Preclinical and Clinical Development of Cyclin-Dependent Kinase Modulators

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In the last decade, the discovery and cloning of the cyclin-dependent kinases (cdks), key regulators of cell cycle progression, have led to the identification of novel modulators of cdk activity. Initial experimental results demonstrated that these cdk modulators are able to block cell cycle progression, induce apoptotic cell death, promote differentiation, inhibit angiogenesis, and modulate transcription. Alteration of cdk activity may occur indirectly by affecting upstream pathways that regulate cdk activity or directly by targeting the cdk holoenzyme. Two direct cdk modulators, flavopiridol and UCN-01, are showing promising results in early clinical trials, in which the drugs reach plasma concentrations that can alter cdk activity in vitro. Although modulation of cdk activity is a well-grounded concept and new cdk modulators are being assessed for clinical testing, important scientific questions remain to be addressed. These questions include whether one or more cdks should be inhibited, how cdk inhibitors should be combined with other chemotherapy agents, and which cdk substrates should be used to assess the biologic effects of these drugs in patients. Thus, modulation of cdk activity is an attractive target for cancer chemotherapy, and several agents that modulate cdk activity are in or are approaching entry into clinical trials. [J Natl Cancer Inst 2000;92:376–87]

INTRODUCTION TO CELL CYCLE REGULATION

After activation of several mitogenic signaling cascades, cells traverse the cell cycle in several tightly controlled phases (Fig. 1). G1 phase separates M and S phases. In this period, cells commit to enter the cell cycle and prepare to duplicate their DNA (1). After G1 phase, cells enter S phase, the period of DNA synthesis (genome duplication). After S phase, cells enter G2 phase, the period in which cells can repair errors that might occur during DNA duplication and thus prevent passing these errors to daughter cells. During G2 phase, cells prepare to enter M phase, the period in which chromatids and then daughter cells separate. After M phase, cells can enter G1 phase again or enter G0 phase, a replicatively quiescent phase. In G0 phase, the cells usually have a diploid amount of DNA, which represents the differentiated functioning cell not committed to the cell cycle.

The progression of cells from G1 to S phase is accompanied by the phosphorylation of the retinoblastoma gene product (Rb protein), a tumor suppressor gene active in the control of G1 phase (2,3). Phosphorylation of Rb protein by serine/threonine kinases known as cyclin-dependent kinases (cdks) inactivates Rb (4). The cdks, key regulators of the cell cycle, consist of catalytic subunits that form complexes with proteins known as cyclins. There are at least nine cdks (cdk1–cdk9) (4–7). The cdks that are clearly involved in cell cycle control are cdk1 through cdk7. Although structurally related to cdk1 through cdk7, cdk8 and cdk9 are important transcriptional regulators (5,6). There are at least 15 cyclins (cyclin A through cyclin T) (8–10). Cyclin expression varies during the cell cycle, and indeed the periodic expression of different cyclins defines the start of each phase of the cell cycle and also marks the transitions between the various phases. Cyclins and their cognate cdk catalytic subunits noncovalently form 1:1 complexes to produce the cdk holoenzyme. The holoenzyme is activated by the phosphorylation of specific residues in the cdk catalytic subunit. This phosphorylation can be catalyzed by cdk7/cyclin H, which is also known as the cdk-activating kinase (11,12).

Specific cdks operate in distinct phases of the cell cycle. Complexed with their respective D-type cyclin partners, cdk4 and cdk6 are responsible for the cell’s progression through G1 phase (Fig. 1). A complex of cdk2 and cyclin E is responsible for the cell’s progression from G1 phase to S phase. A complex of cdk2 and cyclin A is required for the cell’s progression through S phase, and a complex of cdk1 (also known as cdc2) and cyclin B is required for mitosis (1). These complexes are in turn regulated by a stoichiometric combination with small inhibitory proteins called endogenous cdk inhibitors. The INK4 (inhibitor of cdk4) family includes p16ink4a, p15ink4b, p18ink4c, and p19ink4d, and its members specifically inhibit cyclin D-associated kinases. Members of the kinase inhibitor protein family p21waf1, p27kip1, and p57kip2 bind and inhibit the activity of complexes of cyclin E and cdk2 and complexes of cyclin A and cdk2 (13–15). Although members of the kinase inhibitor protein family were initially thought to exclusively regulate G1 and S phases, several reports (16–18) demonstrated that these proteins can also regulate the G2/M-phase transition.

DNA synthesis (S phase) begins with the cdk4- and/or cdk6-mediated phosphorylation of Rb protein (which is complexed with the transcriptional factor E2F). Phosphorylated Rb is released from its complex with E2F. The released E2F then promotes the transcription of numerous genes required for the cell to progress through S phase, including thymidylate synthase and dihydrofolate reductase, among others (19,20). Additional information about cell cycle regulation can be found in several reviews (21–24).

The vast majority of human cancers have abnormalities in some component of the Rb pathway (Fig. 2) because of hyperactivation of cdks resulting from the overexpression of positive...
cofactors (cyclins/cdk) or a decrease in negative factors (endogenous cdk inhibitors) or Rb gene mutations (Fig. 2). Therefore, a pharmacologic cdk inhibitor that could be used in "mechanism-based therapy" would be of great theoretical interest as a treatment for many neoplasms (25). This possibility is intriguing because, for cancer patients, the loss of endogenous cdk inhibitors confers poor prognosis. For example, loss of p27kip1 protein predicts a poor outcome in patients with breast, prostate, lung, colon, or gastric carcinoma [reviewed in (26)]. Loss of p16ink4a is clearly associated with poor prognosis in patients with non-small-cell lung cancer or melanoma (26). However, the results with p21waf1 are inconclusive; loss of p21 may be prognostic in certain cancers, but inconsistent results were obtained for breast cancer (26). If validated in larger clinical studies, these markers could be incorporated in the routine pathologic examination of many tumors to determine prognosis.

