



Development of cyclin-dependent kinase modulators as novel therapeutic approaches for hematological malignancies

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The majority of hematopoietic malignancies have aberrancies in the retinoblastoma (Rb) pathway. Loss in Rb function is, in most cases, a result of the phosphorylation and inactivation of Rb by the cyclin-dependent kinases (cdks), main regulators of cell cycle progression. Flavopiridol, the first cdk modulator tested in clinical trials, is a flavonoid that inhibits several cdks with evidence of cell cycle block. Other interesting preclinical features are the induction of apoptosis, promotion of differentiation, inhibition of angiogenic processes and modulation of transcriptional events. Initial clinical trials with infusional flavopiridol demonstrated activity in some patients with non-Hodgkin's lymphoma, renal, prostate, colon and gastric carcinomas. Main side-effects were secretory diarrhea and a pro-inflammatory syndrome associated with hypotension. Phase 2 trials with infusional flavopiridol in CLL and mantle cell lymphoma, other schedules and combination with standard chemotherapies are ongoing. The second cdk modulator tested in clinical trials, UCN-01, is a potent protein kinase C inhibitor that inhibits cdk activity *in vitro* as well. UCN-01 blocks cell cycle progression and promotes apoptosis in hematopoietic models. Moreover, UCN-01 is able to abrogate checkpoints induced by genotoxic stress due to modulation in chk1 kinase. The first clinical trial of UCN-01 demonstrated very prolonged half-life (~600 h), 100 times longer than the half-life observed in preclinical models. This effect is due to high binding affinity of UCN-01 to the human plasma protein alpha-1-acid glycoprotein. Main side-effects in this trial were headaches, nausea/vomiting, hypoxemia and hyperglycemia. Clinical activity was observed in patients with melanoma, non-Hodgkin's lymphoma and leiomyosarcoma. Of interest, a patient with anaplastic large cell lymphoma refractory to high-dose chemotherapy showed no evidence of disease after 3 years of UCN-01 therapy. Trials of infusional UCN-01 in combination with Ara-C or gemcitabine in patients with acute leukemia and CLL, respectively, have commenced. In conclusion, flavopiridol and UCN-01 are cdk modulators that reach biologically active concentrations effective in modulating CDK *in vitro*, and show encouraging results in early clinical trials in patients with refractory hematopoietic malignancies. Although important questions remain to be answered, these positive experiences will hopefully increase the therapeutic modalities in hematological malignancies. *Leukemia* (2001) 15, 1–9.

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Overview of cell cycle regulation and rationale for cdk modulators for cancer therapy

Mitogenic signals commit cells to entry into a series of regulated steps allowing traverse of the cell cycle. Synthesis of DNA (S phase), and separation of two daughter cells (M phase) are the main features of cell cycle progression (Figure 1). The time between the S and M phases is known as G2 phase. This phase is important to allow cells to repair errors that occur during DNA duplication, preventing the propagation of these

errors to daughter cells. In contrast, the G1 phase represents the period of commitment to cell cycle progression that separates M and S phases as cells prepare for DNA duplication upon mitogenic signals.¹ An important step in the G1/S transition is the inactivation and phosphorylation of the retinoblastoma gene product (Rb), a tumor suppressor gene product important for G1 control, leading to the release of the transcriptional factor E2F1 and the activation of E2F-responsive genes necessary for progression to S phase (such as cyclin E, thymidine kinase).^{2–5} This phosphorylation occurs by the activation of serine/threonine kinases, known as cyclin-dependent kinases (cdks).⁶ These key regulators of the cell cycle are enzymes that periodically form complexes with proteins known as cyclins (Figure 1). There are at least nine different cdks (cdk1–cdk9)^{6–9} and at least 15 different cyclins (cyclin A to T).^{10,11} These complexes are in turn regulated by a stoichiometric combination with small inhibitory proteins (see Figure 1). These proteins are called cyclin-dependent kinase inhibitors (CKIs). There are two families of CKIs: the INK4 (inhibitor of cdk4) family, p16^{ink4a}, p15^{ink4b}, p18^{ink4c} and p19^{ink4d}, that specifically inhibit cyclin D-associated kinases and the KIP (kinase inhibitor protein) family, p21^{waf1}, and p27^{kip1} and p57^{kip2}, that bind and inhibit the activity of cyclin E/cdk2 and cyclin A/cdk2 complexes.¹² For further insight into cell cycle regulation, excellent reviews of cell cycle control have recently been published.^{6,13,14}

