Flavopiridol: pleiotropic biological effects enhance its anti-cancer activity

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Flavopiridol has potent anti-proliferative properties due to its direct action of binding to the ATP-binding pocket of cyclin-dependent kinases (cdks), and due to its indirect action reducing levels of other cyclins and cdk inhibitors, contributing to its pleiotropic effects. Flavopiridol is a potent apoptotic agent due to its ability to cause cell death in cycling as well as non-cycling tumor cells; to down-regulate important cell survival proteins, such as survivin, through inhibition of the phosphorylation of Thr34; to increase sensitivity for S phase cells to drug treatment by modulating E2F-1 transcription factor activity in tumor cells; to induce both caspase-dependent and -independent mitochondrial cell death pathways; and to inhibit the activation of p-Akt which in turn inhibits activation of NF-κB. Flavopiridol possesses several important anti-angiogenic activities including induction of apoptosis of endothelial cells; inhibition of the hypoxic induction of vascular endothelial growth factor and/or its production under hypoxic conditions through inhibition of HIF-1α transcription; and decreased secretion of matrix metalloproteinases that is linked with significant inhibition of invasive potential in Matrigel assays. Taken together, the anti-proliferative and anti-angiogenic properties of flavopiridol may contribute to its anti-tumor activities observed in several preclinical animal models of human cancers including prostate, lymphoid, head and neck, colon, and glioma. These promising preclinical observations opened the way for phase I and II clinical trials. Given the low toxicity profile of flavopiridol used as a single agent in patients, combination therapy now offers numerous opportunities in the near future to improve the efficacy of flavopiridol in the treatment of refractory cancers. *Anti-Cancer Drugs* 15:411–419 © 2004 Lippincott Williams & Wilkins.

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Introduction

An effective anti-cancer agent should target features associated with the unrestrained growth of tumor cells such as their insensitivity to positive and negative growth control signals, their insensitivity to apoptotic signals, and their ability to invade tissues and to sustain angiogenesis. Flavopiridol has pleiotropic effects as an anti-cancer agent associated with its ability to induce growth arrest, to induce apoptosis in both cycling and non-cycling tumor cells, to inhibit cell migration, and to inhibit angiogenic activity. Flavopiridol inhibits tyrosine and serine kinases, particularly cyclin-dependent kinases (cdks) [1–4]. Importantly, clinical treatment of patients with flavopiridol has not been associated with the development of drug resistance. This is in contrast with the protein tyrosine kinase inhibitor STI-571 (Gleevec) that, although very successful in the clinic, has been linked with eventual emergence of drug-resistant tumor cells [5]. The aim of this review will focus on the current understanding of the molecular mechanisms associated with the anti-cancer activity of flavopiridol in several different test systems. In addition, the use of flavopiridol in clinical trials alone or in combination with other treatments will be summarized.

Flavopiridol (NSC649890; (–)*cis*–5,7-dihydroxy-2-(2-chlorophenyl)-8[4-(3-hydroxy-1-methyl)-piperidinyl]-4H-1-benzopyran-4-1) is a semi-synthetic flavonoid derived from the bark of rohitukin, an indigenous plant from India [1]. It was first discovered in the late 1980s in a screen of natural products that inhibited phosphorylation of the epidermal growth factor receptor (EGFR) [1]. Screening in the mid-1990s for activity on the National Cancer Institute (NCI) panel of 60 human cancer cell lines showed that flavopiridol was a potent inhibitor of cell growth [1,2]. Cdks regulate the cell cycle and consist of a catalytic subunit (cdk) complexed together with their respective regulatory unit (cyclin). The ATP-binding pocket on the catalytic subunit in the cdk/cyclin complex, when phosphorylated, activates the kinase activity. Flavopiridol inhibition of cdk activity is direct, in that it targets the ATP-binding pocket of the catalytic subunit that can be competitively blocked by ATP [6].

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However, in addition to inhibition of cdks (cdks 1, 2, 4/6, 7 and 9) at submicromolar concentrations, flavopiridol inhibits the activity of several other protein kinases contributing to its pleiotropic effects [1,2,7,8]. At higher concentrations flavopiridol can inhibit signal transducing kinases protein kinase A (PKA), PKC and Erk-1, the receptor tyrosine kinase EGFR, and receptor associated protein kinases such as c-Src [2,3]. Thus, the ability of flavopiridol to inhibit several different classes of cellular kinases involving many different signaling pathways underlies the complex mechanisms of action of this drug that may account, in part, for its potent anti-cancer activity.