**Targets for Intervention in the Cell Cycle**

Several strategies for therapeutic intervention could modulate cdk activity (Fig. 3). These strategies are divided into direct efforts that target the catalytic cdk subunit or indirect efforts that target the regulatory pathways that govern cdk activity. Compounds that directly target the catalytic cdk subunit are chemical inhibitors (small molecule cdk inhibitors); these compounds provide the opportunity for rational design of drugs that interact specifically with the adenosine 5'-triphosphate (ATP)-binding site of cdks (27–29). Compounds may inhibit cdk activity by targeting the regulatory pathways that modulate the activity of cdks; by altering the expression and synthesis of the cdk/cyclin subunits or the cdk inhibitory proteins; by modulating the phosphorylation of cdks; by targeting cdk-activating kinase (cdk7); by affecting cdc25 and wee1/myt1 (Fig. 3); or by manipulating the proteolytic machinery that regulates the catabolism of cdk/cyclin complexes or their regulators.

**Indirect Cdk Modulators**

**cdk Inhibition by Overexpression of Endogenous cdk Inhibitors**

When the cdk inhibitor p16ink4A was introduced into a lung cancer cell line with defective endogenous p16ink4A, the cells

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**Fig. 1.** Schematic representation of the cell cycle showing pathways for various cyclin-dependent kinases (cdks). PCNA = proliferating cell nuclear antigen.

**Fig. 2.** Inactivation of the retinoblastoma (Rb) pathway in human cancer. P = phosphate; cdk = cyclin-dependent kinase. The inactivation of the Rb pathway may occur by mutation of the Rb gene itself or by the inactivation of Rb by phosphorylation with the cdks. Cdks can be activated by an increase in the amount of the catalytic enzyme; by an increase in the amount of cofactor (cyclins), or by a decrease in the amount of endogenous cdk inhibitors (i.e., p16ink4A).

**Fig. 3.** Possible targets for cyclin-dependent kinase (cdk) inactivation. CK1 = endogenous cdk inhibitor; ATP = adenosine 5'-triphosphate; PO4− = phosphate; T160 = threonine-160; T14 = threonine-14; Y15 = tyrosine-15. 1 = cyclin; 2 = CDK (cdk); 3 = CKI (cdk inhibitor); 4 = ATP; 5 = CDK7; 6 = Cdc25, a dual threonine-tyrosine phosphatase that removes phosphates from the negative phosphorylation sites, T14 and Y15; 7 = wee1/myt1, protein kinases that phosphorylate the negative phosphorylation sites, T14 and Y15. Compounds can be designed to decrease the amount of cofactors (1) or of catalytic subunit (1). Moreover, compounds may increase the expression of endogenous cdk inhibitors (3). Activation of wee1/myt1 (7) or inhibition of cdk7 (5) and cdc25 (6) leads to loss of cdk activity. Direct competitors of ATP at the cdk-binding site (4) may specifically modulate cdk activity.
were arrested in G1 phase (30). This effect occurs only in cells with functional Rb (31). When both p53 and p16INK4a were introduced into cells, apoptotic cell death followed (32). When p21WAF1 or p27KIP1 were introduced, *in vitro* and *in vivo* antitumor effects and G1/S arrest were observed (33,34). When p27 was introduced in several preclinical tumor models, apoptotic cell death was induced, but the relationship between the putative cdk inhibition of p27KIP1 and its induction of apoptotic cell death is unclear (35,36).

Several small molecule chemical inhibitors of cdks appear to modulate the expression of cdk inhibitors. For example, lovastatin blocks cells in G1 phase by induction of p21WAF1 and p27KIP1, which leads to the loss of cdk activity (37,38). Rapamycin blocks lymphocytes in G1 phase by preventing the interleukin 2-stimulated degradation of p27KIP1 (39). However, it is unclear whether the cell cycle inhibitory effects of these small molecule chemical inhibitors can be solely explained by the induction of p27KIP1.

**cdk Inhibition by Peptidomimetic-Based Approaches**

Another strategy to block cell cycle progression by loss of cdk activity is to use small peptides that mimic the effects of endogenous cdk inhibitors. Several carriers have been tested that introduce peptides into cells, including a 16-residue segment derived from the *Drosophila* antennapedia protein. When this 16-residue transmembrane carrier was linked to the third ankyrin repeat of the p16INK4A protein and inserted into cells, RB-dependent G1 arrest and cell senescence were observed (40). Hybrids containing the antennapedia peptide and different p21WAF1 peptides were constructed. In a breast-derived cell line, the chimera containing the carboxyl-terminal peptide of p21, amino acids 141–160, had a higher specificity for cdk4/cyclin D than for cdk2/cyclin E and arrested the cells in G1 phase (41). In contrast, *in vitro* the chimera containing amino-terminal peptides of p21, amino acids 17–33 and 63–77, inhibited both cdk1 and cdk2, and cells transduced with this chimera were arrested in all phases of the cell cycle (42).

Another approach to inhibiting cdk activity is to develop peptides that bind to cdks and inhibit cdk kinase activity (43,44). Colas et al. (43) demonstrated that a 20-amino acid peptide, identified by use of a combinatorial library, specifically binds cdk2 and inhibits its activity at low nanomolar concentrations *in vitro*. This peptide could act by blocking the interaction of the catalytic subunit with substrates or cyclin cofactors (43). Chen et al. (44) have shown that 8-amino acid peptides derived from the putative cyclin-cdk2-binding region of p21WAF1 (PVKRLFG) and E2F1 (PVKRLDL) linked to N-terminal residues derived from human immunodeficiency virus Tat protein or antennapedia protein can block cells in S phase. This effect was associated with a loss of cdk2 activity. Although all of the cells tested with these chimeras showed clear evidence of G1/S-phase arrest, immortalized/transformed cells were more prone to apoptotic cell death.

