Flavopiridol Induces Apoptosis *via* Mitochondrial Pathway in B16F10 Murine Melanoma Cells and a Subcutaneous Melanoma Tumor Model

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ABSTRACT Flavopiridol is a cyclin-dependent kinase (CDK) inhibitor that promotes cell cycle arrest. We aimed to examine the anti-proliferative effects of the flavopiridol and oxaliplatin combination on p16INK4A deficient melanoma cells B16F10 and also its apoptotic effects on a subcutaneously injected B16F10 allograft melanoma tumor model. Flavopiridol and oxaliplatin treated B16F10 cell viability was determined by MTT assay. C57BL6 mice were injected with B16F10 cells and treated with flavopiridol after tumor implantation. BRAF and BCL2L1 mRNA expression levels were measured using reverse transcription-polymerase chain reaction (RT-PCR). Caspase 9 and caspase 3/7 activity were determined by activity assay kits. Proliferating cell nuclear antigen (PCNA) and B-cell lymphoma 2 (BCL-2) protein expression levels were analyzed immunohistochemically. Flavopiridol and oxaliplatin decreased cell death. Flavopiridol enhanced caspase 3/7 and caspase 9 activities *in vitro* and *in vivo* in a dose dependent manner *via* the mitochondrial apoptotic pathway. Even though there was a significant increase in Bcl-2 staining, PCNA staining was decreased in flavopiridol-administered mice. Decreased PCNA expression showed antiproliferative effects of flavopiridol which might be the result of cell-cycle arrest. Flavopiridol can be used as a cell cycle inhibitor, which induced apoptosis through the mitochondrial pathway, independently from BCL2 in B16F10 cells and B16F10 injected C57BL6 allografts.

KEY WORDS: malignant melanoma, flavopiridol, apoptosis, proliferation

INTRODUCTION

Malignant melanoma is a neoplasm of melanocytes and is considered a serious health problem due to both annual incidence and death rates (1). Malignant melanoma can develop in any tissue involving melanocytes including the skin, eye, and mucosal epithelium of the internal ear. Patients with malignant melanoma who have high risk of developing metastatic disease may benefit from adjuvant therapy.

There have been some agents developed to inhibit specific mutations in malignant melanoma cells. Understanding the role of these mutations and activation mechanisms has led to the identification of survival pathways and resistance mechanisms (2).

BRAF is a member of the RAF kinase family of the serine/threonine kinases in the ERK/MAPK pathway. BRAF gene activating mutations (V600E and V600K) are found in approximately 50% of melanomas (3). The constitutive activation of BRAF kinase enhances the RAS–RAF–MEK–ERK pathway and thereby induces melanoma cell survival and proliferation. Vemurafenib is a mutant BRAF inhibitor drug that has been approved by the FDA (4). After vemurafenib, trametenib (5), a MEK inhibitor and dabrafenib (6), a BRAF inhibitor, have been announced for use in treatment of malignant melanoma. KIT mutations have been another molecular change identified in many melanomas. However, KIT inhibitors such as imatinib have not been useful in patients with activating mutations of the c-KIT gene (7).

Almost 10% of patients with malignant melanoma had a family history of the disease, which is linked to a genetic cause on the short arm of chromosome 9. The deleted locus on chromosome 9 hosts the CDKN2A gene, and the germ-line mutations of this gene has been associated with familial malignant melanomas (8,9). The CDKN2A locus has two overlapping genes, p14ARF and p16INK4a, which are entirely different tumor suppressor proteins (10,11). While p16INK4a regulates RB activation, p14ARF regulates the p53 pathway. P16INK4a binds and inhibits the CyclinD/CDK4/6 complex and thereby prevents RB phosphorylation and causes G1/S cell cycle arrest (12). MDM2 functions as a negative regulator of the cell cycle by ubiquitinating p53, which is a well-known tumor suppressor protein. p14ARF inactivates MDM2 and thus activates p53 (13). The loss of p16INK4a and/or p14ARF directly affects cell cycle control (14).