Anti-proliferative activities

Through direct inhibition of cdk activity, flavopiridol arrests cells either at the G_1/S or G_2/M phases of the cell cycle. Regulation of the cell cycle also requires negative regulators such as the cdk inhibitor $p16^{ink4a}$ that specifically inhibits cyclin D-associated kinases with cdk4/6 or $p21^{Cip1}$ and $p27^{Kip1}$ that inhibit cyclin E/A-associated kinases with cdk2. Flavopiridol also acts indirectly to inhibit cell proliferation by decreasing levels of expression of cyclin D_1, $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ [4,9–15]. Decreased expression of cyclin D_1 serves to decrease cdk4 and its kinase activity, resulting in accumulation of hypophosphorylated Rb, favoring growth arrest. Similarly, the $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ cdk inhibitors that regulate progression of cells from G_1 to S phase through inhibition of cdk2 are decreased, resulting in G_1 growth arrest. Another effect of reduced $p21^{Waf1/Cip1}$ expression enhances sensitivity of cells to undergo flavopiridol-induced apoptosis, since $p21^{Waf1/Cip1}$ can negatively regulate drug-induced changes in mitochondria [16]. The indirect effect of flavopiridol on reducing levels of gene expression is associated with reduced mRNA transcript levels [4,7,11]. This is due to inhibition by flavopiridol of the transcription elongation factor b (P-TEFb) or cdk9/cyclin T1 that results in decreased transcription by RNA polymerase II [7,8]. In particular, flavopiridol selectively affects the transcription of genes that have short mRNA half-lifes, a characteristic of MDM2 and cyclin D_1 genes [11,17,18].

In summary, flavopiridol has potent anti-proliferative properties due to its direct action of binding to the ATP-binding pocket of cdks, and due to its indirect action reducing levels of other cyclins and cdk inhibitors favoring growth arrest.

The mechanisms by which flavopiridol induces apoptosis are diverse. Although the cytostatic activity of flavopiridol was appreciated originally [1], its potent cytotoxic activity in many different cell types is now well known [2,4]. Importantly, flavopiridol can induce apoptosis in non-cycling cells [19]. This is a highly attractive feature of the drug, considering that all solid malignancies contain hypoxic zones populated with non-proliferating tumor cells. However, administration of flavopiridol during S phase of the cell cycle greatly potentiates apoptosis [20,21]. At least one study has related this increase in sensitivity of transformed cells to drug treatment during S phase to the activity of the E2F-1 transcription factor, concomitant with the inhibition of cyclin A-dependent kinase activity by flavopiridol [13]. These results suggest that modulation of E2F-1 activity produced by drug-induced inhibition of cyclin A kinase activity is a critical event in the apoptotic response of tumor cells.

Apoptosis is controlled by several anti-apoptotic proteins including Bcl-2, Bcl-x_L, Mcl-1, XIAP and survivin [22]. Flavopiridol has been shown to down-regulate the expression of all five of these anti-apoptotic proteins, thus favoring entry into apoptosis [2,14,22–27]. Survivin, a member of inhibitors of apoptosis (IAPs), regulates cell survival as well as serves as a mitotic checkpoint. Flavopiridol inhibits phosphorylation of Thr34 on survivin, resulting in loss of protein expression and enhanced apoptosis [22].

In the majority of studies to date, apoptosis induced by flavopiridol treatment appears to be independent of p53 status of the cancer cell lines used [1,2,11]. Moreover, apoptosis induced by flavopiridol generally has been associated with classic markers of mitochondrial-induced damage such as release of cytochrome *c*, DNA laddering, activation of caspases-9, -3 and -8, PARP cleavage, and cleavage of Bid; all of these events can be attenuated with pre-treatment of cells with pan-caspase-3 inhibitors [21,25,28–31]. In addition, flavopiridol can also induce a caspase-independent pathway of apoptosis in the absence of cytochrome *c* release from mitochondria via release of apoptosis-inducing factor (AIF) and independent of Bcl-2 overexpression [11,32]. In other studies, flavopiridol treatment induced release of both cytochrome *c* and AIF from mitochondria, thus triggering both caspase-dependent and -independent cell death pathways [12,31].

