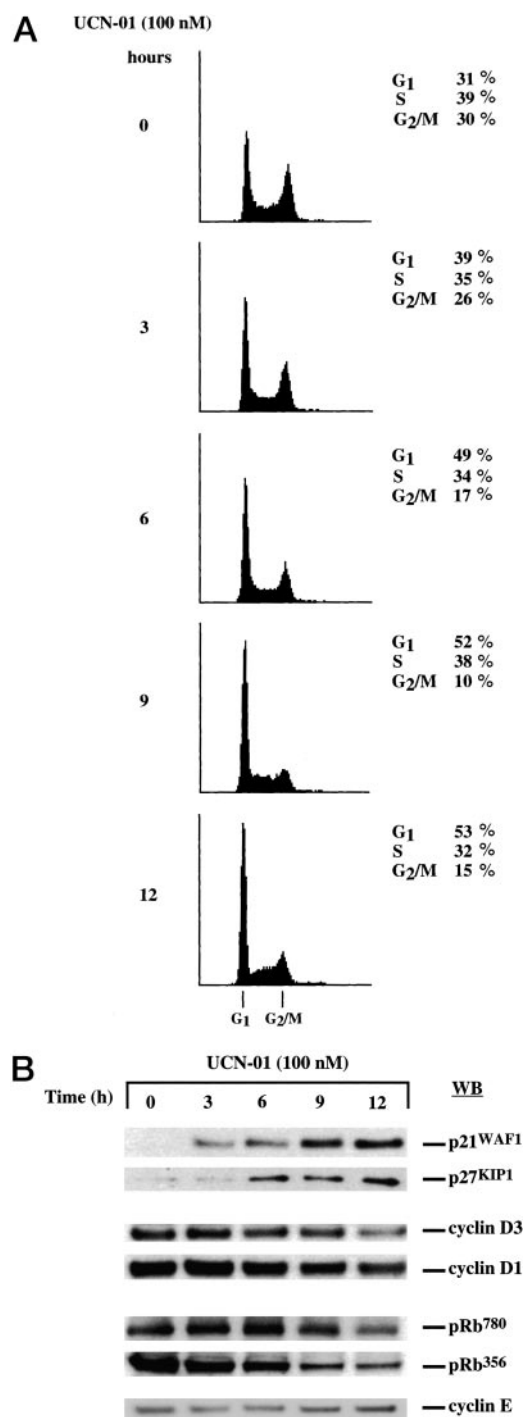


Fig. 4 Cell cycle kinetics of UCN-01-treated HN12 cells. Exponentially growing HN12 cells were exposed to 100 nM UCN-01 for the indicated times, harvested, fixed, and DNA stained with propidium iodide. DNA content of the cells was obtained by flow cytometry, and representative histograms for the indicated times are shown with values for each phase of the cell cycle (A). Parallel samples were obtained, and 25 μ g of total cell lysates were resolved in polyacrylamide-SDS gels, and the indicated proteins were analyzed by Western blot using appropriate antibodies as described in "Materials and Methods" and detected by enhanced chemiluminescence (B). This figure is a representative of three similar independent experiments. Levels of cyclin E are indicated and served as a protein load control.

