

UCN-01 induces cytotoxicity toward human CLL cells through a p53-independent mechanism

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Objectives. UCN-01, a novel protein kinase C inhibitor, is currently being tested in phase I clinical trials after being noted to induce apoptosis in lymphoid cell lines. We sought to study the *in vitro* activity of UCN-01 against human chronic lymphocytic leukemia (CLL) cells and potential mechanisms of action for inducing this cytotoxicity.

Methods. Detailed *in vitro* studies were performed from tumor cells derived from patients with CLL cells following attainment of written informed consent.

Results. The 50% loss of viability (LC_{50}) in mononuclear cells from CLL patients ($n = 10$) following exposure to UCN-01 for 4 days was $0.4 \mu\text{M}$ (95% CI ± 0.21 ; range 0.09–1.16). Loss of viability in human CLL cells correlated with early induction of apoptosis. Exposure of CLL cells to 0.4 and $5.0 \mu\text{M}$ of UCN-01 resulted in decreased expression of p53 protein. We therefore investigated the dependence of UCN-01 on intact p53 by exposing splenocytes from wild-type (p53^{+/+}) and p53 null (p53^{-/-}) mice, which demonstrated no preferential cytotoxicity when compared to the marked differential induced by F-Ara-A and radiation.

Conclusion. UCN-01 has significant *in vitro* activity against human CLL cells that appears to occur independent of p53 status. While demonstration of *in vitro* cytotoxicity does not establish *in vivo* efficacy, the findings described support the early introduction of UCN-01 into clinical trials for patients with B-CLL. © 2001 International Society for Experimental Hematology. Published by Elsevier Science Inc.

B-cell chronic lymphocytic leukemia (CLL) is the most common leukemia in the western hemisphere, with approximately 10,000 new cases diagnosed each year [1]. The overall prognosis relative to other forms of leukemia in the absence of therapy is good, with even the most advanced-stage CLL patients having a median survival of three years [2]. However, unlike most other forms of acute and chronic leukemia, substantial therapeutic progress has not been made over the past 40 years in either prolongation of survival or the introduction of curative therapy. Fludarabine as com-

pared to alkylator-based regimens has led to a higher rate of complete responses (27% vs 3%) and duration of progression-free survival (33 vs 17 months) [3]. Despite this advance, the majority of CLL patients still fail to attain a complete remission with fludarabine. Furthermore, all patients with CLL treated with fludarabine eventually relapse, making its role as a single agent purely palliative. Identification of new therapies that abrogate molecular features associated with drug resistance in CLL will be necessary if further progress is to be made in the treatment of this disease.

There are many reasons for drug resistance in CLL, including the cellular overexpression of *bcl-2*, increased *bcl-2*:*bax* ratio, 17p13 deletions (p53 locus), and cytokine deregulation (interleukin-4 (IL-4) or basic fibroblast growth factor) [4–12]. Of these, the most extensively studied and uniformly predictive factor for poor response to therapy and inferior survival in CLL patients is aberrant p53 function as

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characterized by point mutations or chromosome 17p13 deletions [4–7]. Indeed, virtually no responses to either alkylator or purine analog therapy have been documented in multiple single-institution case series for those CLL patients with abnormal p53 function [4–7]. Introduction of a therapeutic agent with the ability to overcome the drug resistance associated with p53 mutation in CLL would be a major advance for the treatment of this disease.

Herein we describe UCN-01, a staurosporine analog currently under evaluation in phase I clinical trials, which demonstrates marked *in vitro* cytotoxicity toward human CLL cells through a p53-independent pathway.

Methods

Patients, cell separation, and culture conditions

Written, informed consent was obtained to procure cells from patients with previously diagnosed B-cell chronic lymphocytic leukemia as defined by the modified NCI criteria [13]. Mononuclear cells were isolated from the peripheral blood utilizing density gradient centrifugation (Ficoll-Paque Plus, Pharmacia Biotech, Piscataway, NJ, USA). Cells were immediately cultured (1×10^7 cells/mL) in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin-G, 100 μ g/mL streptomycin, and 2 mM L-glutamine (Life Technologies, Grand Island, NY, USA) and incubated at 37°C in a 5% CO₂ incubator.