**cdk Inhibition by Depletion in cdk/Cyclin Subunits**

Antisense technology has been used to deplete messenger RNAs (mRNAs) for cdk and/or cyclins (45–47). When cyclin D1 was depleted from tumor cell lines, a substantial antiproliferative effect was observed that was synergistic with different standard chemotherapeutic agents (45).

Several compounds can inhibit tumor progression by the modulation of cdk/cyclin subunits. In breast carcinoma cell lines, antiestrogens, such as tamoxifen, inhibit the expression of cyclin D and other cell cycle-related proteins and inhibit cdk activity (48). In breast carcinoma cell lines, retinoids, such as all-trans-retinoic acid and 9-cis-retinoic acid, inhibit the expression of multiple cell cycle regulators, including cyclin D1, cyclin D3, cdk2, and cdk4 (49). In some model systems, rapamycin, an inhibitor of FKBP (FK-506-binding protein)/mTOR (mammalian target of rapamycin), was also associated with a decline in cyclin D1 protein (39). Although treatment of cells with any of these compounds may lead to the decline of cyclin proteins and the perturbation of other cell cycle-related proteins, it is unclear how these compounds act. Perhaps the changes result from a direct interaction between the drug and the pathways that regulate the production of cyclin/cdk or result from the G1-phase arrest and/or Rb dephosphorylation, which are observed with these compounds.

**cdk Inhibition by Modulation of Proteosomal Machinery**

Sequential turnover of certain cell cycle regulators, including cyclins and p27KIP1, is mediated by the 20S proteasome, which promotes proteolytic degradation through the ubiquitin/proteasome pathway. Increased turnover of cyclins with the associated loss of cdk activity may lead to cell cycle arrest with or without apoptotic cell death. Inhibiting 20S proteasome-mediated degradation could lead to accumulation of cdk inhibitors, such as p27KIP1, and to cell cycle arrest with or without apoptotic cell death (50). An important unresolved issue is the net effect and/or specificity of modulating proteasomal pathways. Nonspecific proteasome modulation could alter many signaling pathways (by the accumulation of proteins that activate or inhibit cdks) and thus could have a final effect on cells that is difficult to predict.

**cdk Activation by Modulation of Upstream Phosphatases/Kinasess**

The abrogation (overriding) of intact cell cycle checkpoints by upstream phosphatases and/or kinases could induce the “inappropriate acceleration” of certain phases of the cell cycle. For example, when the G2-phase checkpoint is activated by genotoxic stress (i.e., γ-irradiation), the G2 period is extended to allow DNA repair. In the presence of agents that abrogate (override) this checkpoint, premature mitosis occurs, resulting in apoptotic cell death (51). Thus, abolishing the G2 checkpoint might sensitize cells to agents that would normally cause cells to pause or arrest in G2 phase (51,52).

**DIRECT cdk MODULATORS (SMALL MOLECULAR cdk INHIBITORS)**

Chemical (small molecular) cdk inhibitors can be subdivided into the following eight families (Fig. 4): 1) purine derivatives (isopentenyladenine, 6-dimethylaminopurine, olomoucine, rosovitiine, CVT-313, and purvalanol and its derivatives), 2) butyrolactone I, 3) flavopiridols (flavopiridol and deschloroflavopiridol), 4) staurosporines (staurosporine and UCN-01), 5) toyocamycin, 6) 9-hydroxyellipticine, 7) polysulfates (suramin), and 8) paullones. Not all small molecular cdk inhibitors are specific for cdks. In fact, staurosporine, UCN-01, suramin, 6-dimethylaminopurine, and isopentenyladenine are relatively nonspecific protein kinase inhibitors. In contrast, flavopiridol, bu-
tyrolactone I, olomoucine, roscovitine, CVT-313, paullones, and paullone derivatives are clearly more selective for cdks. Butyrolactone I, olomoucine, roscovitine, CVT-313, purvalanol, and paullone derivatives are relatively selective for cdk1 and cdk2 but are relatively inactive for cdk4 and cdk6. Flavopiridol can inhibit all cdks tested (53–55).

Olomoucine, Roscovitine, and Other Purine Derivatives

The first cdk inhibitor discovered was dimethylaminopurine (56). This compound was initially shown to inhibit mitosis in sea urchin embryos without inhibiting protein synthesis. Later, dimethylaminopurine was shown to inhibit cdk1 activity (IC\textsubscript{50} [concentration that inhibits activity by 50%] \approx 120 \mu M) but to be relatively nonspecific (27). Isopentenyladenine, a derivative of dimethylaminopurine, was somewhat more potent and selective for the cdks (IC\textsubscript{50} \approx 55 \mu M) (57). Other active purine derivatives have been identified in screening campaigns for more specific and potent cdk inhibitors. Olomoucine potently inhibited cdk1 and cdk2 activities (IC\textsubscript{50} = 7 \mu M) (27,57). Roscovitine, a derivative of olomoucine, is a more potent cdk inhibitor (IC\textsubscript{50} values for cdk1/cdk2 = 0.7 \mu M) (27).

