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Advances in Brief

In Vitro Sensitivity of T-Cell Lymphoblastic Leukemia to UCN-01 (7-Hydroxystaurosporine) Is Dependent on p16 Protein Status: A Pediatric Oncology Group Study[1]

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Abstract

p16 regulates the cell cycle pathway by inhibiting the cyclin Ds-cyclin-dependent kinase (CDK) 4/6-mediated phosphorylation of retinoblastoma protein (pRb). Previously, we reported that most primary T-cell acute lymphoblastic leukemia (T-ALL) harbored p16 inactivation and hyperphosphorylated pRb without cyclin Ds or CDK4/6 alterations. Therefore, inhibiting CDK4/6 may be an ideal therapeutic approach for p16 (−) T-ALL. UCN-01 (7-hydroxystaurosporine) is a potent antitumor agent that exerts its effects through the inhibition of CDKs. We now report that p16 protein expression status of T-ALL cells influences their sensitivity to UCN-01. In 36 primary T-ALL cells, the IC_{50}s of UCN-01 in the 27 p16 (−) cells (43 ± 52 nM) was significantly lower than that in the 9 p16 (+) cells (258 ± 260 nM). Our results suggest that agents like UCN-01 may be useful as a p16-selective therapy for T-ALL.

Introduction

The p16/p15-cyclin Ds/CDKs[3]-pRb pathway plays a critical role in cell cycle progression (1, 2). Protein complexes of D-type cyclins and CDK4/6 induce the phosphorylation of the pRb and subsequent G_1-S phase transition. The ability of cyclin Ds-CDK4/6 protein complexes to phosphorylate pRb is prevented by CDK inhibitor proteins including p16 (3) and p15 (4). Deregulation of any of these components, such as gene alterations of p16 and p15, amplification/overexpression of CDKs or cyclins, and alteration/deletion of the *pRb* gene, may result in inappropriate G_1-S progression and tumorigenesis. Such alterations of the p16/p15-cyclin Ds/CDKs-pRb pathway have been found frequently in various human tumors (1, 5), suggesting that deregulation of this pathway is involved in the pathogenesis of cancer. Consistent with these findings, in our previous study of 124 primary T-ALL samples, we found that p16 and p15 are inactivated at high frequency through a number of mechanisms including gene deletion, mutation, promoter hypermethylation, or transcriptional and translational inactivation (6). Overall abrogation rates for p16 and p15 were 93% (115 of 124) and 99% (123 of 124), respectively. No alterations were evident in cyclin Ds or CDK4/6, and pRb was hyperphosphorylated in the majority of samples investigated. These findings strongly support that both p16 and p15 are specific targets of cell cycle deregulation in T-ALL and that the inactivation of both genes is most likely essential for the pathogenesis of this disease. Furthermore, these findings suggest that inhibition CDK4/6 may provide an ideal therapeutic approach for p16 (−) T-ALL. In this regard, UCN-01 (7-hydroxystaurosporine) appears to be of particular interest. It has been shown to have anticancer activity *in vitro* and *in vivo* against a broad spectrum of human cancers (7–10). Although UCN-01 was originally characterized as an inhibitor of protein kinase C (11), its anticancer activity is more likely to result from a modulation of the cell cycle rather than the direct effect of protein kinase C inhibition (8). Studies have demonstrated that UCN-01 has an inhibitory effect on CDKs such as CDK2, CDK4, and CDK6. Thus, it prevents phosphorylation of pRb and arrests tumor cells in G_1 (7, 12, 13). Therefore, it is believed that UCN-01 exerts its antitumor effects at least in part through the inhibition of CDK activity. In line with this, pRb status influences the effect of UCN-01 on normal and tumor cells; pRb (−) cells are significantly more resistant than are pRb (+) cells (14, 15). Taken together, the high rate of inactivation of the CDK inhibitor p16, with the sparing of downstream components including CDK/cyclin and pRb in T-ALL, prompted us to speculate that UCN-01 may have therapeutic potential for T-ALL. Furthermore, we hypothesized that p16 (−) T-ALL cells may be more sensitive to UCN-01 than p16 (+) T-ALL cells, in which alterations other than p16 may be involved in the deregulation of cell cycle progression. To test these hypotheses, we investigated the cytotoxicity of UCN-01 against 36 primary T-ALL samples and correlated the results with their p16 protein status. The results demonstrate for the first time the impact of p16 protein status on the effectiveness of UCN-01 in primary human cancer.