Treatment of mice and harvested cells

Homozygous typed p53-deficient mice (C57BL/GJ-Trp53^{tm1Tyj}; p53^{-/-}) (The Jackson Laboratory, Bar Harbor, ME, USA) and wild-type mice (C57BL/6NCR; p53^{+/+}) mice (Frederick Cancer Research and Development Center, Frederick, MD, USA) were obtained. Cells from both spleens and thymus glands were harvested from the mice and were immediately cultured (5×10^6 cells/mL) using conditions similar to those described above. The genotypes of the mice were verified by 1) amplification of the p53 exon 6/intron 6/exon 7 fragment in the p53 wild-type mouse and the lack of product in the p53 knockout mouse; and 2) amplification of a neo/p53 exon 7 hybrid product in the p53 knockout mouse, and the lack of hybrid product in the p53 wild-type mouse as previously described [14,15].

UCN-01 and F-ara-a (active metabolite of fludarabine) was obtained from the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute.

Viability assays

Viability assays of isolated mononuclear cells from CLL patients were performed utilizing the MTT assay as previously described by our group [14]. Briefly, cells (1×10^6 per well) were placed in a 96-well flat-bottom plate and the test drug or medium alone was added. All human experiments were performed in quadruplicate. Cells were incubated for 96 hours, incubated with MTT (Sigma Chemical Company, St. Louis, MO, USA) for 24 hours, washed, and protamine sulfate (Sigma Chemical Company, St. Louis, MO, USA) added. Plates were then allowed to air dry. The precipitated MTT formazan was solubilized with dimethylsulfoxide (DMSO) and the optical density at 540 nm was obtained utilizing an Anthos Reader 2001 (Anthos Labtec Inc., Frederick, MD, USA) with a Bio-

lise-Windows program. Cell viability was expressed as the absorption ratio of drugged cells to control sample.

Viability assays on isolated cells from the p53^{-/-} and p53^{+/+} mice were performed utilizing the trypan blue exclusion assay. All experiments were performed in duplicate. Cells were counted with a hemocytometer and viability was expressed as % alive = alive/(alive + dead) \times 100. The relative effects of UCN-01 and F-ara-a after 24 hours of exposure were expressed as the viability with drug/viability in medium.

Isolation of CLL cells was performed by incubating cells overnight and then removing nonadherent cells. These were then removed, washed, and resuspended in MACS Buffer (phosphate-buffered saline (PBS) with 0.5% bovine serum albumin and 2 mM EDTA). Separation using MACS CD3 microbeads and depletion columns (Miltenyi Biotec, Auburn, CA, USA), as directed by the manufacturer, was performed. The effluent cells were 99% CD3⁻, as assessed by flow cytometry utilizing FITC-conjugated CD3 antibody (Becton-Dickinson, Franklin Lakes, NJ, USA) and the appropriate isotype-negative control.

Apoptosis assays

Following 24 and 96 hours of incubation with 0.4 and 5.0 μ M UCN-01 in supplemented RPMI and 10% FBS, apoptosis studies were performed utilizing the TdT/PI method and Annexin/PI method as previously outlined [14].

Protein extraction and Western blot analysis

The *bcl-2*, *bax*, and p53 protein expression were analyzed by Western blot after incubation in either medium or two concentrations of UCN-01 (0.4 μ M and 5.0 μ M) for 4, 24, and 48 hours. Whole-cell lysates were prepared by pelleting 1.25×10^8 PBS-washed mononuclear cells in a microcentrifuge, aspirating the supernatant, and adding 0.5 mL of cold lysis buffer as described previously [14]. This cell suspension was incubated at 4°C for 40 minutes with constant agitation, then centrifuged for 15 minutes at 14,000 rpm at 4°C. The supernatant was recovered, aliquoted, and frozen at -80°C.