Understanding cell cycle regulation and cell death will be beneficial for identifying the pathogenesis and the appropriate treatment of malignant melanoma. Programmed cell death, apoptosis, represents a complex signaling mechanism consisting of several intracellular and extracellular pathways. Binding of ligands to specific death receptors triggers the extracellular pathway. This receptor-ligand interaction is followed by intracellular caspase cascade. The initiator caspase 8 activates caspase 3 and 7 that induces endonucleases, resulting in DNA fragmentation (15).

On the other hand, the intrinsic or mitochondrial pathway is triggered by the release of cytochrome-c from mitochondria in response to several intracellular disorganizations such as DNA damage, loss of mitochondrial transmembrane potential, increased oxidative stress, serum starvation, and activation of oncogenes (16). The mitochondrial translocation of pro-apoptotic Bcl-2 family members such as BAX, BID, and BAK proteins assists the release of cytochrome-c (17,18). When cytochrome-c is released into cytosol it forms an oligomeric complex with APAF-1 (apoptosome) (19,20). When the apoptosome complex is formed, it recruits the initiator pro-caspase 9. Activated caspase 9 induces caspase 3 and 7, which lead to DNA fragmentation (21).

The Bcl-2 family of proteins has both pro- and anti-apoptotic characteristics. While Bcl-2, Bcl-XL, Mcl-1, Bcl-W, and Bcl-2L10 proteins contain four BH domains and prevent cell apoptosis, Bim, Bid, Bad, Bik, Puma, and Noxa proteins induce apoptosis (22). Alternative splicing of Bcl-2L1 (Bcl-X) mRNA forms two functionally distinct mRNAs. Bcl-XL is the longest isoform and acts as an inhibitor of apoptosis. On the other hand, Bcl-XS is the shortest isoform, which binds and inhibits Bcl-2 and then inhibits survival. Bcl-XL directly binds and closes the VDAC mitochondrial channel where BAX and BAK allow cytochrome c transition to cytosol (23).

Irregular CDK activity is frequently observed in malignant melanoma cells; thus, CDK inhibition is a rational approach to lead cell cycle arrest and then apoptosis (24,25). Flavopiridol is a semisynthetic flavanoid obtained from *Dysoxylum binectariferum* (26). Flavopiridol is reported to inhibit *in vitro* cell growth through CDKs (CDK2, CDK4, CDK6) in G1/S or G2/M of the cell cycle (27,28). There have also been several studies showing flavopiridol`s apoptotic effects on melanoma cell lines (29). Combination studies of the flavopiridol and platin groups or other chemotherapy agents also show preclinical and clinical effects in terms of inhibiting the cell cycle and triggering apoptosis (30-32).

Proliferating cell nuclear antigen (PCNA) is a sliding clamp of DNA polymerase delta which catalyze DNA synthesis and take part in DNA repair (33). Thus, it is important to find PCNA decrease in order to get antiproliferative results of an applied agent.

The therapeutic advantage of DNA damaging agents relies on their ability to inhibit the cell cycle and enhance apoptosis in tumor tissue. It is thus important to evaluate the proliferative and apoptotic response against antineoplastic agents *in vitro* and *in vivo*. In this study, we aimed to examine the antiproliferative effects of the flavopiridol and oxaliplatin combination on the p16INK4A deficient melanoma cell line B16F10 and also its apoptotic effects on an allograft tumor model as a single agent.

MATERIALS AND METHODS

Cell lines and cell culture

The murine melanoma cell line B16F10 was obtained from American Type Culture Collection. Cells were maintained in the Dulbecco's modified Eagle (DMEM) medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin (all from Hyclone, USA). Cells were grown in a humidified incubator containing 5% CO_2 and 95% air at 37°C. B16F10 cells do not express p16INK4A and p14ARF genes.

Reagents

Flavopiridol was obtained from Enzo (USA). A 10 mg/mL stock solution was prepared in dimethyl sulfoxide (DMSO). Oxaliplatin was purchased from Tocris (USA). A 86.33 mg/mL stock solution was prepared in DMSO.