Materials and Methods

Patient Population and Isolation of Primary T-ALL Cells. Heparinized bone marrow or peripheral blood samples were obtained from 36 T-ALL patients, 32 of whom were enrolled in Pediatric Oncology Group ALL Biology protocol #9400. Peripheral blood mononuclear cells from two healthy donors were also obtained. Mononuclear cells were isolated from the samples by Ficoll-Hypaque density gradient centrifugation (Pharmacia Fine Chemicals, Piscataway, NJ). The content of lymphoblasts in the samples was generally >80%. Thirty-four samples were obtained from patients at the time of diagnosis, and two were from those at relapse phase. Six of the 36 samples have been examined previously and reported as to their *p16* gene and expression status (6).

DNA and RNA Analysis. Genomic DNA and total RNA were isolated from 20 to 40 × 10^6 T-ALL cells using the Trizol reagent (Life Technologies, Inc., Gaithersburg, MD). The *p16* gene was examined by semiquantitative PCR, PCR-single strand conformational polymorphism, and DNA sequence analysis as described previously (16–18). Total RNA (2 μg) was reverse transcribed into cDNA using the Superscript Preamplification System (Life Technologies, Inc.) for reverse transcription-PCR of *p16* and *p15*. Two μl of

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[3] The abbreviations used are; CDK, cyclin-dependent kinase; pRb, retinoblastoma protein; T-ALL, T-cell acute lymphoblastic leukemia; PHA, phytohemagglutinin.

cDNA were amplified under standard conditions as described previously (19). Amplification of glyceraldehyde-3-phosphate dehydrogenase was performed as a control.

Western Blot Analysis. For Western blot analysis, we used 20–50 μg of protein in cell lysate prepared from 2.5 to 5.0 $\times$ 10^6 cells. Western blot analysis of p16, pRb, and β-actin was performed as described previously (19).

Material. UCN-01 was a generous gift from the Drug Synthesis and Chemistry Branch, Development Therapeutic Program, Division of Cancer Treatment (National Cancer Institute, Bethesda, MD).

Effect of UCN-01 on T-ALL Cell Lines, Primary T-ALL Cells, and Normal T Cells. T-ALL cell lines, CEM and Molt-4, were maintained as described (20). For cell count experiments, 96-well plates were seeded with 1 $\times$ 10^5 exponentially growing cells in 200 μl of 10% FCS supplemented RPMI 1640, exposed to 0, 200, or 500 nM UCN-01, and harvested at 24, 48, and 72 h. Cell numbers were counted by hemocytometer, and the viability was assessed by trypan blue exclusion. Proliferation was also assessed by [^{3}H]thymidine incorporation. T-ALL cell lines, primary T-ALL cells and normal T-cells, were plated at 0.5 $\times$ 10^6 cells/ml into 96-well plates in 200 μl of complete RPMI 1640 containing increasing concentrations of UCN-01 for 3 days. Cells were pulsed for 6 h with 1.6 μCi of [^{3}H]thymidine, and incorporation was determined with the use of an automated microtiter harvester and scintillation counting. Normal T cells were assessed in the presence of 1 μg/ml PHA to stimulate proliferation.