Protein was quantified in each supernatant by the BCA method (Pierce, Rockford, IL, USA). Varied amounts (2–100 μ g) of each sample were utilized for each protein studied based upon varying expression of protein in human CLL cells. Once identified, a single loading concentration was examined for each protein. Rainbow-colored protein molecular weight markers (Amersham Life Science, Arlington Heights, IL, USA) and samples were loaded onto 10–14% polyacrylamide gels and electrophoresced. The proteins were transferred to a 0.45 μ m nitrocellulose membrane (Schleicher and Schuell, Keene, NH, USA) using an electroblot apparatus (Hoefer, San Francisco, CA, USA). After transfer of the proteins, the nitrocellulose membranes were blocked for 1 hour in TBS-T (Tris Buffered Saline–0.05% Tween) (JT Baker, Phillipsburg, NJ, USA) containing 5% skim milk. The membranes were incubated with either 1 μ g/mL of monoclonal mouse anti-human *bcl-2* antibody clone 124 (Dako, Carpinteria, CA, USA), 2 μ g/mL polyclonal rabbit anti-human *bax* (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or 0.1 μ g/mL of monoclonal p53 (Ab6) (Oncogene Research Products, Cambridge, MA, USA) diluted in TBS-T with 5% skim milk. The blots were incubated with horseradish peroxidase-conjugated anti-mouse IgG (H and L chains) (Pierce, Rockford, IL, USA), horseradish peroxidase-conjugated antigoat IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or horseradish peroxidase-conjugated anti-rabbit IgG (Caltag Lab-

oratories, Burlingame, CA, USA) diluted 1:5000 (goat) or 1:2000 (rabbit) with 5% skim milk in PBS and revealed with chemiluminescent substrate (Pierce Super-Signal chemiluminescent, Pierce, Rockford, IL, USA) for 1.5 minutes. Autoradiography was performed with X-ray film (Kodak, Rochester, NY, USA). Gel loading equivalence was confirmed either by reprobing with 1 $\mu\text{g}/\text{mL}$ polyclonal goat anti-human Actin (I-19) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or by exposing the nitrocellulose gel to Fast Green Stain to reveal total protein banding pattern. Protein bands were quantified by computer densitometry.

Statistics

Groups of data were compared using paired or nonpaired Student's *t*-tests (two-sided) as appropriate. Nonparametric data were analyzed utilizing the Wilcoxon signed-rank test. JMP Statistics software (SAS institute, Trumbull, CT, USA) or Quatropro software (Novell Inc., Orem, UT, USA) were utilized to perform these analyses.

Results

UCN-01 is cytotoxic toward CLL cells

Peripheral mononuclear cells from 10 CLL patients were exposed to varying concentrations of UCN-01 (0.01, 0.033, 0.1, 0.33, 1, 3.3, 10, 33, 100 μM) for 4 days. All of the patients with CLL demonstrated in vitro response to UCN-01 with a mean dosage of 0.4 μM (95% CI \pm 0.21; range 0.09–1.16) required to produce 50% cytotoxicity at 4 days. Subsequent annexin-V/PI and TdT analysis demonstrated that loss of viability occurred through induction of apoptosis (data not shown).

To assure that the cytotoxicity observed in CLL cells was not secondary to UCN-01 altering viability of normal T cells and monocytes in the mononuclear isolate, we performed parallel experiments on four patients, comparing the LC₅₀ of monocyte- and CD3-depleted CLL cells to CLL cells that were not depleted. Concentrations of UCN-01 (0–100 μM) and control F-ara-a (0–100 μM) were tested for 4 days as described above. The average LC₅₀ for CD3- and monocyte-depleted CLL cells vs nondepleted CLL cells was similar for both UCN-01 (0.22 vs 0.33 μM , *p* = 0.4) and F-ara-a (1.19 vs 1.35 μM , *p* = 0.8). The similar loss of viability between these parallel experiments suggests that UCN-01 and F-ara-a both induce cytotoxicity by direct effect on CLL cells, as opposed to accessory T cells and monocytes.

Interleukin-4 induces

resistance to UCN-01 in human CLL cells

In vitro incubation of B-CLL cells with IL-4 has been demonstrated to induce drug resistance to chlorambucil, fludarabine, and prednisone. We sought to determine if UCN-01 was also affected by IL-4 incubation. Cells from an additional six patients with B-CLL were exposed to varying concentrations (0.01–100 μM) of UCN-01 \pm 10 ng/mL of IL-4. Incubation with IL-4 for 4 days increased the LC₅₀ of UCN-01 significantly (*p* = 0.03) in all of the six CLL cell samples tested from a mean of 0.85 μM (range 0.04–1.68;

95% ci \pm 0.55) to 3.13 μM (range 0.64–6.24; 95% ci \pm 2.03). These data suggest that IL-4 incubation increases CLL cell in vitro drug resistance to UCN-01.