Animal studies

Eight weeks old C57BL6 mice weighing 25 g were purchased from the Laboratory of Animal Breeding and Experimental Research Center of Gazi University. Local Institutional Committee for the Ethical Use of Animals of Gazi University approved experimental procedures. Animals were fed with standard diet and water. C57BL6 mice received an injection of 5×10^6 B16F10 cells in 200 mL phosphate-buffered saline (PBS) subcutaneously. Twenty-four C57BL6 mice bearing B16F10 tumors were separated into four groups, six mice per group. Groups received intraperitoneal injections of DMSO, 2.5 mg/kg, 5.0 mg/kg and 10.0 mg/kg of flavopiridol at day 12 and 15 after tumor implantation. Mice from the control and flavopiridol groups were sacrificed by cervical dislocation after the last day of drug application. Tumor tissues were retrieved for analysis.

Cell viability

Cell viability was determined by MTT assay. Cells were seeded in DMEM at 7×10^3 cells/200 µL per well in 96 well plates for 24h at 37°C. When cells were attached after 24 h, cells were treated with DMSO (control group), only flavopiridol (50 nM; 100 nM; 200 nM; 400 nM), only oxaliplatin (40 µM; 80 µM) and a combination of flavopiridol and oxaliplatin for 24 and 48 hours. After incubation time, a 10 µL MTT solution (0.5 mg/mL) was added to each well. After 4h of MTT incubation at 37°C, a 100 µL crystal dissolving buffer was added and the plates were gently shaken on an orbital shaker for 5 min. The absorbance at 570 nM was measured with a microplate reader. Each treatment was repeated four times. The mean absorbance of four wells was used as an indicator of relative cell growth.

RNA extraction and cDNA synthesis

3×10^6 B16F10 cells were seeded in 6 well plates and incubated with DMSO (control group) and flavopiridol (50 nM; 100 nM; 200 nM) for 24 and 48 hours. B16F10 injected C57BL6 mice also received 2.5, 5.0 and 10.0 mg/kg flavopiridol and sacrificed. *In vitro* total RNA was extracted with the "High Pure RNA Isolation kit" (Roche, Germany) protocol. *In vivo* total RNA was extracted from subcutaneous tumors (approximately 50-100 mg) using the Trizol reagent. The RNA-containing pellet was treated with 1-5 U RNase-free DNase per µg RNA and incubated at 37°C for 30 min before washing with 75% ethanol. The amount and quality of the RNA of each sample were determined by measuring the absorbance at 260 and 280 nm using the Nanodrop spectrophotometer (NanoDrop ND-1000, NanoDrop Technologies, USA). Total RNA (1 µg) was reverse transcribed in a 20-µL reaction mixture using random hexamers and the Transcriptor First-Strand cDNA Synthesis kit (Roche, Germany) according to manufacturer instructions. cDNA was used as a template for real time quantitative PCR analysis.

Quantitative real-time polymerase chain reaction PCR (reverse transcription (RT)-PCR) analysis

BRAF and BCL2L1 mRNA expression levels were measured using RT-PCR with the LightCycler 480 (Roche, Germany). β-actin (ACTB) was used as a housekeeping gene in order to normalize BRAF and BCL2L1 expression levels. Probes and primers spanning exon-exon boundaries for each gene assay were designed using the Universal Probe Library (UPL). Primer and UPL probe numbers are presented in Table 1. The reaction mixture was prepared in 96 well plates containing 1 X LightCycler Taq-Man Master reaction mixture. Each sample was tested three times.

Caspase 9 protein activity

2×10^6 cells were seeded in 6 well plates and incubated with DMSO (control group) and flavopiridol (50 nM and 100 nM) for 24 and 48 hours. B16F10 injected C57BL6 mice also received 2.5, 5.0, and 10.0 mg/kg flavopiridol and sacrificed the next day after the last injection. After sacrificing, tumors were stored at -80°C for protein activity assays. Protein concentration of each sample was determined using the BCA kit (Thermo, USA). Caspase 9 protein activity of each sample was measured by the "Caspase 9 Colorimetric