Results

Cytotoxicity of UCN-01 to T-ALL Cell Lines and Primary T-ALL. The effect of UCN-01 was first examined on two p16-deleted T-ALL cell lines, CEM and Molt-4 (data not shown). At 200 nM, the growth of both cell lines was inhibited to ~70% of control by day 3. At 500 nM, there was not only a complete arrest of cell growth but also loss of viable cells in both cell lines. Consistent with this finding, DNA synthesis in CEM and Molt-4, as measured by [^{3}H]thymidine incorporation, showed a concentration-dependent inhibition by UCN-01, with IC_{50} of 560 $\pm$ 57.9 and 420 $\pm$ 51.6 nM, respectively (data not shown). UCN-01 cytotoxicity was next examined in 36 primary T-ALL samples. In representative experiments shown in Fig. 1*A*, incubation with UCN-01 resulted in a dose-dependent inhibition of [^{3}H]thymidine incorporation, yielding an IC_{50} of <150 mM in 30 of 36 primary T-ALL samples (Table 1). These 30 T-ALL displayed an average IC_{50} of 35.7 $\pm$ 29.9 *versus* an average IC_{50} of the 6 resistant T-ALL of 402 $\pm$ 194. In comparison, PHA-activated normal T cells from 2 healthy donors were less sensitive to UCN-01 than most T-ALL with IC_{50}s of 315 $\pm$ 23 and 269 $\pm$ 64 nM, the former of which is shown in Fig. 1*A*. Specifically, at concentrations of UCN-01 <125 nM, which inhibited DNA synthesis in most T-ALL cells by >50%, normal T cells were barely affected.

***In Vitro* Sensitivity of Primary T-ALL to the Cytotoxicity of UCN-01 and Their Correlation with pRb and p16 Status.** Previous studies have demonstrated that pRb status influences the cytotoxicity of UCN-01; pRb (−) cells are significantly more resistant to UCN-01 than pRb (+) cells (14, 15). We therefore examined pRb status by Western blot in 32 of the 36 primary T-ALL samples and correlated the results with their sensitivity to UCN-01. As summarized in Table 1, 25 of the 32 samples expressed pRb protein in the predominantly hyperphosphorylated form (*e.g.*, Fig. 2*B, Lanes 3–5*), 4 displayed only hypophosphorylated pRb, and 3 did not express any detectable pRb protein (*e.g.*, Fig. 2*B, Lane 6*). Despite the reported pRb inactivation/UCN-01 resistance relationship in other cancers, no significant correlation between pRb status and UCN-01 sensitivity was observed in T-ALL. For example, 2 (samples 32564 and 32712) of the 3 samples lacking pRb expression were sensitive to UCN-01 with IC_{50}s of 21 and 57 nM, respectively. Overall, 24 of 28 (83%) pRb-inactivated (hyperphosphorylated and no expression) T-ALL samples were UCN-01 sensitive (<150 nM), which was not significantly different from the 2 of 4 (50%) expressing pRb in the functional, hypophosphorylated form (P = 0.15, Fisher's exact test).