Lastly, flavopiridol inhibits activation of the nuclear transcription factor NF-κB that has immediate implications for its potential to enhance sensitivity to radiation and other sequential chemotherapeutic treatments [15]. The mechanism of inhibition of NF-κB was associated with inhibition of activation of Akt [15]. Phosphorylation of Akt (p-Akt) leads to its activation and its ability to regulate diverse biological processes such as proliferation, apoptosis and migration. High levels of expression of p-Akt are linked with cell survival. At least for human leukemia cell lines, flavopiridol treatment in combination with inhibitors of the phosphatidylinositol 3-kinase (PI3K) signaling pathway potentiated apoptosis [31].

Moreover, proteasome inhibitors in combination with flavopiridol also produced synergy for apoptosis in leukemic cell lines that was associated with inhibition of NF-κB activity [14].

In summary, flavopiridol is a potent apoptotic agent due to its ability to cause cell death in cycling as well as non-cycling tumor cells; to down-regulate important cell-survival proteins, such as survivin, through inhibition of the phosphorylation of Thr34 inducing rapid degradation of the protein; to increase sensitivity for S phase cells to drug treatment by modulating E2F-1 transcription factor activity in tumor cells; to induce both caspase-dependent and -independent mitochondrial cell death pathways; and to inhibit the activation of p-Akt which in turn inhibits activation of NF-κB. Both p-Akt and NF-κB proteins, in their respective signaling pathways, are targets themselves for chemotherapy interventions in human cancer [33,34].

Anti-angiogenic activities

The processes associated with angiogenesis and tumor-induced neovascularization include the stimulation of endothelial cells by cytokines secreted by tumor cells such as vascular endothelial growth factor (VEGF) that induces their increased proliferation and migration. Normally the secretion of the VEGF cytokine is greatly up-regulated under conditions of hypoxia. In situations of low O_2, the transcription factor hypoxia inducible factor (HIF)-1α controls the expression of many genes that produce proteins allowing rapid adaptation to the microenvironment, including the production of the potent cytokine VEGF [35]. Invasion of tumor cells is promoted by secretion of extracellular matrix degrading enzymes, such as the matrix metalloproteinases (MMPs).

One hallmark of an anti-angiogenic agent is its ability to induce apoptosis of endothelial cells. In early studies, flavopiridol induced apoptosis in human umbilical vein endothelial cells (HUVECs) whether they were cycling or non-cycling [36]. One possible mechanism of action of flavopiridol treatment in HUVECs may be through the down-regulation of survivin. As noted above, flavopiridol brings about the phosphorylation of Thr34 on survivin, resulting in loss of protein expression and enhanced apoptosis [22]. It has been noted that survivin is overexpressed in endothelial cells during the early proliferation and remodeling stages of angiogenesis, and this is associated with resistance to apoptosis [38–40]. The down-regulation of survivin by antisense treatment inhibited the ability of VEGF to promote cell survival inducing apoptotic-induced collapse of three-dimensional capillaries *in vitro* [41]. Future studies will be needed to test this hypothesis. Using a different model system, flavopiridol tested *in vivo*, using the Matrigel model of angiogenesis, showed a 70% decrease in the formation of blood vessels [42].

One extremely potent angiogenic factor is VEGF, a mitogen for endothelial cells, that can be produced by normal as well as tumor cells in response to hypoxia. Flavopiridol was shown to inhibit the induction of VEGF in human monocytes under hypoxic conditions [43]. Induction of VEGF production in human neuroblastoma cell lines by inducers of HIF-1α such as hypoxia (1% O_2), picolinic acid (PA) and iron-chelating agents such as desferrioxamine (DFX) were all inhibited by flavopiridol treatment [44]. Similarly, induction of VEGF in human U87MG and murine GL261 glioma cell lines by cobalt chloride ($CoCl_2$) treatment under normoxic conditions could be significantly decreased with simultaneous addition of flavopiridol to the culture medium (Newcomb *et al.*, in preparation). The mechanism of action of flavopiridol to inhibit VEGF production was due to decreased expression of HIF-1α. Thus, this data indicate that flavopiridol can inhibit the induction of HIF-1α that, in turn, prevents the production of VEGF under a variety of conditions. A recent study of the 2000 compounds in the NCI chemical repository identified only three drugs that could inhibit hypoxia-mediated induction of HIF-1α, and they were identified as camptothecin analogs and topoisomerase inhibitors [45]. Now, flavopiridol can be added to this list. The potential to down-regulate HIF-1α expression makes this drug an extremely attractive agent for tumors with high angiogenic activity, such as renal cell carcinomas and glioblastomas.