The crystal structures of cdk2 complexed with isopentenyladenine, olomoucine, or roscovitine showed that all three inhibitors bind to the ATP site (29,58). CVT-313, another purine analogue, was identified by use of a combinatorial library strategy and the crystal structure of cdk2. Similar to previous analogues, CVT-313 was specific for cdk1 and cdk2 with IC\textsubscript{50} values of 4.2 and 1.5 \mu M, respectively (59).

A combinatorial approach was then used to modify the purine scaffold of 2-fluoro-6-chloropurine, with isopentenyladenine, olomoucine, or roscovitine to cdk2. The more membrane-permeable purvalanol-A was tested on the National Cancer Institute’s (NCI’s) 60-cell line anticancer drug screen panel. The average IC\textsubscript{50} value of purvalanol-A was 2 \mu M, demonstrating that it was a more active antiproliferative agent than purvalanol-B (60). Cell cycle studies of purvalanol-A on human fibroblasts showed that it arrested cells in G\textsubscript{i}/S phase and G\textsubscript{2}/M phase, compatible with the putative inhibitory properties in cdk1 and cdk2, respectively (60).

Paullones

With the use of the antiproliferative in vitro profile of flavopiridol in NCI’s anticancer drug screen panel and the computational algorithm COMPARE, several members of the paullone family were identified (61). Kenpaullone (NSC 664704) potently inhibited cdk1/cyclin B (IC\textsubscript{50} = 0.4 \mu M), cdk2/cyclin A (IC\textsubscript{50} = 0.68 \mu M), cdk2/cyclin E (IC\textsubscript{50} = 7.5 \mu M), and cdk5/p35 (IC\textsubscript{50} = 0.85 \mu M) but had much lower activity toward other kinases (28). Kenpaullone competitively inhibits the binding of ATP, with an apparent K\textsubscript{i} (i.e., inhibitory constant) for cdk1/cyclin B of about 2.5 \mu M. Molecular modeling studies demonstrated that kenpaullone may bind to the ATP-binding site with residue contacts similar to other cdk2 inhibitors (28). Cell cycle effects of kenpaullone were characterized with the MCF10A breast epithelial cell line. Cells were synchronized in G\textsubscript{i}/G\textsubscript{1} phase by serum starvation and then stimulated to re-enter the cell cycle in the presence of vehicle or kenpaullone at its approximate IC\textsubscript{50} concentration (30 \mu M). Twenty hours later, vehicle-treated cells entered S phase. However, cells exposed to kenpaullone were arrested at the G\textsubscript{i}/S boundary. A similar effect was obtained with another paullone analogue, 10-bromo-kenpaullone (NSC 672234) (28).

CONSEQUENCES OF CDK INHIBITION

Cell Cycle Arrest

Initial cell cycle studies by van den Heuvel and Harlow (62) demonstrated that ectopic expression of cdk1-dominant negative
alleles was able to block U2OS osteosarcoma cell lines at the G_{2}/M-phase boundary. In contrast, expression of dominant negative alleles of cdk2 or cdk3 blocked cells in S phase (62). Thus, roles for each cdk in human cell cycle began to be assigned.

Ectopic expression of endogenous cdk inhibitors (such as p16, p21, or p27) or peptidomimetics derived from p21, p16, or E2F1, as described above in detail, demonstrates the feasibility of using this method to arrest cells in the cell cycle.

As described above, several small molecular cdk inhibitors, including roscovitine, olomoucine, purvalanol, and flavopiridol, arrest cells at either the G_{1}/S- or the G_{2}/M-phase boundaries (53,59,60,63,64). It is unclear why these agents arrest some cells in G_{1}/S phase and other cells in G_{2}/M phase or both.

**Apoptotic Cell Death**

The antiproliferative effects of olomoucine, flavopiridol, and roscovitine were accompanied by the induction of apoptotic cell death in certain cell types (64–66). In one study (66), the ability of flavopiridol and olomoucine to induce apoptotic cell death varied, depending on the growth status of the cells. That is, flavopiridol or olomoucine protected postmitotic nondividing PC12 neuronal cells from apoptotic cell death after the withdrawal of nerve growth factor. However, flavopiridol did not protect cycling PC12 cells from apoptotic cell death after the withdrawal of nerve growth factor (66). Similarly, cdk4 and cdk6 proteins from dominant negative alleles, but not cdk2 or cdk3 proteins from dominant negative alleles, protected neurons from apoptotic cell death after the withdrawal of nerve growth factor (67). Thus, susceptibility of PC12 cells to flavopiridol- and olomucine-induced apoptotic cell death may vary, depending on the growth state of the cells.

HeLa cervical carcinoma cells treated with staurosporine and tumor necrosis factor-α were protected from apoptotic cell death by cdc2, cdk2, or cdk3 encoded by dominant negative alleles. However, only cdk2 encoded by a dominant negative allele protected cells from apoptotic cell death induced by ectopic expression of topoisomerase-IIα (68). Finally, certain apoptotic stimuli induce the caspase-mediated cleavage in endogenous cdk inhibitors (p21/p27) or cdk-inhibitory proteins (wee1 and cdc27) leading to activation of cdk5 (69–71). Thus, cell cycle arrest and/or apoptosis induced by the inhibition of cdk5 depends on several factors, including the mechanism of inhibition, the type of cells, and the proliferation status of the cells.

**Differentiation**

During differentiation, cells exit the cell cycle and lose cdk2 activity. Lee et al. (72) tested whether the chemical cdk2 inhibitors flavopiridol and roscovitine could induce a differentiated phenotype by exposing NCI-H358 lung carcinoma cells to a cdk2 antisense construct, flavopiridol, or roscovitine. They observed that all three cdk2 inhibitors could induce mucusin differentiation with the loss of cdk2 activity.