UCN-01 exposure does not

alter *bcl-2* and *bax* protein expression

The *bcl-2* protein is overexpressed in the majority of CLL patients and is associated with in vitro drug resistance. To assess if UCN-01 may be favorably altering expression of this apoptosis-related protein, we incubated mononuclear cells from CLL (*n* = 5) patients with UCN-01 (5 μM) or media with subsequent assessment of *bcl-2* expression at 1 hour, 4 hours, and 24 hours. Figure 1 demonstrates the lack of change in *bcl-2* protein expression with UCN-01 exposure as compared to media. This was observed in all five patients. Similarly, no alteration was noted in *bax* protein expression in three CLL patients (data not shown).

UCN-01 causes a decrease in *p53* protein expression

The normal CLL cellular response to DNA damage induced by alkylator and/or purine analog exposure is an increase of *p53* protein expression. To determine if UCN-01 was inducing apoptosis via a similar pathway, we incubated mononuclear CLL cells from three patients with 5.0 μM of UCN-01 or medium with subsequent assessment of *p53* protein expression at 1, 4, and 24 hours. Figure 2 depicts a representative Western blot demonstrating a decrease in *p53* protein expression with UCN-01 exposure at 24 hours as compared to medium-only control samples.

UCN-01 induces apoptosis independent of *p53* status

The observation that *p53* protein expression was actually decreased in response to UCN-01 exposure suggested that this agent might be operating in a unique, *p53*-independent fashion. To test this hypothesis, we explored whether the

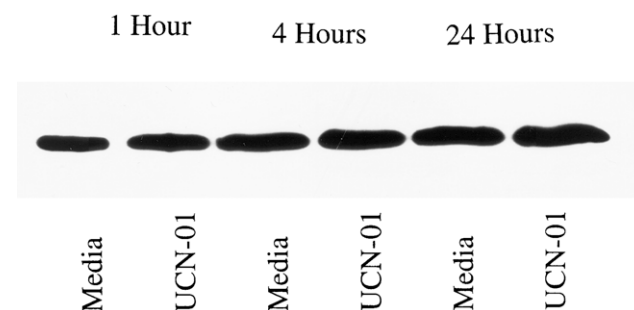


Figure 1. Expression of *bcl-2* protein in human CLL cells at 1, 4, and 24 hours following incubation with medium or 5.0 μM of UCN-01. The cells were obtained from CLL patients following informed consent, isolated, and cultured at $5 \times 10^6/\text{mL}$ in medium or the above stated concentrations of UCN-01. Cell lysates were prepared and protein concentration was quantified utilizing the BCA method (Pierce). Two micrograms of protein/lane from the CLL cell lysates was loaded onto a 10% SDS-PAGE gel and electrophoresced. *bcl-2* protein was detected utilizing an anti-*bcl-2* monoclonal antibody (Dako). Lane-equivalent loading was certified by assessment with Fast Green Staining (not shown).

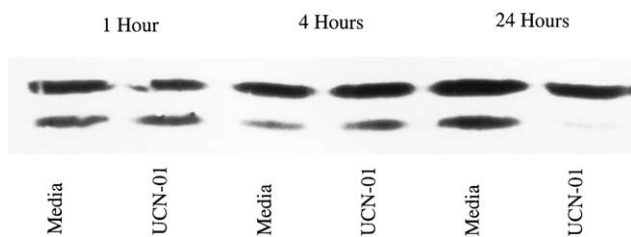


Figure 2. Expression of p53 protein in human CLL cells at 1, 4, and 24 hours following incubation with medium or 5.0 μM of UCN-01. The cells were obtained from CLL patients following informed consent, isolated, and cultured at $5 \times 10^6/\text{ml}$ in medium or the above stated concentrations of UCN-01. Cell lysates were prepared and protein concentration was quantified utilizing the BCA method (Pierce). Twenty-five micrograms of protein/lane from the CLL cell lysates were loaded onto a 10% SDS-PAGE gel and electrophoresced. The p53 protein was detected utilizing an anti-p53 monoclonal antibody (Oncogene). Lane-equivalent loading was certified by assessment with Fast Green Staining (not shown).