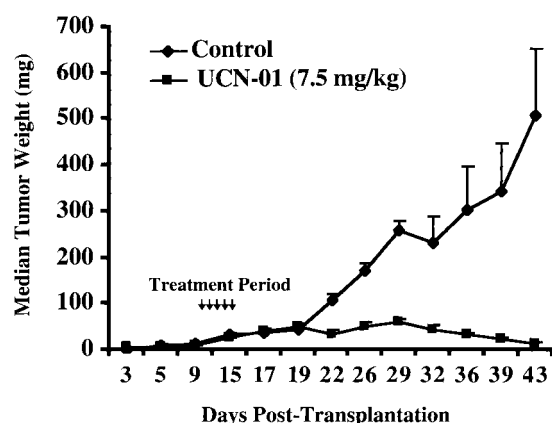


Fig. 5 Reduced growth of HNSCC xenografts by UCN-01. HN12 cells were used to establish xenografts in athymic *nu/nu* mice, and tumor-bearing animals were treated on day 10 i.p. for 5 consecutive days (i.p./qd $\times$ 5) with either UCN-01 (7.5 mg/kg; $n = 10$) or equivalent volume of 2% sodium citrate (pH 3.5; control; $n = 10$). The treatment period is indicated (days 10–15). Tumor size in both groups was assessed twice weekly, and tumor weight was calculated as described in “Materials and Methods.” The results are expressed as mean tumor weight (mg) $\pm$ SE. Student’s *t* test was used to determine the difference between the treated control group ($P < 0.00001$). Data are from a representative independent experiment that was repeated three times with similar results.

UCN-01 Exhibits Potent Antitumor Properties against HNSCC Tumor Xenografts. As HNSCC cells were sensitive to the antiproliferative effects of UCN-01, we wanted to next determine whether UCN-01 possessed antitumor activity *in vivo* in HNSCC models. To address this issue, we established xenografts with HN12 cells in athymic *nu/nu* mice as described previously (35). Animals were then treated with either UCN-01 (7.5 mg/kg/day) or vehicle [2% sodium citrate (pH 3.5)] i.p. for 5 consecutive days, and tumor growth was monitored for up to 43 days after implantation. As illustrated in Fig. 5, the antitumor properties of UCN-01 became apparent on day 22 after implantation, and this effect was sustained over the course of the study. At the end of the study (day 43), whereas vehicle-treated tumors weighed 508 ± 147 mg, a $>4000\%$ increase over day 9 (initiation of treatment), the UCN-01-treated group demonstrated minimal growth over the same period (63% over day 9), with a tumor weight of 16 ± 2 mg on day 43. Student *t* test analyses demonstrated a very significant statistical difference ($P < 0.00001$) by day 29, and this difference was sustained for the remainder of the study. Drug toxicity, as assessed by weight loss, was minimal in the UCN-01-treated group (reduction $<10\%$) during the treatment period. Those animals that were affected (3 of 10) made full weight recovery within 72 h on completion of treatment (day 8). Thus, the administration of UCN-01 at tolerable doses caused a significant antitumor effect in HN12 xenografts after only one cycle of UCN-01.

Assessment for Apoptosis and Cell Cycle-related Proteins in UCN-01-treated HN12 Xenografts. To investigate the mechanism(s) whereby UCN-01 may be inducing this antitumor effect in HN12 xenografts and to identify surrogate markers for drug efficacy, several apoptotic and cell cycle markers were assessed in control and treated tissue samples. We

first examined if the antitumor activity of UCN-01 was attributable to apoptosis (as shown in Fig. 2) and, for this, we carried out TUNEL staining on frozen xenograft tissue sections excised from control and treated groups at the end of the treatment period (day 5 posttreatment). As shown in Fig. 6A, tissue sections excised from animals on completion of the UCN-01 treatment demonstrated an increase in apoptotic cells, as manifested by positively labeled TUNEL staining, compared with corresponding vehicle control tissues. To extend these results to other HNSCC cell lines and to verify that the observed accumulation of sub-G₁ DNA content (Fig. 2) in these cells is the result of apoptosis, we used the TUNEL technique to label fragmented DNA in HN12, HN30, and HaCaT exposed to 300 nM UCN-01 for 24 h and staining visualized by confocal microscopy. Clear evidence of apoptosis was observed in all cell lines (data not shown). Furthermore, in parallel samples, the apoptosis observed in HNSCC was additionally verified by DNA gel fragmentation assays (data not shown). These data indicate that UCN-01 *in vitro* and *in vivo* has the capacity to induce apoptosis, even in HN30 cells, which were previously shown to be insensitive to other known therapeutic agents (35).