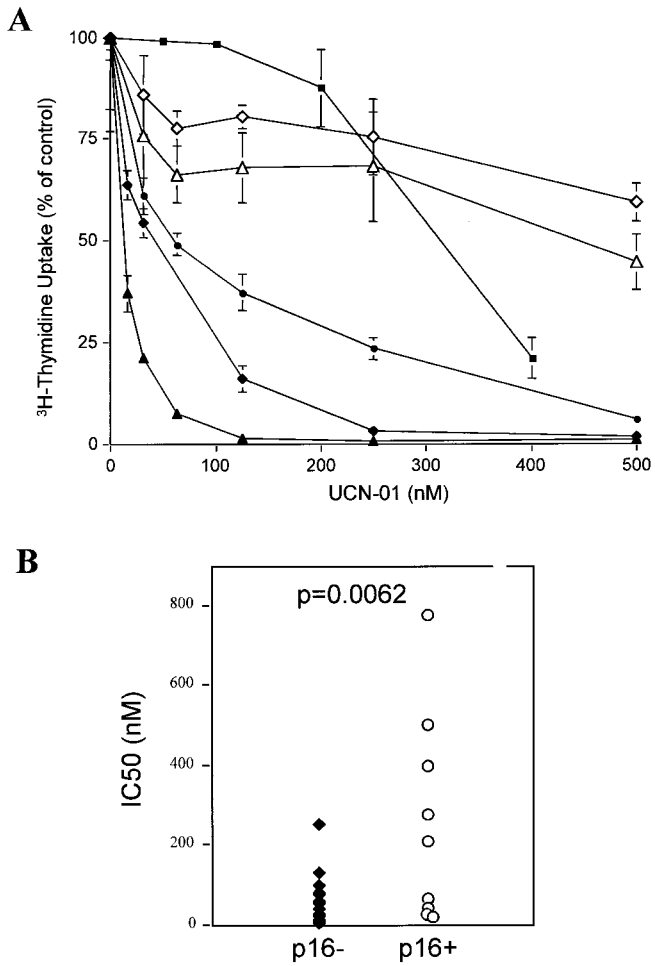

Fig. 1. Toxicity of UCN-01 in primary T-ALL and normal T-cells. *A*, T-ALL cells were treated with increasing concentrations of UCN-01 for 3 days and then pulsed with [^{3}H]thymidine for 6 h. Each experimental condition was performed in triplicate, and the data are reported as the means; *bars*, SD. ▲, ●, and ♦, p16 (−) T-ALL cells (nos. 33174, 31380, and 1009) in which p16 is inactivated at DNA, RNA, and protein levels, respectively. △ and ◇, p16 (+) T-ALL cells (nos. 30612 and 32779); ■, normal T-cells. *B*, comparison of the IC_{50}s of UCN-01 in p16 (−) *versus* p16 (+) primary T-ALL cells. Statistical analysis was performed using the Mann-Whitney test.

We next examined the p16 status of the 36 primary T-ALL samples and correlated their status with sensitivity to UCN-01. Twenty-seven of the 36 primary T-ALL samples showed inactivation of p16 at the DNA, RNA, and protein level (DNA alterations in 21 with 20 homozygous deletions and 1 frameshift mutation, no mRNA expression in 4, no protein expression in 2; Table 1 and *e.g.*, Fig. 2*A, Lanes 7–10*). The remaining 9 expressed wild-type p16 protein (*e.g.*, Fig. 2*A, Lanes 2, 4,* and *5*). When correlated with p16 status, T-ALL cells lacking p16, in general, were more sensitive to UCN-01 than those retaining p16 protein. In representative experiments shown in Fig. 1*A*, treatment with 250 nM UCN-01 resulted in >75% inhibition of [^{3}H]thymidine incorporation in p16 (−) T-ALL cells but <30% inhibition in p16 (+) cells. At 500 nM, DNA synthesis was almost negligible in p16 (−) T-ALL cells but remained significant (>50% of control) in p16 (+) cells. Sensitivity to UCN-01 was independent of the mechanism of p16 inactivation, with no significant differences in the IC_{50}s among three types of the mechanisms: gene alteration, transcriptional inactivation, and translational inactivation (Table 1 and Fig. 1*A*). As shown in Fig. 1*B*, whereas the IC_{50}s of UCN-01 for most p16 (−) T-ALL cells were <150 nM (26 of 27; 96%), those for the 5

Table 1 *p16 status of 36 primary T-ALL cells and their sensitivity to UCN-01*[a]

	Patient no.	p16 status[b]	pRb status[c]	IC50 (nM)
1	9439	mut	hyper	27
2	9844	deletion	ND	27
3	30745	deletion	hypo	6
4	31195	deletion	hyper	37
5	31384	deletion	hyper	2
6	31421	deletion	hyper	54
7	31922	deletion	hyper	11
8	32103	deletion	hyper	96
9	32459	deletion	hyper	37
10	32564	deletion	No pRb	21
11	32663	deletion	hyper	126
12	32691	deletion	hyper	5
13	32697	deletion	hyper	80
14	987	deletion	hyper	25
15	32810	deletion	hyper	58
16	32973	deletion	hyper	5
17	33174	deletion	ND	7
18	33224	deletion	hyper	39
19	33241	deletion	ND	10
20	33271	deletion	hyper	73
21	30496[d]	deletion	hyper	9
22	31263	mRNA−	hyper	2
23	31380	mRNA−	hyper	52
24	32712	mRNA−	No pRb	57
25	32749	mRNA−	hyper	250
26	1009	protein−	hyper	36
27	9895	protein−	hyper	16
1	30570	p16+	hyper	778
2	30612	p16+	No pRb	400
3	31390	p16+	hyper	26
4	31474	p16+	hypo	277
5	958[d]	p16+	hyper	210
6	32172	p16+	hyper	67
7	32779	p16+	hypo	500
8	1000	p16+	hypo	42
9	33223	p16+	ND	22

[a] T-ALL cells were treated increasing concentrations of UCN-01 for 3 days and then pulsed with [^{3}H]thymidine for 6 h.