observed effects of UCN-01 were dependent upon intact p53. Splenocytes from four wild-type and three typed p53 null-type mice were exposed to varying concentrations of UCN-01 (0.01, 0.1, 0.33, 1 μM). The absolute molecular status of the wild-type and p53 null-type mice was secondarily confirmed by identifying the presence or absence of the p53 gene and neogene as previously described [14,15]. The cytotoxicity as assayed by trypan blue following 24 hours of incubation at each concentration of UCN-01 is summarized in Table 1. Viability of the p53 null (mean 81%; 95% ci \pm 4.3) and wild-type (mean 90%; 95% ci \pm 2) mice splenocytes following 24 hours of incubation in medium was similar. A noticeable decline in splenocyte viability was noted with incremental increases in UCN-01 concentrations without preferential cytotoxicity to p53^{+/+} as compared to the p53^{-/-} splenocytes. This nonpreferential toxicity was also observed in the thymocytes (data not shown). In contrast to this, the viability of the p53^{-/-} splenocytes (89%) was minimally affected by 500 cGy of irradiation as opposed to almost complete loss of viability (22%) in the p53^{+/+} splenocytes. Incubation with fludarabine at varying concentrations yielded a similar result to that observed after irradiation. Following 24 hours of incubation with F-ara-a, the p53^{-/-} splenocytes had a LC₅₀ of >100 μM as opposed to a LC₅₀ of 3.95 μM in p53^{+/+} cells. This observation parallels that observed in the clinic, where CLL patients with p53 mutations rarely respond to fludarabine-based therapy.

UCN-01 chemosensitizes CLL cells to fludarabine

CLL cells from six patients were exposed to varying concentrations of F-ara-a (0.01–100 μM) and a fixed concentration of UCN-01 (10 ηM). Such concentrations of free UCN-01 have been obtained at levels below the maximally tolerated dose in currently ongoing UCN-01 trials [16]. In the previous studied patients outlined, along with the six patients tested for these studies, UCN-01 concentrations of 10

Table 1. Splenocytes from wild-type (p53^{+/+}) and null (p53^{-/-}) murine splenocytes demonstrate similar in vitro sensitivity to UCN-01

Condition	% Viability, p53 ^{+/+} wild-type	% Viability, p53 ^{-/-} null-type
Media	79.2	83.4
0.01 μM UCN-01	84.4	75.4
0.1 μM UCN-01	73.5	72.0
0.33 μM UCN-01	42.4	38.8
1.0 μM UCN-01	29.0	20.1

The cells were obtained from spleens of typed and PCR-confirmed p53^{+/+} and p53^{-/-} mice. These cells were cultured at $1 \times 10^6/\text{mL}$ in media (control) and varying concentrations of UCN-01 for 1 day. The viability was then assessed utilizing the trypan blue exclusion test. Experiments were performed in triplicate.

ηM were not cytotoxic to CLL cells. The mean LC₅₀ of F-ara-a alone in the CLL samples was 1.95 μM (range 0.36–7.82; 95% ci \pm 2.31). Incubation with the 10 ηM concentration of UCN-01 significantly ($p = 0.036$) lowered the LC₅₀ of F-ara-a to 1.47 μM (range 0.21–6.54; 95% ci \pm 1.99). These data demonstrate that the PKC inhibitor UCN-01 chemosensitizes human CLL cells to the cytotoxic effects of F-ara-a.

Discussion

These data demonstrate that UCN-01 induces in vitro cytotoxicity and apoptosis in human CLL cells. UCN-01 does not alter *bcl-2* or *bax* protein expression but does cause p53 protein to decrease in contrast to observations with p53-dependent agents such as fludarabine and chlorambucil [17]. These results prompted us to test the hypothesis that UCN-01 induces apoptosis independent of p53 gene status, a finding that has previously been documented only in cell lines [18–20]. Utilizing p53 knockout mice and sex-matched control wild-type mice, we demonstrated that UCN-01 as a single agent is equally cytotoxic toward p53^{-/-} and p53^{+/+} splenocytes. This contrasts with the effect of either fludarabine or radiation, whose cytotoxic effect was markedly impaired in p53-null as compared to wild-type cells. In sum, these data suggest that UCN-01 has preclinical promise for the treatment of CLL.