To assess the effects of UCN-01 on cell cycle components on these xenografts, parallel immunohistochemical staining demonstrated an increase in protein expression levels of p27^{KIP1} in tumor cells after 5 days of treatment with UCN-01 when compared with similar cells in vehicle control samples (Fig. 6B). Of note, in the control group, the only positive cells for p27^{KIP1} were the endothelial cells (indicated by arrows in Fig. 6B), as expected, which served as an internal staining control. However, the majority of the tumor cells in this group are negative for p27^{KIP1}. Thus, the induction of p27^{KIP1} in response to UCN-01 treatment was restricted to tumor cells. Under these experimental conditions, only a minimal increase in p21^{WAF1} protein levels was detected in the treated tissue samples when compared with control (Fig. 6B). In contrast, cyclin D3 protein levels were observed to be significantly reduced in the xenografts treated with UCN-01 when compared with control (Fig. 6B). Collectively, these findings suggest that the observed antitumor effect of UCN-01 in HN12 xenografts may be because of the activation of cellular apoptotic processes together with the altered expression of G₁-S transition proteins, which may lead to cell cycle arrest and apoptosis. Furthermore, these observations suggest that the expression levels of p27^{KIP1} and cyclin D3 may represent attractive surrogate markers for the biochemical activity of UCN-01 in tumors from patients treated with UCN-01.

DISCUSSION

The poor prognosis characterizing squamous lesions of the head and neck may be attributable, in part, to the overall complexity of the genetic alterations that characterize this disease (1), thus compromising the task of choosing a curative treatment strategy. In this regard, altered and deregulated cdk activity is now believed to play a major role in the pathogenesis of many neoplasias, including HNSCC, thus providing a suitable cellular target for therapeutic intervention (33, 44). These observations and the lack of effective treatment strategies in advanced HNSCC prompted us to evaluate the use of UCN-01 in HNSCC *in vitro* and *in vivo* xenograft models, with the aim