Approximately 90% of human neoplasms have abnormalities in some component of the Rb pathway due either to hyperactivation of cdks as a result of amplification/overexpression of positive cofactors, cyclins/cdks, or downregulation of negative factors, endogenous CKIs, or mutation in the Rb gene product.^{1,5} Of interest, hematopoietic malignancies show clear evidence of aberrations in the Rb pathway, for instance, cyclin D1 translocation, in mantle cell lymphoma; loss in endogenous cdk inhibitors such as in leukemias and hyperactivation of cdk4/cdk6 in human T cell leukemia virus type 1 leukemias, among others.^{15–22} Thus, it is theoretically reasonable to test cdk modulators in human clinical trials in patients afflicted with hematopoietic neoplasms.

Several strategies have been conceived to modulate cdk activity for therapeutic intervention. These strategies may be divided into efforts to directly target the catalytic cdk subunit, or to manipulate cdk activity indirectly by affecting the regulatory pathways that govern cdk activity.^{23,24} The first approach or 'direct' chemical inhibitors represents the most immediate opportunity to allow rational design of small molecule cdk inhibitors to interact specifically with the ATP binding site of cdks.^{23–28} Examples of this group include flavopiridol congeners, polysulfates, butyrolactone 1, 9-hydroxyellipticine, toyocamycin derivatives, paullones and purine derivatives. The second approach utilizes agents that 'indirectly' modulate the activity of the cdks by altering the expression and synthesis of the cdk/cyclin subunits or the cdk inhibitory proteins (CKIs); by modulating the phosphorylation

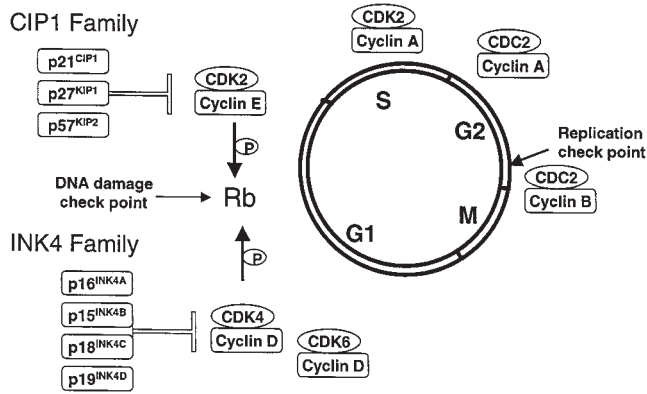


Figure 1 Schematic representation of cell cycle regulation.

state of the cdk's by targeting CAK, *cdc25*, *wee1/myt1* and by manipulating the proteolytic machinery that regulates the catabolism of cdk/cyclin complexes or their regulators (see review for further detail²³).

Flavopiridol

Preclinical pharmacology

Mechanism of action

Flavopiridol, (L86-8275 or HMR 1275) is a semisynthetic flavonoid derived from rohitukine, an alkaloid isolated from a plant indigenous to India (Figure 2). Initial studies from our laboratory revealed that flavopiridol could induce cell cycle arrest during either G1 or G2/M, concordant with the ability of flavopiridol to inhibit both *cdk2* and *cdk1*, respectively.²⁹ Studies utilizing purified cdk's showed that flavopiridol inhibits cdk's 1, 2 and 4 in a competitive manner with respect to ATP, with a *Ki* of 41 nM.²⁹⁻³³ When the crystal structure of *cdk2* and deschloro-flavopiridol was resolved, it was evident that flavopiridol binds to the ATP binding pocket, with the benzopyran occupying the same region as the purine ring of ATP.³⁴ This observation corroborated our prior biochemical studies with the compound.³¹ Flavopiridol inhibits all cdk's thus far examined (*IC*₅₀ ~100 nM), with somewhat less potent effect on *cdk 7* (*cdk* -activating kinase), (*IC*₅₀ ~300 nM).³⁰⁻³²

Another interesting aspect of flavopiridol's cell cycle regulat-

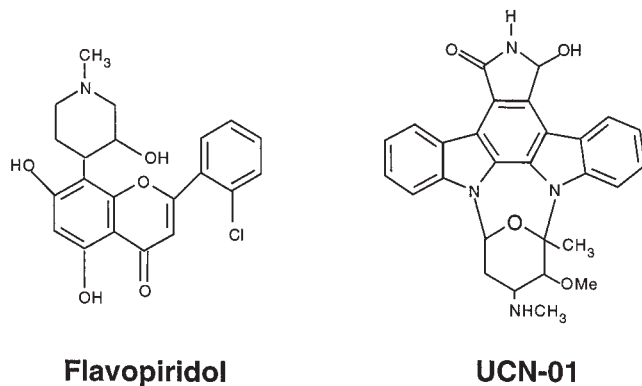


Figure 2 Chemical structures of flavopiridol and UCN-01.