The mechanism by which UCN-01 promotes such toxicity in nondividing cells is uncertain. One mechanism of cytotoxicity observed following UCN-01 exposure in rapidly dividing cells is the abrogation of the G₂ cell-cycle checkpoint. Inappropriate activation of CDC2 by a variety of agents including pentoxifylline, caffeine, and related staurosporine results in either a partial or complete loss of the cellular G₂M checkpoint function with activation of Cdc2 kinase. [21–23,9–11]. Abrogation of this checkpoint in a variety of p53-deficient cell lines in combination with radiation or chemotherapy has been noted to produce loss of the G₂M fraction with induction of apoptosis. This mode of cytotoxicity in UCN-01 [23] appears directly related to Cdc2

activation as its effect can be reversed with inactivation of this enzyme by heat in Cdc2 temperature-sensitive FT-210 cell lines. Wang et al. [18] recently identified that UCN-01 also abrogates the G2 checkpoint in a MCF-7 breast cancer cell line with transfected E6 papillomavirus-induced p53 inactivation. These transfected cells when combined with UCN-01 were in fact more sensitive to the effects of radiation and chemotherapy as compared to control MCF-7 cell lines. However, no direct comparisons between UCN-01 cytotoxicity observed in the related cell lines were made. Work by Graves et al. [24] has recently demonstrated that UCN-01 causes dephosphorylation at the serine 216 residue of Cdc25 phosphatase, which increases the activity of this enzyme with subsequent activation of Cdc2 through dephosphorylation of the threonine 14 and tyrosine 15 residue of this second enzyme. Activation of Cdc25 in this study by UCN-01 was demonstrated to be mediated by inhibition of Chk1, but not Cds1 protein kinase. Indeed, Chk1 is responsible for phosphorylation of the serine 215 residue of Cdc25 phosphatase. The clinical relevance of these findings outside of research with transformed cell lines is uncertain, as the majority of human tumors have relatively long tumor doubling times with a small proportion of actively dividing cells at any given time point. Our data also demonstrate that UCN-01 can promote apoptosis in human CLL cells and nonproliferating G₀₋₁ splenocytes in a p53-independent fashion, suggesting that additional mechanisms of cytotoxicity for this agent are probable and remain to be defined. One possibility is the observation by Forbes et al. [25], who noted that activation of protein kinase C in human CLL cells by phorbol esters prevents apoptosis to etoposide and methylprednisolone. The apoptosis protecting effect is prevented by coinubation with H-7, another protein kinase C inhibitor. Furthermore, H7 induced apoptosis in human CLL cells, suggesting that inhibition of this enzyme might be an important therapeutic target for CLL. Work by others with other staurosporine derivatives in vivo have demonstrated preliminary clinical activity [26]. Based on the data presented herein and that previously published, exploration of agents that inhibit protein kinase C in CLL appears warranted.

The introduction of UCN-01 in phase I studies led to the observation of a markedly prolonged terminal half-life relative to that observed in the preclinical animal models [27,16]. Subsequent studies have demonstrated that this prolonged half-life is due to α -1 acid glycoprotein-specific binding, which leads to a small proportion of free UCN-01 in human plasma. A similar prolonged half-life has been noted with other staurosporine analogs currently being explored in the clinic [26]. Noteworthy, however, is that the dose-limiting toxicity of UCN-01 is not myelosuppression [28,29]. This enhances the attractiveness of this agent for study in patients with CLL who often have baseline compromised bone marrow reserve as a consequence of the underlying disease. Current development plans at the National

Cancer Institute and in company-sponsored studies in Japan are additional phase I studies that administer UCN-01 over a short period of time to increase free plasma drug concentrations. Regardless of the eventual clinical viability of UCN-01, the data described herein provide further support for therapies that target protein kinase C in CLL. Additionally, studies to characterize the selectivity of UCN-01 for different normal mononuclear subsets will be required.

In summary, our study demonstrates that UCN-01 has a marked in vitro activity in CLL cells. Furthermore, UCN-01 induces apoptosis independent of p53 status, a known predictor of drug resistance in CLL. While demonstration of in vitro cytotoxicity does not establish in vivo efficacy, these data do provide support for initiating phase II studies of UCN-01 in patients with CLL.

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