MMPs are important enzymes associated with degradation of extracellular matrix molecules to create a permissive environment for tumor cell invasion and migration. Breast cancer cell lines treated with flavopiridol showed decreased secretion of MMP2 and MMP9, concomitant with inhibition of migration in Matrigel invasion chambers [28]. Human kidney cells (A293) treated with flavopiridol down-regulated expression of MMP9 that was associated with inactivation of NF-κB [15]. Similarly, flavopiridol treatment of human U87MG and murine GL261 glioma cells down-regulated MMP2 secretion, simultaneously with inhibition of migration in Matrigel invasion chambers (Newcomb *et al.*, in preparation). Since NF-κB regulates expression of gene products such as VEGF, cyclin D_1 and MMPs, and flavopiridol is known to suppress NF-κB activation, one mechanism for flavopiridol inhibition of VEGF and MMP secretion, together with decreased migration, may be through modulation of NF-κB activity [15].

In summary, flavopiridol possesses several important anti-angiogenic activities including induction of apoptosis of endothelial cells; inhibition of the hypoxic induction of VEGF and/or its production under hypoxic conditions

through inhibition of HIF-1α transcription; decreased secretion of MMPs that is linked with significant inhibition of invasive potential in Matrigel assays. Taken together, the anti-proliferative and anti-angiogenic properties of flavopiridol may contribute to its anti-tumor activities observed in animal models tested to date.

Anti-tumor activities in preclinical models

Flavopiridol has shown anti-tumor activity on a broad range of human tumor cell lines implanted as human xenografts in the subrenal capsule assay or subcutaneous (s.c.) or intracranial (i.c.) implant models [1,2,4,46–48]. Table 1 summarizes the results of *in vivo* studies reported in the literature for human and animal cell lines engrafted into mice treated with flavopiridol, alone or in combination with other therapies, and the observed percent inhibition of tumor growth. Early studies using the subrenal capsule assay indicated that the most effective schedule for drug administration inducing anti-tumor growth of human xenografts was more effective when given daily rather than at weekly intervals [1]. Anti-tumor effects were observed regardless of the route of administration: intravenous (i.v.), intraperitoneal (i.p.) or oral dosing (p.o.) [1].

Activity against human prostate was observed against two cell lines PRXF1137 and PRXF1369, where flavopiridol treatment alone induced 27–33% regression (R) of tumor volume compared with controls, respectively, with oral dosing of 10 mg/kg/day on days 1–4 and 7–11 [49]. More recently, the prostate cell lines PC-3 and DU145 were tested with flavopiridol alone as a monotherapy or in combination with antisense (AS) treatment using oligodeoxynucleotides (ODNs) against the cdk inhibitor $p27^{KIP1}$ [46]. The rationale behind this approach was based on the observation that AS $p27^{KIP1}$ treatment induces non-cycling growth arrested tumor cells to enter S phase, thus synergizing with many chemotherapeutics [46]. Treatment of animals with xenografts was followed for 80 days. AS treatment alone either produced no growth delay for DU145 model or a small effect (30%) in the PC-3 prostate model. Animals receiving 4 weekly cycles of flavopiridol at 5 mg/kg/day alone showed approximately 50% reduction in tumor volumes at day 40 compared with control groups. However, for both PC-3 and DU145 models there was up to 80% inhibition of tumor volume at day 60 when animals received flavopiridol given first with daily i.p. dosing for 4 weekly cycles followed by AS $p27^{KIP1}$ ODNs given i.v. on the last 2 days of each cycle [46]. The synergy between the schedule of administration maybe related to the fact the tumors cells were driven into S phase by AS $p27^{KIP1}$ ODNS as shown by BrdU incorporation studies. Given that flavopiridol is an S-phase-specific drug [20], this sequence of administration should be considered for future clinical applications.