[b] mut, mutation; deletion, homozygous deletion; mRNA−, samples had neither detectable p16 mRNA nor protein despite retaining wild-type *p16* gene; protein−, samples had no detectable p16 protein despite expressing wild-type of *p16* mRNA; p16+, samples expressed wild-type p16 protein.

[c] hyper, hyperphosphorylated pRb; hypo, only hypophosphorylated pRb; ND, not determined.

[d] Relapse patient.

of 9 p16 (+) cells were >200 nM. Overall, the IC_{50}s of UCN-01 for the 27 p16 (−) T-ALL cells (mean, 43 ± 52 nM; range, 1.7–250 nM) were significantly lower than those for the 9 p16 (+) cells (mean, 258 ± 260 nM; range, 22–788 nM; $p = 0.0062$; Mann-Whitney test). Presented in another way, 26 of the 27 (96%) T-ALL samples with an inactivated p16 were UCN-01 sensitive, whereas only 4 of 9 (44%) samples harboring an intact p16 were UCN-01 sensitive ($P = 0.002$, Fisher's exact test). Similar results were obtained when we limited our comparative analysis of p16 to only those samples for which pRb data were also available, *i.e.*, 23 of 24 (96%) p16 (−) samples were UCN-01 sensitive as compared with 3 of 8 [38%) p16 (+) samples, $P = 0.002$, Fisher's exact test].

Of the 36 T-ALL samples, 31, including the 9 expressing p16 protein, were also investigated for the expression status of *p15*, another CDK inhibitor, by reverse transcription-PCR. None of the 31 expressed *p15* mRNA, consistent with our previous study (Ref. 6; data not shown).

Discussion

Recent studies have demonstrated the impact of the deregulation of the cell cycle pathway in the pathogenesis of various types of cancer (1, 5). Thus, modulation of cell cycle progression in the cancer cells is considered an ideal therapeutic approach. UCN-01 is one such anticancer drug that has shown clear antiproliferative effect against a broad spectrum of human cancer. More importantly, encouraging results have been observed in early clinical trials showing activity in several neoplasms including lymphoma (21). In this study, we found that UCN-01 inhibited cell growth and DNA synthesis of T-ALL cells. Most importantly, our results suggest that p16 protein expression status of primary T-ALL influences their sensitivity to UCN-01; p16 (−) T-ALL cells were more sensitive to UCN-01 than P16 (+) cells. Although p16 inactivation mechanisms may vary, such as gene alteration and transcriptional and translational inactivation, sensitivities to UCN-01 were similar among the three subgroups. Our results suggest that agents like UCN-01 may be useful as a p16-selective therapy for T-ALL. In addition, because p16 inactivation is a frequent event not only in T-ALL but also in various types of tumors, targeting the CDK with agents like UCN-01 should be a promising therapeutic strategy for numerous human cancers. Moreover, given the easy access of T-ALL cells and almost universal inactivation of both p15 and p16 in T-ALL (6), primary T-ALL is valuable for preclinical screening of inhibitors for CDK4 and CDK6.