ory properties is the depletion of cyclin D1, an oncogene that is upregulated in many human neoplasias and, when overexpressed harbors bad prognosis.^{35,36} Carlson *et al*³⁷ showed that when MCF-7 human breast carcinoma cell line were exposed to flavopiridol, cyclin D1 protein levels decreased within 3 h. This effect was followed by decline in cyclin D3 with no alteration in cyclin D2 or cyclin E protein levels, the remaining G1 cyclins, leading to loss in *cdk4* activity.³⁷ Cyclin D1 depletion was a consequence of downregulation of cyclin D1 mRNA associated with specific decline in cyclin D1 promoter measured by luciferase reporter assay.³⁷ Thus, the cyclin D1 transcriptional repression observed with flavopiridol is consistent with the effects of flavopiridol on the mRNA expression of cell cycle genes from yeast cells (see below) and also underscores the conserved effect of flavopiridol on eukaryotic cyclin transcription.³⁸ In summary, flavopiridol could induce cell cycle arrest by at least three different mechanisms (see Figure 3): first, by the direct inhibition of cdk's, competitive with respect to ATP; second, by preventing necessary threonine 160 phosphorylation of cdk's due to inhibition of *cdk7/cyclin H*,^{32,33} and finally, in the case of the G1, by downregulation of cyclin D1 and cyclin D3, important co-factors for *cdk4* and *cdk6* activation.

Several lines of evidence determined that flavopiridol has antiangiogenic properties, Brusselbach *et al*³⁹ exposed HUVEC human endothelial cells to flavopiridol. Apoptosis was present even in cells that were non-cycling. Kerr *et al*⁴⁰ tested flavopiridol in an *in vivo* angiogenesis model demonstrating a decrease blood formation in the mouse Matrigel model of angiogenesis.⁴⁰ Furthermore, Melillo *et al*⁴¹ demonstrated that hypoxia-induced VEGF from human monocytes is suppressed by the presence of low nM concentrations of flavopiridol. This effect occurs at the mRNA and protein levels and is due to decrease in VEGF mRNA stability.⁴¹ Thus, it is conceivable that flavopiridol's antitumor activity may also be mediated by antiangiogenic effects. The relationship between these antiangiogenic properties and cdk modulation are being investigated.

Another interesting feature of flavopiridol is the induction of a differentiated phenotype. Lee and coworkers⁴² exposed NCI-H358 lung carcinoma cell lines to flavopiridol and roscovitine, both *cdk2* inhibitors, or *cdk2* antisense constructs;

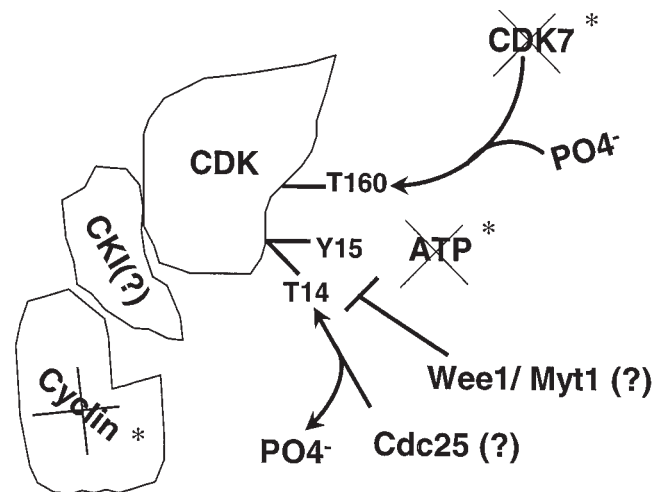


Figure 3 G1/S arrest by flavopiridol. Asterisk denotes proven targets for flavopiridol-induced G1 arrest. In the case of cyclins, only cyclin D1 and cyclin D3 are affected by this compound.

clear evidence of mucinous differentiation along with loss in cdk2 activity was observed. However, each treatment had different effects on cell cycle regulatory expression despite similar differentiated phenotype.⁴²