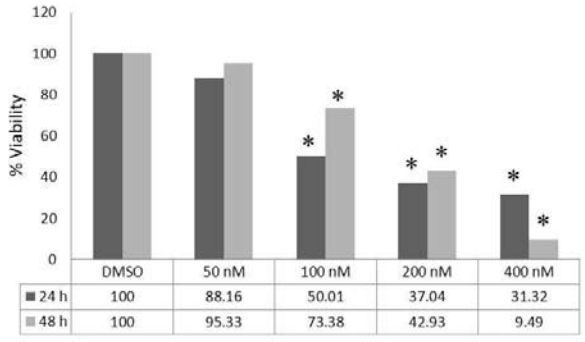
Assay Kit" (BioVision, Palo Alto, CA) according to the kit protocol.

Caspase 3/7 protein activity

2×10^6 cells were seeded in 6 well plates and incubated with DMSO (control group) and flavopiridol (50 nM and 100 nM) for 24 and 48 hours. B16F10 injected C57BL6 mice also received 2.5, 5.0, and 10.0 mg/kg flavopiridol and sacrificed the next day after the last injection. After sacrificing, tumors were stored at -80°C for protein activity assays. Protein concentration of each sample was determined using the BCA kit (Thermo, USA). Caspase 3/7 protein activity was measured using the "Anaspec Sensolyte Homogeneous AFC Kaspaz 3/7 Assay Kit" (Anaspec, USA), according to the assay protocol.

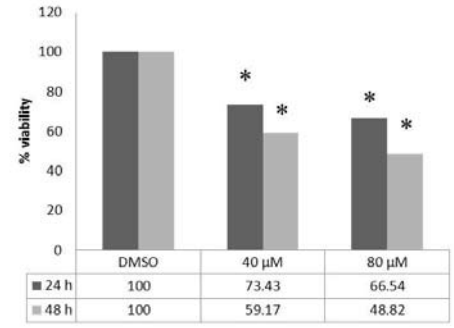
Immunohistochemistry

After the animals were sacrificed their tumors were excised, fixed in formalin, and embedded in paraffin for immunohistochemical staining. Paraffin sections were rehydrated in a series of xylene and ethanol washes. Antigen retrieval was carried out using a preheated target retrieval solution (pH 6.0) (Dako, Carpinteria, CA, USA). For immunohistochemistry, Vectastain Elite ABC kits (Vector Laboratories) were used according to manufacturer's instructions for blocking, dilution of primary antibody, and labeling. Sections were incubated with primary antibody against PCNA (Abbomax, #500-2854) and Bcl-2 (Dako, #102230) overnight at 4°C. 3.3-diaminobenzidine was prepared fresh from tablets (Sigma-Aldrich). Positive cells for each antibody of all mice from each group were counted using microscopy with estimation of intensity of staining (<10 weak, 11-50% medium, and 51-80% strong).



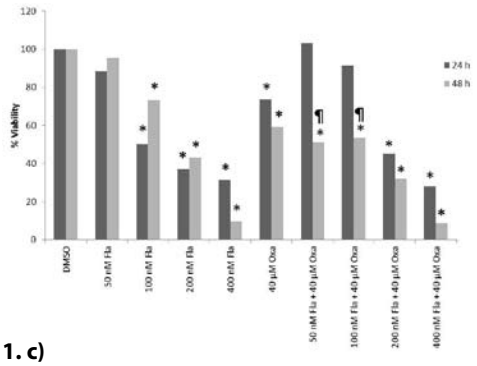
	DMSO	50 nM	100 nM	200 nM	400 nM
24 h	100	88.16	50.01	37.04	31.32
48 h	100	95.33	73.38	42.93	9.49

1. a)