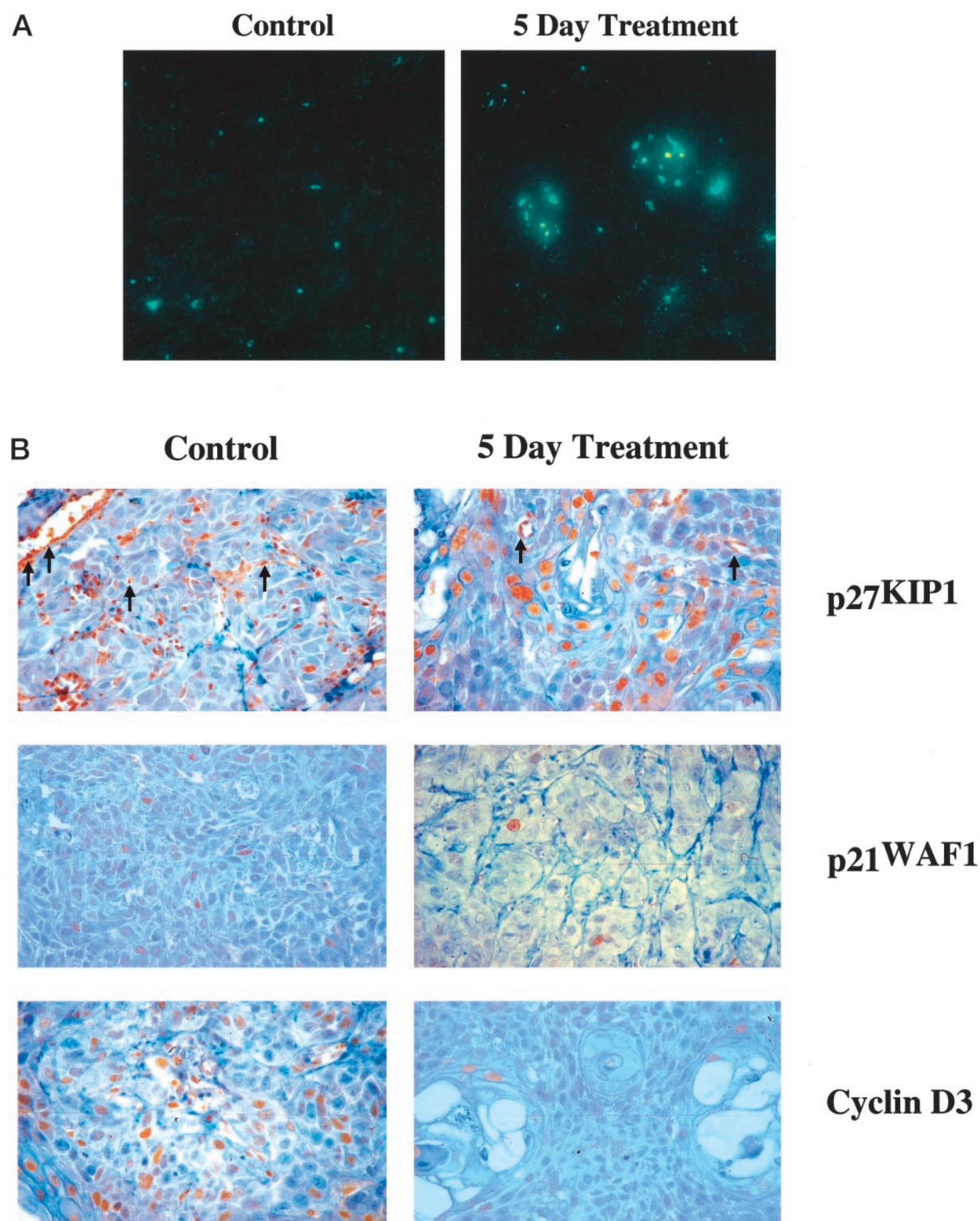


Fig. 6 *In vivo* assessment of apoptosis and differentially expressed cell cycle-related proteins in UCN-01-treated xenografts. HN12 tumor xenografts from control and UCN-01-treated animals (7.5 mg/kg i.p. for five consecutive days) were obtained at the end of the treatment period (day 15), and frozen tissue sections were labeled for DNA strand breaks using the TUNEL assay as described in "Materials and Methods" and analyzed using fluorescent light. Sections are shown at $\times 200$ magnification (A). Similarly obtained tissues were processed for paraffin embedding, and tissue sections were analyzed for the relative levels of p27^{KIP1}, p21^{WAF1}, and cyclin D3, using appropriate antibodies (B). Endothelial cells, including blood vessels, stained positively for p27^{KIP1} in both UCN-01 and control-treated animals are indicated (arrows). All sections are shown at $\times 400$ magnification.

of assessing the potential clinical benefit of this agent for the treatment of HNSCC patients.

In this study, we found that HNSCC cell lines were sensitive to the antiproliferative properties of UCN-01, with an average IC_{50} concentration of 49 nM, which is very similar to that estimated for the NCI 60 cancer cell line panel (37 nM) and to those reported elsewhere (14, 42, 45). Furthermore, growth inhibition correlated with the ability of UCN-01 to cause cells to arrest at the G_1 -S phase of the cell cycle. This likely results from the reduction of G_1 cdk activity (cdk2, cdk4) by the substantial increase in endogenous cdk inhibitors, p21^{WAF1} and p27^{KIP1}, together with their increased association with cdks, as demonstrated here and elsewhere (15, 43), and reported for the first time by the reduced expression of cyclin D3. Furthermore, the ability of UCN-01 to block cell cycle progression in G_1 -S correlated strongly with the accumulation of unphosphorylated pRb at threonine 356 and serine 780, targets for cdk2, cdk4, and cdk6, respectively (36), and indicating a functional pRb/ G_1 checkpoint in these cells. Thus, loss of pRb phosphorylation reflected the inhibitory effect of UCN-01 on cdks and may represent an important molecular determinant for the response of this compound. In line with this observation, recent work suggests that tumor cell lines null for pRb are less sensitive to effective levels of UCN-01 (20, 46). However, loss of pRb expression in HNSCC progression is a rare occurrence and thus reflected in the lack of appropriate model cell lines null for Rb, but alterations in the Rb pathway (i.e., up-regulation in cdk6, cyclin D1, and loss in p15^{INK4B}/p16^{INK4A}), a frequent occurrence in HNSCC (23), shows that the use of UCN-01 for treating this cancer type may be a reasonable approach. Thus, these findings suggest that this drug may be targeting early G_1 processes. However, the exact mechanism by which UCN-01 leads to pRb hypophosphorylation is still unclear. Furthermore, the requirements of pRb for the G_1 -S arrest by UCN-01 needs to be explored in appropriate models.