To compare the effects of several cdk inhibitors on cDNA expression from yeast cells, Gray *et al*³⁸ cells exposed *S. cerevisiae* for 2 h to compound 52 (a potent cdk2/cdk1 inhibitor) and to flavopiridol. Almost 50% of affected transcripts were shared by compound 52 and flavopiridol. These transcripts belong to genes which regulate progression of cell cycle, phosphate and cellular energy metabolism; and GTP or ATP binding proteins.³⁸ Similar transcriptional effects of flavopiridol on cyclin genes were observed in human cells (see above). Recently, Chao *et al*⁴³ demonstrated that flavopiridol inhibits P-TEFb (also known as cdk9/cyclin T) *in vitro* with a Ki of 3 nM. Interestingly, in contrast with all cdk tested so far, flavopiridol was not competitive with ATP in this reaction. P-TEFb is a required cellular cofactor for the human immunodeficiency virus (HIV-1) transactivator, Tat. As expected from P-TEFb inhibition, flavopiridol blocked Tat transactivation of the viral promoter *in vitro*. Furthermore, flavopiridol blocked HIV-1 replication in both single round and viral spread assays with an IC₅₀ of less than 10 nM.⁴³ Thus, this biochemical effect of flavopiridol should be tested in clinical trials of patients with HIV-related malignancies, including HIV lymphomas.

In an effort to test synergistic effects with other compounds, cytotoxic assays with flavopiridol in combination with standard chemotherapeutic agents were performed.^{44,45} Synergistic effects were demonstrated when flavopiridol followed treatment of A549 lung carcinoma cells with paclitaxel, cytarabine, topotecan, doxorubicin, or etoposide. In contrast, in the case of 5-fluorouracil, flavopiridol needs to be present for 24 h previously to be synergistic. Synergistic effects with cisplatin were not schedule-dependent.⁴⁵ In other models, the synergism between flavopiridol with cisplatin and/or γ -irradiation was not demonstrated, however, flavopiridol demonstrated even more potent effects on tumor cells lines that overexpress MDR.⁴⁶

Effects of flavopiridol on hematopoietic preclinical models

Another important feature of flavopiridol's action is the induction of apoptosis. Studies in several laboratories demonstrated that hematopoietic cell lines such as SUDHL-4 (B cell lymphoma) and Jurkat (T cell leukemia/lymphoma) and CLL cell lines were exquisitely sensitive to flavopiridol-induced apoptosis.⁴⁷⁻⁵¹ Furthermore, flavopiridol inhibits the proliferation of several mantle cell lymphomas cell lines, cell lines that carry overexpression of cyclin D1 due to translocation of cyclin D1 with the heavy chain immunoglobulin promoter.^{52,53} The potent antiproliferative effect of flavopiridol was associated with decline in cyclin D1 expression with loss in cdk activity, loss of clonogenicity and apoptosis.⁵³ These results have stimulated the development of clinical trials of flavopiridol in this specific refractory lymphoma.^{23,54} Another interesting property of flavopiridol is the ability of this compound to induce apoptosis in DNA-damage insensitive cell lines such as HN-30 squamous head and neck cell line.⁵⁵ Again, the apoptotic effect was independent of p53 status and was associated with the depletion of cyclin D1.⁵⁵

The mechanism(s) by which flavopiridol induces apoptosis have not yet been clarified. Flavopiridol did not modulate topoisomerase I/II activity, a known effector of apoptosis.⁴⁹

Moreover, in some hematopoietic cell lines neither BCL-2/BAX nor p53 appeared to be involved,^{49,51,56,57} while in other hematopoietic models, bcl-2 may be downregulated.^{50,51} Similar p53-independent apoptosis was observed when flavopiridol was tested against a panel of squamous head and neck cell lines.⁵⁵ Preliminary evidence from our laboratory demonstrated that flavopiridol-induced apoptosis in leukemia cell lines is associated with early activation of the MAPK protein kinase family (MEK, p38 and JNK).⁵⁶ This activation may lead to activation of caspases.⁵⁶ As seen in this and other models, caspase inhibitors prevent flavopiridol-induced apoptosis.^{48,56} Recently, Kitada *et al*⁵¹ showed that in a panel of B-CLL cell lines, flavopiridol downregulated the anti-apoptosis proteins XIAP, Mcl-1 and BAG-1. The mechanism(s) of downregulation and relevance for apoptosis are still unknown. It is unclear whether the putative flavopiridol-induced inhibition of cdk activity is required for induction of apoptosis. Intense efforts to understand the mechanism of flavopiridol-induced apoptosis are currently being carried out by several investigators.