Several different leukemia and lymphoma xenograft models have been assessed for their sensitivity towards flavopiridol [50]. The best anti-tumor activity was found using a daily drug treatment, either i.v. or i.p. bolus injection, that resulted in peak plasma levels of 7 μmol/l with a decay over 8 h to approximately 100 nmol/l. Although the drug was relatively short lived, it appeared to promote tumor regression or inhibition of tumor growth. For HL60 leukemia, 80–100% of animals showed complete tumor regression after a single cycle of flavopiridol treatment [50]. In contrast, the SUDHL-4 follicular lymphoma and the AIDS-associated AS283 lymphomas were considerably more resistant when

Table 1 Effect of flavopiridol on various human and animal tumors

Tumor	Treatment	Inhibition (%)	Reference
Human			
PRXF1337, prostate	9 × 10 mg/kg/day q2 week p.o.	R, 27%	49
PRXF1369, prostate	9 × 10 mg/kg/day q2 week p.o.	R, 33%	49
PC-3, prostate	20 × 5 mg/kg/day i.p.	GD, 50%	46
	8 × AS ODN 17 mg/kg i.v.	GD, 30%	
	20 × F + 8 × AS ODN	GD, 80%	
DU145, prostate	20 × 5 mg/kg/day i.p.	GD, 50%	46
	8 × AS ODN 17 mg/kg i.v.	GD, 0%	
	20 × F + 8 × AS ODN	GD, 80%	
HL60, leukemia	5 × 7.5 mg/kg/day i.v.	R, 80%	50
	5 × 7.5 mg/kg/day i.p.	R, 100%	50
SUDHL-4, lymphoma	10 × 7.5 mg/kg/day i.v.	R, 25%	50
AS283, lymphoma	10 × 7.5 mg/kg/day i.v.	R, 0%	50
HN12, HNSCC	5 × 5 mg/kg/day i.p.	GD, 60%	51
RKO, colon	7 × 5 mg/kg/day q2 week i.p.	GD, 70%	42
Hct116, colon	5 × 10 mg/kg/2 × week i.p.	GD, 0%	47
	5 × CPT11–100 mg/kg i.p.	GD, 40%	
	5 × CPT-11 + F (3 mg/kg)	GD, 85%	
Mouse			
GL261 glioma s.c.	9 × 5 mg/kg/3 × /week i.p.	GD, 65%	48
GL261 glioma i.c.	5 × 5 mg/kg/day q1 week i.p.	GD, 50%	48

Abbreviations: R = regression; GD = growth delay; p.o. = by mouth; i.p. = intraperitoneal; i.v. = intravenous; s.c. = subcutaneous; i.c. = intracranial; AS ODN = antisense p27 oligodeoxynucleotides; schedule was F + AS ODN = flavopiridol given daily for 4 weekly cycles with AS ODN given on last 2 days of each cycle; HNSCC = head and neck squamous cell carcinoma; CPT-11 = camptothecin; CPT-11 + F = CPT-11 given first and followed by flavopiridol at 7 or 16 h.

Table 2 Flavopiridol in clinical trials

Disease	Treatment	Phase	Source
Single agent			
refractory neoplasms	72-h infusion q2 week	I	52
solid tumors	72-h infusion q2 week	I	53
mantle cell lymphoma	72-h infusion q2 week	I	54
metastatic renal cancer	72-h infusion q2 week	II	55
non-small cell lung cancer	72-h infusion q2 week	II	56
gastric carcinoma	72-h infusion q2 week	II	57
colorectal cancer	72-h infusion q2 week	II	58
advanced neoplasms	1-h infusion daily × 5 q3 week 1-h infusion daily × 3 q3 week 1-h infusion daily q3 week	I	59
refractory neoplasms	1-h infusion daily × 5 q3 week 1-h infusion daily × 3 q3 week 1-h infusion daily q3 week	I	60
mantle cell lymphoma	1-h infusion daily × 3 q4 week	II	61
advanced cancers	1-h infusion weekly × 3 q4 week	I	NCI
head and neck cancers	1-h infusion daily × 5 q1 week	II	NCI
multiple myeloma	1-h infusion daily × 3 q3 week	II	NCI
B cell malignancies	4-h infusion weekly × 4 q6 week	I	NCI
In combination with other agents			
advanced solid tumors	paclitaxel + 24-h infusion F	I	62
hematologic cancer	imatinib mesylate + 1-h infusion F	I	NCI
pancreatic cancer	1-h F + radiation + gemcitabine	I	NCI
breast cancer	Herceptin + 24-h infusion F	I	NCI
lymphoid disorders	1-h F + fludarabine + rituximab	I	NCI
advanced cancer	24-h F + leucovorin + 5-flurouracil	I	NCI
advanced solid tumors	docetaxel + 1-h infusion F	I	NCI
advanced solid tumors	irinotecan + cisplatin + 1-h F	I	NCI
advanced metastatic	24-h F + irinotecan + leucovorin + 5-FU	I	NCI
hematologic cancer	1-h F + cytarabine + mitoxantrone	I/II	NCI
esophageal cancer	paclitaxel + 24-h infusion F	II	NCI

Compiled from the literature, the National Cancer Institute (www.nci.nih.gov) and the National Institutes of Health (www.clinicaltrials.gov). Detailed information on dosing and scheduling of the combination drug treatments can be found on these websites. Abbreviations: F=flavopiridol; 5-FU=5-fluorouracil.

treated as large established xenografts, despite two cycles of drug treatment. However, due to the response detected in the preclinical model, hematologic malignancies are frequently treated with flavopiridol alone or in combination with other therapies (Table 2).