The effects of UCN-01 on G_1 progression have been documented (15, 20, 42, 43) and include the ability to inhibit cdk activity (14) and the induction of expression p21^{WAF1} and p27^{KIP1}, as observed in this report and others (15, 20, 43). Although UCN-01 can directly target cdk *in vitro*, this effect occurs at concentrations much higher (> 600 nM) than the ones necessary for loss in pRb phosphorylation and cell cycle arrest (30–100 nM), as observed previously (14). In contrast, the induction of both endogenous cdk inhibitors p21^{WAF1} and p27^{KIP1}, and their association with cdks, occurs at the same low nM concentrations and also occurs either before or at the onset of loss in cdk activity/Rb dephosphorylation as observed in Fig. 4. Of interest, this ability of UCN-01 to promote p21^{WAF1}/p27^{KIP1} expression is independent of the functional status of p53 because this tumor suppressor gene is mutated in HN12 and HaCaT but normal in HN30 (see below). On the other hand, a recent study (47) has shown that UCN-01 may stimulate the IFN regulatory factor 1, which in turn up-regulates p21^{WAF1} in lung cancer cells. Whether this is also the case in HNSCC cells is under investigation. Of note, the up-regulation of p21^{WAF1} and p27^{KIP1} in HNSCC cells exposed to UCN-01 was accompanied by depletion of cyclin D3, an important cofactor of G_1 cdks (48, 49). Thus, although the precise mechanism by which UCN-01 regulates the expression of p21^{WAF1}/p27^{KIP1} and cyclin D3 is

still unknown, these observations may explain collectively the loss in cdk activity and Rb dephosphorylation observed at low nM concentrations of UCN-01.

Intriguingly, prolonged treatment (20–24 h) of cells with UCN-01 resulted in the accumulation of cells with a sub- G_1 content of DNA, and complementary approaches demonstrated that treated cells undergo programmed cell death. The mechanism whereby UCN-01 may be inducing apoptosis in HNSCC cells is unclear but in other model systems, e.g., B-cell chronic lymphocytic leukemia, apoptosis induced by this drug may be related to loss of certain antiapoptotic proteins such as XIAP, Mcl-1, and BAG-1 (50). Nonetheless, this effect is very likely to be independent of p53 function, a key molecular determinant of chemoresistance or chemosensitivity in primary tumors. The p53 status of cell lines used in this study has been documented and includes those that expressed wild-type p53 (HN30) or various altered forms of this tumor suppressor gene (HN12 and HaCaT; Refs. 51, 52). In this regard, the sensitivity of HN30 cells to UCN-01 was of particular interest. We have previously reported that these cells are insensitive to apoptosis provoked by some of the available cancer therapies, e.g., γ -irradiation and bleomycin (34). Similar effects were also observed in MCF-7 cell lines harboring no endogenous p53 because of ectopic expression of the human papillomavirus type-16 E6 protein and demonstrated enhanced cytotoxicity with the combination between DNA-damaging agents, such as cisplatin and UCN-01, compared with the isogenic wild-type MCF-7 cell line (53). Thus, UCN-01 may be considered a suitable alternative for use for enhancing the effectiveness of anticancer agents, particularly when treating p53-defective tumors (13, 54).

We have recently completed the first Phase I clinical trial of UCN-01 in humans (6), and a very surprising finding resulting from this study was the remarkably long half-life of the drug in patients. This effect was subsequently determined to be because of the avid binding to α -1-acid glycoprotein (6, 55, 56), but despite this unfavorable feature of this drug in humans, unbound-free drug ($\sim$ 110 nM) was readily detected in patients at dose levels easily achieved in humans (6). In this study, antiproliferative effects in HNSCC were obtained at concentrations $\leq$ 100 nM. Thus, the concentrations applied in this study are applicable to what could be obtained *in vivo*.