Initial studies with flavopiridol administered in a protracted fashion demonstrated that flavopiridol has a cytostatic effect in colorectal (colo 205) and prostate (LnCap/DU-145) carcinoma xenograft models when administered frequently.⁵⁸ These results stimulated the initial clinical trials of flavopiridol in humans as a 72-h continuous infusion every 2 weeks^{23,59} (and see below).

Recently, studies from our laboratory have shown that flavopiridol has notable activity in leukemia/lymphoma xenografts when administered as a bolus intravenous schedule, rendering animals tumor free in the HL-60 and AIDS-lymphoma models while infusional flavopiridol in similar models only achieved tumor growth delays.⁴⁷ This significant anti-tumor effect is due to frank apoptosis and was associated with peak flavopiridol plasma concentration $\sim 5-8 \mu\text{M}$.⁴⁷ Based on these results, a phase I trial with bolus flavopiridol administration is currently being explored at the NCI (see below).

Human clinical trials with flavopiridol

Flavopiridol phase I clinical trials: Two clinical trials of infusional flavopiridol administered by a 72-h continuous infusion every 2 weeks have recently been completed.^{59,60} Seventy-six patients were treated in the NCI phase I trial. Dose-limiting toxicity (DLT) was secretory diarrhea with a maximal tolerated dose (MTD) of 50 mg/m²/day \times 3 (see Table 1). In the presence of anti-diarrhea prophylaxis, patients were able to be treated at higher doses, allowing a second MTD, 78 mg/m²/day \times 3. DLTs at the higher dose level (Table 1) consisted of reversible hypotension and a significant pro-inflammatory syndrome (fever, fatigue, local tumor pain and modulation of acute phase reactants).⁵⁹ In an effort to understand the pro-inflammatory syndrome, we determined prospectively the plasma concentration of different cytokines before and during flavopiridol infusion. A dose-dependent significant increase in IL-6 was observed (A Senderowicz, manuscript in preparation). How this putative cdk inhibitor modulates cytokine expression is being explored in our laboratory. Another interesting side-effect was the induction of dose-dependent reversible lymphocytopenia. No evidence of increased prevalence of infections was observed in this trial. Based on this effect along with the preclinical data in hematopoietic neoplasms, clinical trials of flavopiridol in CLL are ongoing.⁵⁴

Table 1 Phase I trials with cdk modulators

	<i>Flavopiridol</i>	<i>UCN-01</i>
Schedule	72 h CI q 2 weeks	72 h CI q 4 weeks (cycle one) followed by 36 h CI q 4 week (≥ 2 cycle one) ^a
DLT	Diarrhea (62.5 mg/m ² /day) Hypotension (98 mg/m ² /day)	Nausea/vomiting, hyperglycemia and hypoxemia (53 mg/m ² /day)
MTD	50 mg/m ² /day (without prophylaxis) 78 mg/m ² /day (with prophylaxis)	42.5 mg/m ² /day
Other toxicities	Anorexia, pro-inflammatory syndrome	Headache, myalgias
Antitumor activity	Renal, NHL, colon, gastric, prostate	Melanoma, NHL, leiomyosarcoma
Median plasma concentration at MTD	271 nM (50 mg/m ² /day) 344 nM (78 mg/m ² /day)	Total 36.4 μ M (42.5 mg/m ² /day) Free ^b 111 nM (42.5 mg/m ² /day)
Plasma half-life	11.6 h	622 h

DLT, dose-limiting toxicities; MTD, maximally tolerated dose; CI, continuous infusion; NHL, non-Hodgkin's lymphoma.

^aCycle one, 72 h CI every 4 weeks and \geq cycle 2: half-total dose over 36 h every 4 weeks.

^bFree salivary UCN-01 concentration.

Some patients, particularly with non-Hodgkin's lymphoma, colon, prostate and kidney cancer showed shrinkage of tumor masses that, in some circumstances, lasted for more than 6 months, including one patient with refractory renal cancer who achieved a partial response. Five patients received flavopiridol for more than 1 year and one patient received flavopiridol for more than 2 years. This potential for 'disease stabilization' noted in this trial is concordant with the preclinical models, where tumor stasis was observed. Plasma flavopiridol concentrations between 300 and 500 nM – active biological concentrations able to inhibit cdk *in vitro* – were safely achieved during our trial.⁵⁹

In a second phase I trial, also exploring the use of a 72-h continuous infusion every 2 weeks, Thomas *et al*⁶⁰ found that the DLT is diarrhea, corroborating the NCI experience. Moreover, plasma flavopiridol concentrations between 300 and 500 nM were also observed.⁶⁰ Interestingly, one patient with refractory metastatic gastric cancer achieved a sustained complete response without any evidence disease more than 2 years after discontinuation of flavopiridol.