Among the 9 p16 (+) T-ALL samples, 5 were resistant to UCN-01, with IC_{50}s of >200 nM, whereas 26 of 27 p16 (−) T-ALL exhibited an IC_{50} of <150 nM. In the 5 "UCN-01-resistant" samples, alterations other than p16 may negate the effect of UCN-01. One possibility is the inactivation of pRb, which was observed in 3 of the 5 samples. These included one sample that lacked pRb expression (no. 30612) and 2 that expressed the hyperphosphorylated form of pRb despite p16 protein expression (nos. 30570 and 958). Although pRb inactivation may be a contributing mechanism to UCN-01 insensitivity in these three T-ALL samples, it does not explain why 2 of 3 remaining p16 (+) samples (for which we have pRb data; nos. 1000, 32172, and 31390) harbor an inactivated pRb and yet remain UCN-01 sensitive. A correlation with pRb inactivation further fails to account for the vast majority of patients in the p16 (−) category that are UCN-01 sensitive but harbor an inactivated pRb. Thus, there may be other mechanisms why these p16 (+) samples were UCN-01 sensitive. It is possible that

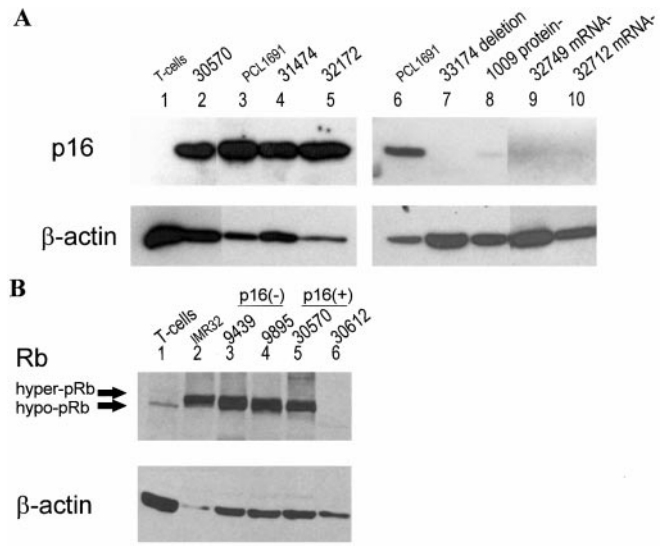

Fig. 2. Expression of p16 and pRb protein in T-ALL samples. Protein expression of p16 (*A*) and pRb (*B*) in T-ALL was determined by Western blot analysis. All levels of protein expression are relative to β-actin. *A: Lane 1*, normal T cells did not express p16 protein; *Lanes 2, 4,* and *5* (nos. 30570, 31474, and 32172), T-ALL samples that had wild-type *p16* gene and expressed p16 protein; *Lanes 3* and *6*, PCL1691 (neuroblastoma cell line) and is shown as a positive control (19); *Lanes 7–10* (nos. 33174, 1009, 32749, and 32712), T-ALL samples that harbored p16 inactivation at different levels. *B: Lane 1*, normal T cells expressed a low level of hypophosphorylated pRb; *Lane 2*, IMR32 (neuroblastoma cell line) displayed only hyperphosphorylated pRb (19) and is shown as a control; *Lanes 3* and *4*, T-ALL samples (nos. 9439 and 9895) harboring p16 inactivation at the DNA and protein levels, respectively; *Lanes 5* and *6*, T-ALL samples (nos. 30570 and 30612) expressing p16 protein. *hyper-pRb*, hyperphosphorylated pRb; *hypo-pRb*, hypophosphorylated pRb.