The exact target(s) for the antiproliferative properties of UCN-01 is still unknown (13). Although UCN-01 inhibits PKC, particularly Ca^{2+} dependent, it is clear that PKC modulation by UCN-01 is not required for the antiproliferative effects as demonstrated in several models (14). To determine whether PKC may be an important target for UCN-01 effects in HNSCC, we determined the expression of a phosphorylated form of α -adducin, a cytoskeletal protein phosphorylated by PKC (40). No loss in phospho- α -adducin was observed at concentrations $\leq$ 300 nM, concluding that PKC is not a likely target for UCN-01 effects in HNSCC. Recently, several groups identified chk1, a protein kinase responsible for the phosphorylation/inactivation of cdc25C, as a target of UCN-01 for the G_2 checkpoint abrogation upon DNA damage (41). As demonstrated in Fig. 3D, HNSCC exposed to UCN-01 demonstrated a loss in serine 216 cdc25C phosphorylation at concentrations $\geq$ 100 nM and a decrease in the total mass of this molecule with a shift in the ratio of the two forms. However, it is unclear how chk1 and/or

cdc25C modulates the p53-independent G₁-S transition in HNSCC. Additional investigation as to the exact target(s) for UCN-01 G₁-S arrest is warranted.

Although UCN-01 has known antitumor properties in several epithelial and hematopoietic xenograft models (11–13), this has not been explored in HNSCCs. On the basis of our encouraging *in vitro* results, we determined the antitumor effects of UCN-01 in the HN12 xenograft model. A very significant antitumor ($P < 0.00001$) effect was observed after only one cycle of UCN-01 (7.5 mg/kg/i.p./qd $\times$ 5) showed a highly significant inhibition of tumor growth that was sustained over the course of the study (43 days). Positive TUNEL staining of tumor tissues after treatment (5 days) suggests that UCN-01 may be acting on cellular intrinsic apoptotic pathways both *in vivo* as well as *in vitro* models of HNSCC. This effect may be selective because staining for apoptotic cells in UCN-01-treated animals of nontumor tissues (spleen, liver, colon) demonstrated comparable intensity to that observed in control animals (data not shown). These findings may have important clinical implications because it is now recognized that one of the most desired outcomes of cytotoxic chemotherapy is the selective induction of apoptosis in cancer cells.

A very important problem in anticancer drug development is the appropriate assessment of pharmacodynamic effects with novel agents, particularly with those drugs that are cytostatic (45). It is also desirable that those pharmacodynamic markers chosen may have predictive value in the response and/or toxicity for these novel agents. In this study, treatment of HNSCC cells with UCN-01 resulted in significant changes in the levels of key cell cycle related proteins (p27^{KIP1} and cyclin D3) both *in vitro* and *in vivo*. In contrast, levels of p21^{WAF1} that were detected *in vitro* did not correlate with those observed *in vivo*, suggesting differences in the regulation of this protein between the two cellular settings (57). Thus, p27^{KIP1} and cyclin D3 may represent potential surrogate markers to study prospectively in clinical trials with this agent.

In summary, the results presented in this report suggests that UCN-01 displays potent antitumor properties in preclinical models of HNSCC and may represent an attractive candidate to use alone or in combination as a treatment modality for this cancer type. Furthermore, determination of p27^{KIP1} and/or cyclin D3 protein levels in tumor or surrogate tissues may help in demonstrating the effect of this agent in patients receiving UCN-01. Together, these data provides the basis for the early assessment of UCN-01 in those patients with refractory HNSCCs.

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Note Added in Proof

PDK1 has recently been demonstrated to be a very sensitive target of UCN-01 [S. Sato *et al.*, Interference with PDK1-Akt survival signaling pathway by UCN-01 (7-hydroxystaurosporine), *Oncogene*, 21: 1727–1738, 2002]. Therefore, the contribution to inhibition of PDK1 to the effects on p21^{WAF1} and p27^{KIP1} will need to be considered in future studies but does

not alter the important capacity of UCN-01 to ultimately modulate several CDK-related targets as demonstrated here.

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