When U937 myelomonocytic leukemia cells were treated with aminopurvalanol, the cells acquired a phenotype characteristic of differentiated macrophages. Moreover, aminopurvalanol, a potent inhibitor of cdk1 and cdk2, appeared to arrest cells at the G_{2}/M boundary and then to induce apoptotic cell death (73). Other investigators (74) observed a similar phenomenon; ectopic expression of p21^{WAF1} or p27^{KIP1} resulted in a “differentiated phenotype” with cells arrested in G_{1} or G_{2} phases and a 4N amount of DNA.

**Transcriptional Effects**

To compare the effects of several chemical cdk inhibitors on the expression of complementary DNA in yeast cells, Gray et al. (60) incubated Saccharomyces cerevisiae with compound 52 and flavopiridol (each at 25 μM) for 2 hours and measured mRNA by oligonucleotide array methods. Two percent to 3% of the 6200 yeast genes examined showed a greater than twofold change in transcript levels in the presence of these agents. Moreover, almost 50% of affected transcripts were affected by both compound 52 and flavopiridol. These genes fell into distinct groups, including genes that regulate progression of cell cycle, genes that regulate phosphate and cellular energy metabolism, and genes that regulate guanosine 5’-triphosphate (GTP)- or ATP-binding proteins. However, more than 40% of the changes in mRNA were not concordant between flavopiridol and compound 52. These discrepancies might be explained 1) by the broad cdk-inhibitory activity of flavopiridol compared with the selective cdk2/cdk1-inhibitory activities of compound 52, 2) by the different intracellular concentrations achieved by these inhibitors, 3) by the distinctive molecular structures of these inhibitors, or 4) from their putative effects on other cellular targets.

**PRECLINICAL PHARMACOLOGY OF FLAVOPIRIDOL**

Of the cdk inhibitors, flavopiridol has advanced the farthest toward clinical applications.

**Mechanism of Action**

Flavopiridol, also known as L86-8275 or HMR 1275, is a semisynthetic flavonoid derived from rohitukine, an alkaloid isolated from a plant indigenous to India (Fig. 4). Table 1 contains a summary of the most important preclinical in vitro effects of flavopiridol. Initially, flavopiridol displayed modest activity in vitro as an inhibitor of tyrosine kinase of the epidermal growth factor receptor and an inhibitor of protein kinase A (IC_{50} = 21 and 122 μM, respectively) (75). However, when flavopiridol was tested in the NCI’s 60-cell-line anticancer drug screen panel, its IC_{50} was 66 nM. This concentration is about 1000 times lower than the concentration required to inhibit protein kinase A and the tyrosine kinase of the epidermal growth factor receptor (75). The antiproliferative effect was not associated with the presence of the epidermal growth factor receptor (75,76). Flavopiridol was shown to arrest cells in G_{1} phase or at the G_{2}/M boundary, raising the possibility that flavopiridol may

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<td><strong>Effect</strong></td>
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<td>Epidermal growth factor receptor tyrosine kinase inhibition</td>
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*IC_{50} = concentration that inhibits growth or activity by 50%; NCI = National Cancer Institute; DTP = Developmental Therapeutics Program; VEGF = vascular endothelial growth factor.
inhibit cdk2 and cdk1 (76). Studies using purified cdks showed that flavopiridol inhibits the activities of cdk1, cdk2, and cdk4; this inhibition is competitively blocked by ATP, with a $K_i$ of 41 nM (53,54,76–78). The crystal structure of the complex of deschloroflavopiridol and cdk2 showed that flavopiridol binds to the ATP-binding pocket, with the benzopyran occupying the same region as the purine ring of ATP (79). This observation was concordant with our earlier biochemical studies with flavopiridol (54). Flavopiridol inhibits all cdks thus far examined (IC$_{50}$ = approximately 100 nM), but it inhibits cdk7 (cdk7-activating kinase) less potently (IC$_{50}$ = approximately 300 nM) (53,54,77).

In addition to directly inhibiting cdks, flavopiridol causes a decrease in the level of cyclin D1, an oncogene that is overexpressed in many human neoplasias. Neoplasms that overexpress cyclin D1 have a poor prognosis (80–82). When MCF-7 human breast carcinoma cells were incubated with flavopiridol, levels of cyclin D1 protein decreased within 3 hours (83). This effect was followed by a decline in the levels of cyclin D3 with no alteration in the levels of cyclin D2 and cyclin E, the remaining G1 cyclins, and then 2 hours later, loss of cdk4 activity occurred. Thus, depletion of cyclin D1 appears to lead to the loss of cdk4 activity (83). The depletion of cyclin D1 is caused by depletion of cyclin D1 mRNA, not by shortening the half-life of the protein. Depletion of cyclin D1 mRNA was associated with a specific decline in cyclin D1 promoter, measured by a luciferase reporter assay (83). The transcriptional repression of cyclin D1 observed after treatment with flavopiridol is consistent with the effects of flavopiridol on yeast cells (see above) and underscores the conserved effect of flavopiridol on eukaryotic cyclin transcription (60). In summary, flavopiridol can induce cell cycle arrest by at least three mechanisms: 1) by direct inhibition of cdk activities by binding to the ATP-binding site; 2) by prevention of the phosphorylation of cdks at threonine-160/161 by inhibition of cdk7/cyclin H (77,78); and 3) for G1-phase arrest, by a decrease in the amount of cyclin D1, an important cofactor for cdk4 and cdk6 activation.