Flavopiridol 1-h infusion phase I clinical trial: In September 1998, we began the first phase I trial of a daily 1-h infusion of flavopiridol for 5 consecutive days every 3 weeks. This dose schedule was based on the striking antitumor results of flavopiridol observed in leukemia/lymphoma and head and neck xenografts.^{47,55} The recommended phase II dose is 37.5 mg/m² per day for 5 consecutive days (Table 2). Dose-

limiting toxicities observed at 52.5 mg/m²/day are nausea/vomiting, neutropenia, fatigue, and diarrhea.⁶¹ Other (non-dose-limiting) side-effects are 'local tumor pain' and anorexia. To reach higher flavopiridol concentrations, the protocol was amended to administer flavopiridol for 3 days (initially) and then for 1 day only (Table 2). These protocol modifications allowed the achievement higher flavopiridol concentrations (~4 μ M).⁶¹ Unfortunately, the half-life observed in this trial is much shorter (~3 h) than the infusional trial (~10 h). Thus, micromolar concentrations attained in this trial were only maintained for very short periods of time.⁶¹ This shorter half-life may explain the lack of antitumor activity observed in this trial.

Other flavopiridol clinical trials: A phase 1 trial testing the combination of paclitaxel and flavopiridol demonstrated good tolerability with a dose-limiting pulmonary toxicity.⁶²

Phase II trials of flavopiridol given as a 72-h continuous infusion with the MTD in the absence of anti-diarrheal prophylaxis (50 mg/m²/day) to patients with chronic lymphocytic leukemia, non-small cell lung cancer, non-Hodgkin's lymphoma, and colon, prostate, gastric, head and neck, and kidney cancer, and phase I trials of flavopiridol administered on novel schedules and in combination with standard chemotherapeutic agents are being explored.^{54,63–66}

UCN-01

Preclinical pharmacology

Mechanism of action

Staurosporine is a non-specific protein kinase inhibitor that shows cell cycle arrest in transformed and non-transformed cells at 1–100 nM.⁶⁷ UCN-01 (7-hydroxystaurosporine; see Figure 2) is a staurosporine analogue with higher specificity against several PKC isoenzymes, particularly against the Ca²⁺-dependent protein kinase C (cPKCs) with an IC₅₀ ~30 nM with lower potency against the novel Ca²⁺-independent PKCs (nPKCs) (IC₅₀ ~500 nM) and no effect against the atypical PKCs.^{68–70} In addition to the effects on PKC, UCN-01 shows prominent effects on cell cycle and antiproliferative activity

Table 2 Phase I trial of 1-h infusion flavopiridol⁶¹

Daily for 5 days ^a	
MTD	37.5 mg/m ² /day
DLTs	nausea/vomiting, diarrhea, fatigue, neutropenia
Daily for 3 days	
MTD	50 mg/m ² /day
DLTs	nausea/vomiting, diarrhea, fatigue, hepatotoxicity, neutropenia
Daily for 1 day	
MTD	62.5 mg/m ² /day
DLTs	nausea/vomiting, diarrhea, fatigue, neutropenia
Plasma half-life	3.6 h

^aEvery 3 weeks.

DLT, dose-limiting toxicities; MTD, maximally tolerated dose.

in several human tumor cell lines.^{71–75} In contrast, another highly selective potent PKC inhibitor, GF 109203X shows no antiproliferative effect despite similar capacity to inhibit PKC *in vitro*.⁷² Taken together, these results suggest that UCN-01's antiproliferative effect is probably not explained solely by inhibition of PKC.