Human head and neck squamous cell carcinoma (HNSCC) cell line HN12 showed a significant growth delay (GD) when established tumors were treated with a single cycle of flavopiridol [51]. The tumors remained growth arrested for the 10 weeks of observation. Sampling of tumors from animals on day 5 showed a high rate of apoptosis in drug-treated animals over control tumors, and also reduced levels of cyclin D_1 both *in vitro* and *in vivo* [51]. HNSCC is generally considered a highly refractory neoplasm, since it is detected most often when tumors are at an advanced stage of disease, making them less responsive to available therapies. HNSCCs, like many other advanced and refractory cancers, are in several clinical trials using flavopiridol alone or in combination with other therapies (Table 2).

Colon carcinoma xenograft models have also been used to test efficacy of flavopiridol alone and in combination with camptothecin-11 (CPT-11) [42,47]. For the RKO colon carcinoma cell line, animals received flavopiridol every other day over a 2-week interval and tumor volume was reduced by 70%. Tumors assessed for apoptotic, and proliferative indices did not show any appreciable differences between the control and drug-treated groups. However, there was a 40% inhibition of angiogenic activity as measured by blood vessel density in the flavopiridol-treated group compared with the control tumors. In one other study, the Hct116 colon carcinoma cell line was resistant to flavopiridol treatment alone. However, when it was combined with SN-38, the active metabolite of CPT-11, marked tumor inhibition was observed. The sequence of administration proved to be critical in achieving synergy. CPT-11 was given first followed by flavopiridol 7 or 16 h later for a total of five treatments over the course of 2 weeks [47]. Based on these promising results, a phase I clinical trial using CPT-11 followed by flavopiridol was initiated [47].

The murine glioma GL261 animal model has been widely used as a syngeneic transplant model for both s.c. and i.c. experiments [48]. Recently, flavopiridol was tested as a monotherapy for both the s.c. and i.c. models, and showed a significant inhibition of tumor growth. Apoptosis and percent necrosis was increased in tumors from drug-treated animals compared with controls. To our knowledge, this is the first report of flavopiridol used to treat tumors growing in immunocompetent animals rather than immunocompromised hosts.

In summary, flavopiridol has shown promising anti-cancer activity in several preclinical animal models of human cancers including prostate, lymphoid, HNSCC, colon and glioma. Inhibition of tumor growth was associated, in some cases, with an increase in apoptotic activity or a decrease in angiogenic activity in tumors from drug-treated animals. These observations from the preclinical animal models opened the way for several of the phase I trials conducted at the NCI (see Table 2).

Clinical trials

The experience with flavopiridol as a single agent or in combination with other cytotoxic agents to date in clinical trials is summarized in Table 2 [52–62]. Different schedules of administration of the drug have been used beginning in the initial studies with a 72-h continuous infusion and more recently with multiple short infusions of 1-h given daily with repeating cycles of treatment [4]. Three phase I and four phase II clinical trials of flavopiridol administered as a 72-h continuous infusion every 2 weeks have been completed and published [52–58]. Patients treated had refractory non-Hodgkin's and mantle cell lymphomas, colon, renal, gastric, and non-small cell lung cancers. Disappointingly, flavopiridol has proven largely ineffective as a single agent in the more than 200 patients treated on this dosing schedule, generally at a dose of 50 mg/m^2/day, despite the promising results obtained in the preclinical animal studies (Table 1) [52–58]. Responses have been noted in a few patients with non-Hodgkin's lymphoma, colon, gastric and renal cancers [52,53,55]. Despite the lack of objective clinical responses, the pharmacokinetic data obtained in several of the clinical trials consistently showed that flavopiridol achieved a steady state concentration in the plasma of 200–500 nM [4]. The use of 300 nM flavopiridol in *in vitro* studies is sufficient to induce apoptosis within 72 h of treatment [1–4,10–12]. The numerous trials conducted with flavopiridol indicate that it is well tolerated with few serious side effects and that the most common toxicities were diarrhea and fatigue [52–58].