Another important action of flavopiridol is the selective induction of apoptotic cell death. Hematopoietic cell lines are often quite sensitive to flavopiridol-induced apoptotic cell death (65,84–86), but the mechanism(s) by which flavopiridol induces apoptosis have not yet been elucidated. Flavopiridol does not modulate topoisomerase II activity (65). In certain hematopoietic cell lines, neither BCL-2/BAX nor p53 appeared to be affected (65,85), whereas, in other systems, BCL-2 may be inhibited (86). It is unclear whether the putative flavopiridol-induced inhibition of cdk activity is required for induction of apoptosis.

Cell cycle arrest, but again with clear evidence of apoptotic cell death, was observed with a panel of head and neck squamous cell carcinoma cell lines, including a cell line (HN30) that is refractory to several DNA-damaging agents, such as γ-irradiation and bleomycin (87). The apoptotic effect was independent of p53 status and was associated with the depletion of cyclin D1 (87). These findings have been corroborated in other preclinical models (88–90). Efforts to understand flavopiridol-induced apoptosis are under way.

To determine whether flavopiridol has antiangiogenic properties, Brusellel et al. (91) incubated human umbilical vein endothelial cells (HUVECs) with flavopiridol and observed apoptotic cell death even in cells that were not cycling. In another report, Kerr et al. (92) tested flavopiridol in an in vivo angiogenesis model and found that flavopiridol decreased blood vessel formation in the mouse Matrigel model of angiogenesis. Melillo et al. (93) demonstrated that, at low nanomolar concentrations, flavopiridol blunted the induction of vascular endothelial growth factor (VEGF) by hypoxia in human monocytes. This effect was caused by a decreased stability of VEGF mRNA, which paralleled the decline in VEGF protein. Thus, the antitumor activity of flavopiridol may be supplemented by antiangiogenic effects. Whether the various antiangiogenic actions of flavopiridol result from its interaction with a cdk target or other targets requires further study.

To test for synergistic effects with other compounds, cytotoxic assays of flavopiridol in combination with standard chemotherapeutic agents were performed (94,95). Synergistic effects in A549 lung carcinoma cells were demonstrated when treatment with flavopiridol followed treatment with paclitaxel, cytarabine, topotecan, doxorubicin, or etoposide. In contrast, a synergistic effect was observed with 5-fluorouracil only when cells were treated with flavopiridol for 24 hours before addition of 5-fluorouracil. Synergistic effects with cisplatin were not schedule dependent (95). However, Chien et al. (88) failed to demonstrate a synergistic effect between flavopiridol and cisplatin and/or γ-irradiation in bladder carcinoma models.

Several questions about the antiproliferative activity of flavopiridol remain unanswered. Why does treatment with flavopiridol cause some cells to arrest at the G1/S-phase boundary and other cells to arrest at the G2/M-phase boundary? What role does the depletion of D-type cyclins play in flavopiridol-induced G1 arrest? What is the relationship between cdk inhibition and apoptotic cell death, and what are the targets for flavopiridol-induced apoptotic cell death?

Antitumor Effect in Preclinical Models

Experiments using colorectal (Colo205) and prostate (LnCap/DU-145) carcinoma xenograft models in which flavopiridol was administered frequently over a protracted period demonstrated that flavopiridol is cytostatic (75). This demonstration led to clinical trials in humans of flavopiridol administered as a 72-hour continuous infusion every 2 weeks (96) (see below).

Subsequent studies in some models of human leukemia/lymphoma xenografts demonstrated that flavopiridol administered intravenously as a bolus rendered animals tumor free, whereas flavopiridol administered as an infusion only delayed tumor growth (84). In head and neck (HN-12) xenografts, when flavopiridol was administered as an intraperitoneal bolus daily at 5 mg/kg for 5 days, a substantial growth delay was observed (87). Again, apoptotic cell death and cyclin D1 depletion were observed in tissues from xenografts treated with flavopiridol at peak plasma concentrations of 5–8 μM (84). Based on these results, the feasibility of a phase I trial with the administration of flavopiridol as a 1-hour infusion is currently being explored at the NCI (see below).

Preclinical Pharmacokinetics and Toxicology

Murine plasma concentration–time profiles for flavopiridol exhibited biexponential behavior with mean α and β half-lives of 16.4 and 201.0 minutes, respectively. The mean total-body plasma clearance was 22.6 mL/minute per kg, and the mean oral bioavailability after bolus intragavage was approximately 20%. Pharmacokinetic studies in dogs (75) had very similar results.

The metabolism of flavopiridol was investigated in isolated
liver perfusion models. Flavopiridol was glucuronidated in the liver, and then this flavopiridol metabolite excreted in the biliary tract. This property underlies flavopiridol’s propensity to undergo enterohepatic circulation (97,98). Preclinical pharmacologic and toxicologic evaluations have identified dose-limiting toxic effects as reversible hematopoietic and gastrointestinal effects.

**Human Clinical Trials of Flavopiridol**

Two clinical trials of flavopiridol given as a 72-hour continuous infusion every 2 weeks have been completed (96,99). In the NCI phase I trial of infusional flavopiridol, 76 patients were treated. Dose-limiting toxicity was secretory diarrhea with a maximal tolerated dose of 50 mg/m² per day for 3 days.

In the presence of anti-diarrheal prophylaxis (a combination of cholestyramine and loperamide), patients tolerated higher doses, defining a second maximal tolerated dose, 78 mg/m² per day for 3 days. The dose-limiting toxicity observed at the higher dose level was reversible hypotension and a substantial proinflammatory syndrome (fever, fatigue, local tumor pain, and modulation of acute-phase reactants) (96).