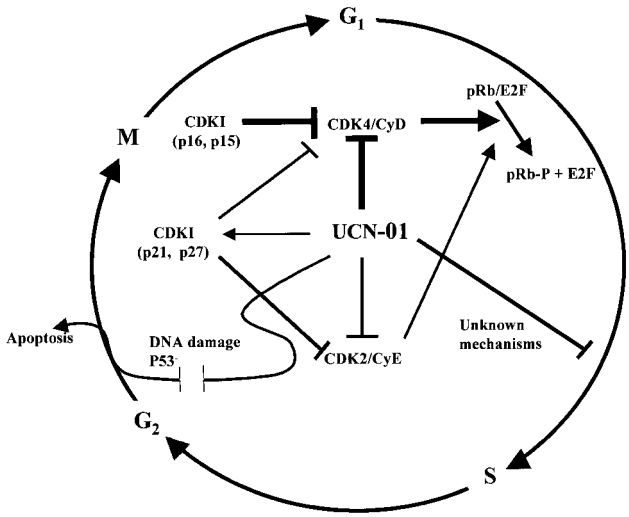

Fig. 3. Schematic representation of the mechanisms by which UCN-01 regulates the G_1 phase of the cell cycle. During the G_1 phase of cell cycle, complexes of CDK/cyclin D (CyD) phosphorylate pRb, releasing E_2F to drive the cell into S phase. The CDK/CyD complex is negatively regulated by CDK inhibitors (CDKI), of which p16 and p15 (*INK4A* and *INK4B*) are prototypes specific for CDK4 and CDK6. When p16 and p15 are inactivated, agents such as UCN-01 can block cell cycle transition by inhibiting CDK4 and CDK6. Additionally, UCN-01 can inhibit CDK2, which when complexed with cyclin E (CyE) also phosphorylates pRb, driving the cell through the cell cycle. UCN-01 can also indirectly augment these effects by stimulating the transcription of *p21* and *p27*, universal CDKIs that inhibit CDK2 as well as CDK4 and CDK6. Although a dependence on pRb has been shown clearly for UCN-01 function in a number of cell types, UCN-01 has also been shown to induce G_1 arrest in the absence of a functional pRb in some cell lines (14, 15) by an as yet-to-be elucidated mechanism(s). Outside of G_1, UCN-01 can abrogate the G_2-phase checkpoint in the presence of DNA damage and an inactivated p53, driving cells into apoptosis (22).

although p16 is expressed in these samples, they may be functionally inactivated by an unknown mechanism. On the other hand, in addition to inhibiting the p16-CDK4/6-Rb pathway, UCN-01 also targets other CDKs such as CDK2 (7). There also appear to be indirect effects of UCN-01, such as the induction of CDK inhibitors, *p21* and *p27* (12). As illustrated in Fig. 3, these activities of UCN-01 might act in concert with each other in pRb hypophosphorylation and the subsequent inhibition of tumor cell growth. It is possible that in the p16 (+) T-ALL, the effects of UCN-01 independent of the p16-CDK-pRb pathway may be unique to each T-ALL, and that such differences may account for the demonstrated variations in their sensitivities to this agent. In addition, although pRb function has been reported to be essential for sensitivity to UCN-01 (14), the inhibition of pRb phosphorylation appears not to be the sole determinant of responsiveness to this agent, because significant G_1 accumulation or cell growth inhibition by UCN-01 were observed in certain pRb (−) cell lines (14, 15). Consistent with these findings, we found that 2 p16 (−)/pRb (−) T-ALL cells (nos. 32564 and 32712) were sensitive to UCN-01.

PHA-stimulated normal T cells were significantly more resistant to UCN-01 than p16 (−) T-ALL cells, especially at the low concentrations (<125 nM) that inhibit DNA synthesis in most p16 (−) T-ALL cells by >50%. Because the p16/p15-cyclin Ds/CDKs-pRb pathway is properly regulated in normal T cells, UCN-01 may have less impact as compared with T-ALL cells in which the pathway is deregulated. Such differential sensitivities to UCN-01 between normal and tumor cells should offer drugs of this type a great advantage when applied clinically.

In conclusion, we have shown that the p16 protein status may be a key factor to determine the cytotoxicity of UCN-01 to T-ALL cells. This is the first study to demonstrate that a molecular alteration can dictate the efficacy of UCN-01 in primary human cancer. Our findings confirm that the identification of the molecular alteration in cancer should facilitate the design of novel and selective anticancer therapeutics. In light of the *in vitro* findings presented here and Phase I study of UCN-01 showing antitumor activity in various malignancies, further clinical trials of UCN-01 and similar agents in p16 (−) cancer including T-ALL are warranted. If such agents prove to be safe and efficacious for p16 (−) cancer in Phase I and II clinical trials, incorporation of CDK inhibitors into the existing therapeutic regimens should be considered in the future to improve the ultimate outcome of these patients.

Acknowledgments

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