Many of the preclinical studies showing anti-tumor activity used a daily schedule of i.v. or i.p. bolus administration of flavopiridol [46,48,50,51]. Building on this favorable observation that short daily infusions favored anti-tumor response, two phase I and one phase II clinical trials of flavopiridol administered as a 1-h continuous infusion given 1, 3 or 5 days a week every 3–4 weeks have been completed and published [59–61]. Approximately 85 patients with non-Hodgkin's and mantle cell lymphomas, esophageal, breast, hand and neck, melanoma, renal, and colon cancers have been treated with the 1-h infusion schedule [59–61]. The pharmacokinetic data obtained showed that flavopiridol administered in this way produced a higher mean peak concentration, from 1.7 to 3.5 μM, compared with 72-h continuous infusion schedule that produced a mean peak concentration from 200 to 500 nM [4,59]. Responses were observed in 35 of 78 (45%) of assessable patients in two large trials: three patients had a partial response in relapsed mantle cell lymphoma; whereas 32 patients had stable disease, with 20 of these patients having relapsed mantle-cell lymphoma [59,61]. These are very encouraging results and future studies are underway to determine the optimal schedule of flavopiridol as a single agent, particularly in hematological malignancies.

At least four NCI phase I and phase II clinical trials are currently accruing patients with recurrent and metastatic cancers of the head and neck, breast, small cell lung cancer, and relapsed or refractory B cell malignancies using flavopiridol as a single agent. These new trials will explore different schedules of short 1- or 4-h infusions of flavopiridol given 1, 3 or 5 days a week as an i.v. bolus repeating every 3–6 weeks (Table 2). The results of these additional trials should add to our information concerning the efficacy of flavopiridol as a monotherapy and whether the drug may have more activity in certain tumors compared with other kinds of advanced cancers, e.g. hematologic malignancies.

The most promising direction for flavopiridol in the clinic is in combination therapies with other cytotoxic agents (Table 2). Synergy between flavopiridol and several other anti-cancer drugs has been reported *in vitro* for paclitaxel, cytarabine, topotecan, doxorubicin, etoposide, 5-fluorouracil and the camptothecin CPT-11 [4,47]. From these initial studies, it was evident that the sequence and timing of the administration of the different drugs were critical factors in achieving maximal potentiating effects. For example, certain drugs (aramycin-C) or treatments (AS p27^{Kip1}) that induce tumor cells to enter S phase favor the administration of flavopiridol second, since it is a potent S phase drug [3,46]. At least one phase I clinical trial using sequential paclitaxel as a 24-h or 3-h infusion on day 1 followed by 24-h infusion of flavopiridol on day 2 has been completed and published [62]. There were 54 patients with various solid tumors and two responses were noted, both in patients with adenocarcinoma of the esophagus.

Because flavopiridol has shown modest activity as a single agent and, more recently, in combination with paclitaxel, many new phase I and II clinical trials are now open, and recruiting patients to further evaluate different schedules and combinations of chemotherapies for the treatment of many different advanced and metastatic malignancies. Detailed information on dosing and scheduling of the combination drug treatments can be found on the following websites: www.nci.nih.gov and www.clinicaltrials.gov. In general, flavopiridol is administered as a 1- or 24-h infusion that can either precede or follow a different

anti-cancer treatment. To cite a few different examples: (i) for hematologic malignancies, imatinib (STI-571/Gleevac) is given orally daily with flavopiridol given as a 1-h infusion on days 2, 9 and 16, repeating every 28 days; (ii) for pancreatic cancer, flavopiridol is given as a 1-h infusion on days 1 and 4, concurrently with daily radiation treatment for 6 weeks, followed 1 month later with gemcitabine given weekly for 3 weeks; (iii) for metastatic breast cancer, trastuzumab (Herceptin monoclonal antibody) is given as a 1-h infusion on days 1, 8 and 15, followed by flavopiridol as a 24-h infusion on days 1 and 8, repeating every 21 days; and (iv) for advanced solid tumors, docetaxel is given as a 0.5-h infusion on days 1, 8 and 15, followed 4 h later on the same days with flavopiridol as a 1-h infusion, repeating every 4 weeks. The results of these and several other currently open trials should help identify the best schedules and combinations of drugs that boost the activity of flavopiridol as an anti-cancer agent.