Tumors in one patient with non-Hodgkin’s lymphoma, one patient with colon cancer, and one patient with kidney cancer decreased in size (minor responses = shrinkage of <50%) for more than 6 months. Moreover, one patient with refractory renal cancer achieved a partial response (shrinkage of >50% of masses) (96). Of 14 patients who received flavopiridol for more than 6 months, five patients received flavopiridol for more than 1 year and one patient received flavopiridol for more than 2 years (96). This potential “disease stabilization,” which may have been noted in this trial, is consistent with preclinical models, where tumor stasis is observed. Appropriate measurements of cytostatic effects are necessary to confirm that cdk inhibition might be related to this clinical outcome. Plasma concentrations of 300–500 nM flavopiridol, which inhibit cdk activity in vitro, were safely achieved during our trial (96).

In a complementary phase I trial also exploring the use of a 72-hour continuous infusion of flavopiridol every 2 weeks, Thomas et al. (99) found that the dose-limiting toxicity is diarrhea, corroborating the experience of the NCI. Moreover, plasma concentrations of 300–500 nM flavopiridol were also observed. It is interesting that there was one patient in this trial with refractory gastric cancer that had metastasized to the liver who was initially treated surgically and subsequently failed to respond to one treatment regimen of 5-fluorouracil. When treated with flavopiridol, this patient achieved a sustained complete response without any evidence of disease for more than 2 years after treatment was completed.

In September 1998, we began the first phase I trial of a daily 1-hour infusion of flavopiridol for 5 consecutive days every 3 weeks. This dose schedule was based on our antitumor results observed in leukemia/lymphoma and head and neck xenografts treated with flavopiridol (see above). At this time, 27 patients have been treated in this phase I trial. The recommended phase II dose is 37.5 mg/m² per day for 5 consecutive days. Dose-limiting toxic effects observed at 52.5 mg/m² per day are nausea/vomiting, neutropenia, fatigue, and diarrhea. Other (non-dose-limiting) side effects are “local tumor pain” and anorexia (Senderowicz AM: unpublished results). To reach higher flavopiridol concentrations, the protocol was amended to administer flavopiridol for 3 days only. Higher peak plasma flavopiridol concentrations (approximately 4 μM) may be obtained with this schedule (Senderowicz AM: unpublished results).

A phase I trial testing the combination of paclitaxel and flavopiridol demonstrated good tolerability with a dose-limiting pulmonary toxicity (100).

Phase II trials of flavopiridol given as a 72-hour continuous infusion to patients with chronic lymphocytic leukemia, non-small-cell lung cancer, non-Hodgkin’s lymphoma, or colon, prostate, gastric, head and neck, or kidney cancer, etc., and phase I trials of flavopiridol administered on novel schedules and in combination with standard chemotherapeutic agents are being explored (101–104).

Several important clinical questions remain to be answered in these trials. Is flavopiridol an “effective” anticancer agent? Which is the best schedule for flavopiridol monotherapy? What is the best method to combine flavopiridol and other agents? Which is the most reliable pharmacodynamic parameter to follow in patients? How should “stable disease” be defined in phase II trials?

**Preclinical Pharmacology of UCN-01**

Staurosporine is a nonspecific protein kinase inhibitor that arrests cell cycle progression in transformed and nontransformed cells at 1–100 nM (105). At similar concentrations, staurosporine inhibits many protein and tyrosine kinases (105). Several analogues of staurosporine have been evaluated to identify compounds with greater specificity for protein kinases.

**Mechanism of Action**

One staurosporine analogue, UCN-01 (7-hydroxystaurosporine; Fig. 4), has potent activity against several protein kinase C isoenzymes, particularly the Ca²⁺-dependent protein kinase C in vitro (110). At similar concentrations, staurosporine has lower potency against the novel Ca²⁺-independent protein kinases C (IC₅₀ = approximately 500 nM) and no effect against the atypical protein kinases C (106–108), similar to the activity of staurosporine. In addition to its effects on protein kinase C, UCN-01 has antiproliferative activity in several human tumor cell lines (109–113). In contrast, another highly selective potent protein kinase C inhibitor, GF 109203X, has a modest antiproliferative activity, despite a similar capacity to inhibit protein kinase C in vitro (110). Thus, these results suggest that the antiproliferative activity of UCN-01 is probably not explained solely by inhibition of protein kinase C. UCN-01 moderately inhibited the activity of immunoprecipitated cdk1 (cdc2) and cdk2 (IC₅₀ = 300–600 nM). However, when intact cells were exposed to UCN-01, “inappropriate activation” of the same kinases occurred (110).

Experimental evidence suggests that DNA damage leads to cell cycle arrest to allow DNA repair. In cells where the G₁-phase checkpoint is not active because of p53 inactivation, irradiated cells accumulate in G₂ phase because the G₂ checkpoint is mediated by the inactivation of cyclin B/cdc2 by wee1 kinase (Fig. 5, A). In contrast, UCN-01 (Fig. 5, B) induces the activation of cdc2/cyclin B and thus promotes cells to enter early mitosis with the onset of apoptotic cell death. These effects could be partially explained by the inactivation of wee1, the kinase that negatively regulates the G₂/M-phase transition or activation of cdc25 phosphatase (114). Thus, although UCN-01 at high concentrations can directly inhibit cdks in vitro, UCN-01 can modulate cellular upstream regulators at much lower concentration, leading to inappropriate cdc2 activation by acting on...
targets that remain to be defined. DNA-damaging agents not only can activate the G2-phase checkpoint but also can activate the S-phase checkpoint (115). Studies from other groups suggest that UCN-01 not only is able to abrogate the G2 checkpoint induced by DNA-damaging agents but also, in some circumstances, is able to abrogate the DNA damage-induced S-phase checkpoint (115).