As demonstrated by different groups, UCN-01 arrest can arrest tumor cells in the G1 phase of the cell cycle^{71,74–78} It appears that this arrest was accompanied by Rb hypophosphorylation and the accumulation of p21^{waf1} and p27^{kip1} and appears to be independent of the p53 and Rb status.^{75,78} In seeking to define the mechanism(s) for cell cycle arrest, UCN-01 displayed moderately potent inhibition of immunoprecipitated cdc2 and cdk2 (IC₅₀ 300–600 nM). However, when intact cells were exposed to UCN-01, 'inappropriate activation' of the same kinases occurred.⁷²

Effects of UCN-01 on hematopoietic preclinical models

A clear antiproliferative effect characterized by loss of G2/M DNA content and clear evidence of apoptosis was readily observed after 300 nM UCN-01 against a panel of leukemia T cell lines (Jurkat, Molt-3, Molt-4 and Hut-78).⁷² These effects were correlated with the inappropriate activation of cdk1 and cdk2 from intact cell lines exposed to UCN-01.⁷² Furthermore, Wang *et al*⁷⁹ exposed asynchronously growing CA-46 Burkitt's lymphoma (a cell line with defective p53 function) to γ -irradiation and these cells, as expected from a cell type defective in the G1/S checkpoint due to loss in p53 function, stopped before mitosis (G2 checkpoint activation).⁷⁹ This pause probably promotes DNA repair in order to prevent cells segregating damaged chromosomes.⁸⁰ The G2 arrest observed after DNA-damaging agents is due to inactivation of the rate-limiting step for progression from G2 to mitosis, cdc2/cyclin B.⁸¹ However, when CA-46 cells are irradiated first, followed by UCN-01, the 'inappropriate activation of cdc2/cyclin B' induced by UCN-01⁷² promotes premature mitosis, also known as 'abrogation of G2 checkpoint' followed by apoptosis.⁷⁹ These effects could be partially explained by the inactivation of wee 1, the kinase that negatively regulates G2/M transition.⁸² Recently, several groups discovered that UCN-01 targets chk1, a regulatory kinase important for G2 checkpoint control after DNA damage.^{83–85} Thus, UCN-01 directly inhibits cdk's, but in cells more potently affects 'upstream' kinases that regulates cdc2 activity. Studies from other groups suggest that not only is UCN-01 able to abrogate the G2 checkpoint induced by DNA-damaging agents but also, in some circumstances, UCN-01 is able to abrogate the S-phase checkpoint induced by similar stimuli.^{86,87}

Other investigators have demonstrated that the potent apoptotic effects observed in B-CLL lines was associated with downregulation of the antiapoptosis proteins XIAP, Mcl-1 and BAG-1.⁵¹ The mechanism(s) of downregulation and relevance for apoptosis is still unknown.

Another interesting feature of UCN-01 is the observed increased cytotoxicity in cells that harbor mutated p53.⁷⁹ In fact, CA-46 (Burkitt's lymphoma) and HT-29 (colon carcinoma) tumor cell lines, both carrying the p53 mutated phenotype, exposed to UCN-01 displayed significant cytotoxicity. Moreover, MCF-7 cell lines harboring no endogenous p53 due to ectopic expression of the human papillomavirus type-16 E6 protein, showed enhanced cytotoxicity with the combination between DNA-damaging agents, such as cispla-

tin and UCN-01, compared with the isogenic wild-type MCF-7 cell line. Another interesting effect of UCN-01 is the synergistic effect with standard chemotherapeutic agents including mitomycin C, 5-fluoruracil, BCNU, camptothecin, among others.^{86,88–93} This *in vitro* experience has stimulated several groups to begin trials of UCN-01 with standard agents (see below).

When UCN-01 is administered by an intraperitoneal or intravenous route antitumor activity is displayed in several human xenografts including MOLT-4, HL-60 leukemia models.^{23,73} Better antitumor effects were observed when UCN-01 was given by protracted exposures. This requirement for longer concentration-time exposure was also observed in *in vitro* models, obtaining the highest antitumor activity when UCN-01 was present for 72 h.⁷¹ Thus, a clinical trial using a 72-h continuous infusion every 2 weeks was conducted (described below).