Conclusions and future directions

Additional preclinical studies are needed to further support the use of flavopiridol combined with new and novel treatment strategies. As noted above, flavopiridol is entering clinical trials in combination with other molecularly targeted agents such as Gleevec, an inhibitor of the Bcr/Abl protein tyrosine kinase, and Herceptin, a humanized monoclonal antibody targeted against the HER2 oncogene. In addition, several other novel anti-cancer combinations have been noted *in vitro* that have potentiated flavopiridol-induced apoptosis. These studies are summarized briefly to give an overview of some of the novel approaches currently being considered. Co-administration of flavopiridol with the tumor promoter and PKC activator phorbol 12-myrisate 13-acetate or the histone deacetylase inhibitor, sodium butyrate, enhanced apoptosis in human leukemia cells [25,30]. The mechanism of action was attributed to inhibition of $p21^{Cip1}$ expression, that in turn, lowered the apoptotic threshold [25,30]. Preclinical studies with AS $p27^{Kip1}$ together with flavopiridol treatment resulted in a significant reduction of prostate xenograft growth *in vivo* and should be considered as a novel clinical trial approach [46]. In this case, the AS treatment induced the tumor cells to enter S phase, a most favorable setting for introduction of the S-phase-specific action of flavopiridol. The PI3 K signaling pathway is implicated in many human cancers. Recently, the use of an inhibitor for PI3 K (LY294002) together with flavopiridol resulted in a dramatic increase in apoptosis in leukemia cells [31]. These results suggested that interrupting the PI3 K pathway also serves to sensitize tumor cells to the actions of flavopiridol by simultaneously disturbing the cell cycle and survival signaling pathways. Another interesting approach is based on the knowledge that regulation of apoptosis and the cell cycle is controlled by the proteasomal degradation pathway. Proteasome inhibitors, such as Bortezomib/PS-341, have entered clinical trials. One molecular target of proteasome inhibitors is NF-κB, noted above to be an important cell survival signaling pathway in tumor cells, similar to PI3 K. The proteasome inhibitor MG-132 used at subtoxic concentrations combined with a pharmacologically active low dose of flavopiridol resulted in a significant synergy of apoptosis in leukemic cell lines [14]. The mechanism of action was related to down-regulation of expression of survival proteins XIAP and Mcl-1, activation of the stress-related kinase JNK involved in mitochondrial damage response, interruption of the NF-κB signaling pathway and decreased expression of $p21^{Cip1}$ [14]. It remains to be determined whether other human cell lines (prostate, colon gastric, head and neck or glioma) would also respond to this novel treatment approach. Lastly, flavopiridol is being tested for its potential as a radiation sensitizer in ovarian, colon and gastric cell lines [63,64]. In a recent study with OCA-I ovarian cells, treatment first with flavopiridol arrested cells in the G_2 phase of the cell cycle, which is the most radiosensitive phase of the cell cycle, thus sensitizing the tumor cells to subsequent radiation treatment. Other mechanisms favoring increased radiosensitivity included down-regulation of the Ku70 and Ku86 proteins involved in DNA repair and down-regulation of cdk9 that would interrupt general cellular transcription processes in the drug treated cell cultures [63]. In contrast, the recent study reported for MKN-74 gastric and HCT-116 colon cell lines showed that the best response was achieved when tumor cells were first exposed to irradiation treatment followed 7 h later with flavopiridol exposure. The sequence dependence was confirmed *in vivo* in nude mice bearing HCT-116 xenografts. A possible mechanism for increased sensitivity to flavopiridol treatment was associated with decreased p21 expression [64]. Future studies will be needed to determine the appropriate scheduling of administration of flavopiridol with radiation to potentiate anti-tumor activity. It remains to be determined which genetic profiles and DNA repair capacities influence the radioresponse of a given solid tumor.

In summary, this data taken together suggests that flavopiridol can be combined with several novel anti-tumor approaches to potentiate drug induced apoptosis and anti-tumor activity. A continuing challenge will be to understand further the mechanisms underlying the pleiotropic biological effects of flavopiridol in different tumors and to relate response, particularly in combination therapy, with the genetic features of the tumor cell involved in regulation of the cell cycle and cell survival pathways. Given the low toxicity profile of flavopiridol used as a single agent in patients, combination therapy offers numerous opportunities to improve the efficacy of flavopiridol in the treatment of refractory cancers.

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