Another pharmacologic feature of UCN-01 is the increased cytotoxicity in cells containing mutated p53 genes (51). In CA-46 Burkitt’s lymphoma and HT-29 colon carcinoma cell lines carrying mutated p53 genes, cytotoxicity results when these cells are exposed to UCN-01. Compared with the isogenic wild-type MCF-7 cell line, the MCF-7 cell line with no endogenous p53 because of the ectopic expression of E6, a human papillomavirus type-16 protein, showed enhanced cytotoxicity when treated with a DNA-damaging agent, such as cisplatin, and UCN-01. The synergistic effect of UCN-01 is enhanced with many chemotherapeutic agents, including mitomycin C, 5-fluorouracil, carmustine, and camptothecin, among others (116–122). Therefore, it is possible that combining UCN-01 with these and other agents could improve its therapeutic index.

Another interesting aspect of UCN-01 is its ability to arrest cells in G1 phase of the cell cycle (109,112,113,123–125). Human epidermoid carcinoma A431 cells contain mutated p53. When incubated with UCN-01, these cells were arrested in G1 phase, Rb was hypophosphorylated, and p21<sup>wafl</sup> and p27<sup>kip1</sup> accumulated (113). However, in another report (125), the antiproliferative effect of UCN-01 was not dependent on the functional status of Rb. Thus, the G1 arrest observed with UCN-01 is apparently independent of the status of p53 and Rb. Further studies on the putative target(s) for UCN-01 in the G1-phase arrest of cells are under way.

Courage et al. (126) found that UCN-01-resistant A549 lung carcinoma cells were sensitive to two other protein kinase C inhibitors, CGP 41251 and Ro 31-8220, and were marginally resistant (about twofold) to etoposide. These UCN-01-resistant cells had lost several protein kinase C isoenzymes, protein kinase C α, ε, and θ. However, the levels of these isoenzymes returned to baseline when these resistant cells were cultured in UCN-01-free medium. Thus, as described above, it is unlikely that the protein kinase C family of signaling proteins is the only target for UCN-01 cytotoxicity (110,126).

Although many important questions have been answered, several questions remain. What is the real target for UCN-01 in G1/S-phase arrested cells? Does protein kinase C play any role in UCN-01-induced cell cycle arrest and/or apoptotic cell death? What is the real target for the G2 checkpoint abrogation?

Spectrum of In Vivo Antitumor Activity

UCN-01 administered by an intravenous or intraperitoneal route displayed antitumor activity in xenograft model systems with breast carcinoma (MCF-7), renal carcinoma (A498), and leukemia (MOLT-4 and HL-60) cell lines (Senderowicz AM: unpublished results). The antitumor effect was greater when UCN-01 was given over a longer period. This requirement for a longer period of treatment was also observed in in vitro models, with highest antitumor activity observed when UCN-01 was present for 72 hours (109). Thus, a clinical trial using a 72-hour continuous infusion every 2 weeks was conducted (described below).

Preclinical Pharmacokinetics and Toxicology

Pharmacokinetics and toxicologic studies using several schedules were done in rats and dogs. When beagle dogs were given a 72-hour continuous infusion of UCN-01, local (site of injection) and gastrointestinal toxic effects were dose-limiting and a steady-state plasma concentration of 330 nM UCN-01 was achieved. Pharmacokinetic parameters were a volume of distribution (6.09 L/kg), a total clearance of 0.6 L/kg per hour with a β half-life of about 12 hours (127).

Phase I Clinical Trials of UCN-01

We recently completed the first phase I trial of UCN-01 in humans (128). Clinical features of UCN-01 observed included the unexpectedly long half-life (approximately 30 days). This half-life was 100 times longer than the half-life observed in preclinical models, which was probably caused by the avid bind-
ing of UCN-01 to α1-acid glycoprotein (129,130). Other clinical features were the relative lack of myelotoxicity or gastrointestinal toxicity (prominent side effects observed in animal models), despite very high plasma concentrations achieved (35–50 μM) (128–131). Dose-limiting toxic effects were nausea/vomiting (amenable to standard antiemetic treatments), symptomatic hyperglycemia associated with an insulin-resistance state, and pulmonary toxicity characterized by substantial hypoxemia without obvious radiologic changes. The recommended phase II dose of UCN-01 given on a 72-hour continuous infusion schedule was 42.5 mg/m² per day (131). One patient with refractory metastatic melanoma developed a partial response that lasted 8 months. Tumors in a few patients with leiomyosarcoma, non-Hodgkin’s lymphoma, or lung cancer were stabilized (≥6 months) (131). The concentration of “free” UCN-01 was assessed in saliva. At the recommended phase II dose, concentrations of free UCN-01 that may cause G2 checkpoint abrogation can be achieved. Table 2 compares important clinical and pharmacologic features of the cdk modulators flavopiridol and UCN-01. Future trials are being explored in which UCN-01 would be given by infusion for shorter periods (132) and/or in combination with DNA-damaging agents.

### Summary

With knowledge of the role of cdks in cell cycle regulation and the discovery that approximately 90% of all neoplasias are associated with “cdk hyperactivation” leading to the inactivation of the Rb pathway (2), novel cdk inhibitors are being developed. The first two modulators of cdk function tested in clinical trials, flavopiridol and UCN-01, have been observed to reach plasma concentrations that can modulate cdk-related functions. Future clinical trials should determine what is the best schedule for administering chemical cdk inhibitors, should determine what is the best combination of chemical cdk inhibitors and standard chemotherapeutic agents, and should demonstrate cdk modulation in tumor samples from patients treated with cdk inhibitors.

### References


**NOTES**

_Editor’s note:_ E. A. Sausville is a participant in the National Cancer Institute’s Cooperative Research and Development Agreement with Hoechst Marion Roussel that manufactures flavopiridol.

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