Human clinical trials with UCN-01

Based on the antitumor activity observed in preclinical models and unique biochemical features including potent antiproliferative effects in p53-defective cell lines and G2 checkpoint abrogation after DNA damage, we recently completed the first phase I trial of UCN-01 in humans (Table 1).⁹⁴ A surprising effect observed in this initial human experience included an unexpected very prolonged half-life (~600 h), 100 times longer than the half-life observed in preclinical models, probably due to very avid binding to the human plasma protein to alpha-1-acid glycoprotein (AAG).^{95,96} DLTs were nausea/vomiting, symptomatic hyperglycemia and pulmonary toxicity (Table 1). The MTD of UCN-01 using a 72 h continuous infusion schedule was 42.5 mg/m²/day.⁹⁷ One patient with refractory metastatic melanoma developed a partial response that lasted 8 months. Few patients with leiomyosarcoma, non-Hodgkin's lymphoma and lung cancer demonstrated significant tumor stabilization (≥ 6 months).⁹⁷ Of interest, one patient with refractory anaplastic large cell lymphoma (T cell phenotype, alk-expressing) who failed multiple chemotherapeutic regimens including high-dose chemotherapy has no evidence of disease 3 years after the initiation of UCN-01. Another patient with refractory large B cell lymphoma who failed prior high-dose combination chemotherapy protocol-EPOCH-2 had rapidly progressive disease after one cycle of UCN-01 with hepatic and bone marrow failure (thrombocytopenia) requiring immediate chemotherapy. After dose-modified EPOCH without cyclophosphamide, his liver function and thrombocytopenia resolved completely with significant improvement in performance status within 2 weeks after combination chemotherapy. Unfortunately, he developed fungemia with a resistant candida *Kruzei* strain and expired. Post-mortem examination revealed a pathological complete response after only one cycle of chemotherapy.⁹⁸ We feel that this refractory large cell lymphoma patient became 'chemotherapy-sensitive' after only one dose of UCN-01. This observation is consistent with the synergistic effect observed in preclinical models of UCN-01 with several chemotherapeutic agents.

In order to determine 'free UCN-01 concentrations' from body fluids in this infusional trial, several efforts were considered. Plasma ultracentrifugation and salivary concentrations of UCN-01 revealed similar results. At the recommended phase II dose (42.5 mg/m²/day over 72 h), concentrations of 'free-salivary' UCN-01 (~111 nM) that may

cause G2 checkpoint abrogation can be achieved. Moreover, to assess if plasma from these patients have the potential to abrogate the G2 checkpoint, irradiated MCF-7 cells were exposed to plasma obtained from patients at the end of the infusion demonstrating clear evidence of G2 checkpoint abrogation (A Senderowicz, manuscript submitted for publication). Furthermore, in order to determine the signaling effects of UCN-01 in tissues, the assessment of protein kinase C activity, as measured by immunoblots that only recognize phosphorylated adducin (a known PKC substrate) was used. Clear decline in phospho-adducin content in patients at the 42.5 and 53 mg/m²/day levels were determined from bone marrow aspirates and tumor cells abrogation (A Senderowicz, manuscript submitted for publication). Thus, we believe that PKC signaling was modulated *in vivo* in this trial despite high AAG binding.

Trials with shorter schedules (3 h) of infusional UCN-01 are being conducted. Interestingly, the toxicity profile of shorter infusions is similar to that observed with the 72-h infusion trial.^{99,100} However, with shorter infusions, more pronounced hypotension was observed.^{99,100} Determination of free UCN-01 and toxicity profiles in these trials are of the utmost importance as higher free concentrations for shorter periods may be more or less beneficial/toxic compared with the free concentrations observed in the 72-h infusion trial.

Based on the unique pharmacological features and anecdotal clinical evidence of synergistic effects in one patient with refractory disease,⁹⁸ several combination trials with standard chemotherapeutic agents recently commenced. A phase I/II trial of gemcitabine followed by 72-h infusional UCN-01 in chronic lymphocytic leukemia started at the NCI. Moreover, a trial of UCN-01 in combination with Ara-C is being conducted at MD Anderson University, Houston, TX, USA. Other studies in combination with cisplatin, 5-fluorouracil, among others, also commenced recently.

Several clinically oriented questions are shared between UCN-01 and flavopiridol, including which is the best schedule (short bolus vs infusion), how to combine with other agents? And what are the best pharmacodynamic endpoints to be followed, along with the specific issue of how to best measure free UCN-01 concentration and how to clinically exploit the G2 checkpoint abrogation induced by UCN-01.

Summary

With the recent understanding of the role of cdk in cell cycle regulation and the discovery that approximately 90% of all neoplasias (including hematopoietic neoplasms) are the result of 'cdk hyperactivation' leading to the abrogation of the Rb pathway, novel cdk modulators are being developed. The first two cdk modulators tested in clinical trials, flavopiridol and UCN-01, demonstrated significant preclinical activity in hematopoietic models. Moreover, in early clinical trials, both compounds demonstrated activity in some patients with NHL. The best schedule to be administered, combination with standard chemotherapeutic agents and demonstration of cdk modulation in tumor samples from patients in these trials are important issues that need to be answered in order to ensure the best possible use of these agents. From a scientific standpoint, these drugs are interesting compounds in continuing to elucidate the connections cdk have to the globally important functions of cell cycle progression, apoptosis, and differentiation.

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