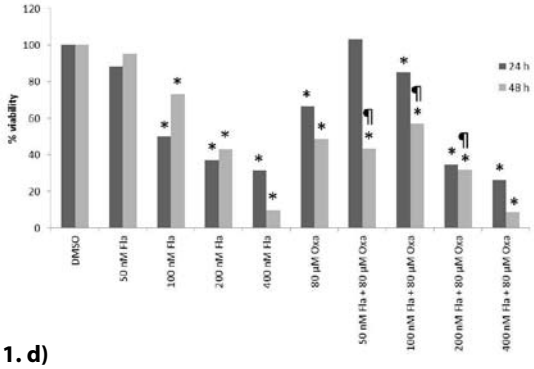


	DMSO	40 µM	80 µM
24 h	100	73.43	66.54
48 h	100	59.17	48.82

1. b)



1. c)



1. d)

Figure 1. a) The proliferation inhibitory effects of flavopiridol on B16F10 cells followed by the MTT assay. Viability rates of flavopiridol were shown in a time- and dose-dependent manner. B16F10 cells were treated with 50, 100, 200, and 400 nM of flavopiridol for 24 and 48 hours. *P<0.05
b) The proliferation inhibitory effects of oxaliplatin on B16F10 cells followed by the MTT assay. Viability rates of oxaliplatin were shown in a time- and dose-dependent manner. B16F10 cells were treated with 40 and 80 µM of oxaliplatin for 24 and 48 hours. *P<0.05
c) The proliferation inhibitory effects of the flavopiridol and 40 µM oxaliplatin combination on B16F10 cells followed by the MTT assay. Viability rates after flavopiridol and oxaliplatin administration were shown in a time- and dose-dependent manner. *P<0.05 Dimethyl sulfoxide (DMSO) versus treated group or combination; πP<0.05 Combination versus flavopiridol treated group.
d) The proliferation inhibitory effects of the flavopiridol and 80 µM oxaliplatin combination on B16F10 cells followed by the MTT assay. Viability rates after flavopiridol and oxaliplatin administration were shown in a time- and dose-dependent manner. *P<0.05 DMSO versus treated group or combination; πP<0.05 Combination versus flavopiridol treated group.

Table 1. Primer sequences and probe numbers

Gene	Forward primer	Reverse primer	UPL Probe No.
ACTB	CTAAGGCCAACCGTGAAAAG	ACCAGAGGCATACAGGGACA	64
BRAF	GCTGGGACACGGACATTT	GCAAAAGTCACAAAATGCTAAGG	55
BCL2L1	GTACCTGAACCGGCATCTG	GGGGCCATATAGTTCCACAA	75

Statistical analysis

Differences in cytotoxicity, immunohistochemical staining values, and protein activity levels were analyzed using SigmaStat software (version 12.0) by using the Student`s t-test and one-way analysis of variance (ANOVA). $P<0.05$ were considered significant. Statistical significance of differences in BRAF and BCL2L1 mRNA expression levels was analyzed by the relative expression software tool (REST) designed for group-wise comparison and statistical analysis of relative expression results.

RESULTS

B16F10 cell proliferation was significantly decreased at 100, 200, and 400 nM concentrations of flavopiridol at 24 and 48 hours. IC_{50} (Inhibition Concentration) value of flavopiridol was determined as 100 nM at 24 hour incubation (Figure 1, a). Oxaliplatin inhibited B16F10 cell proliferation at the concentrations of 40 and 80 μM at 24 and 48 hours (Figure 1, b). However, the combination of flavopiridol and oxaliplatin did not have as much antiproliferative effects as single agents alone (Figure 1, c and d). After the ineffective combination results, we chose to apply flavopiridol as a single agent in all following experiments.

In vivo and *in vitro* BCL2L1 and BRAF relative mRNA expression levels did not differ significantly between the control and flavopiridol groups (Figure 2, a and b). Although *in vitro* BCL2L1 and BRAF mRNA expression levels were decreased with flavopiridol treatment, the difference was not statistically significant (Figure 2, a).

When B16F10 cells were treated with 50 and 100 nM of flavopiridol for 6, 24, and 48 hours, caspase 9 protein activity had a tendency to increase in all groups. Caspase 9 activity was significantly increased at 50 nM concentration at 6, 24, and 48 hours. Also, 100 nM of flavopiridol increased caspase 9 protein activity significantly at 24 hours (Figure 3, a). However, caspase 3/7 protein activity was generally decreased

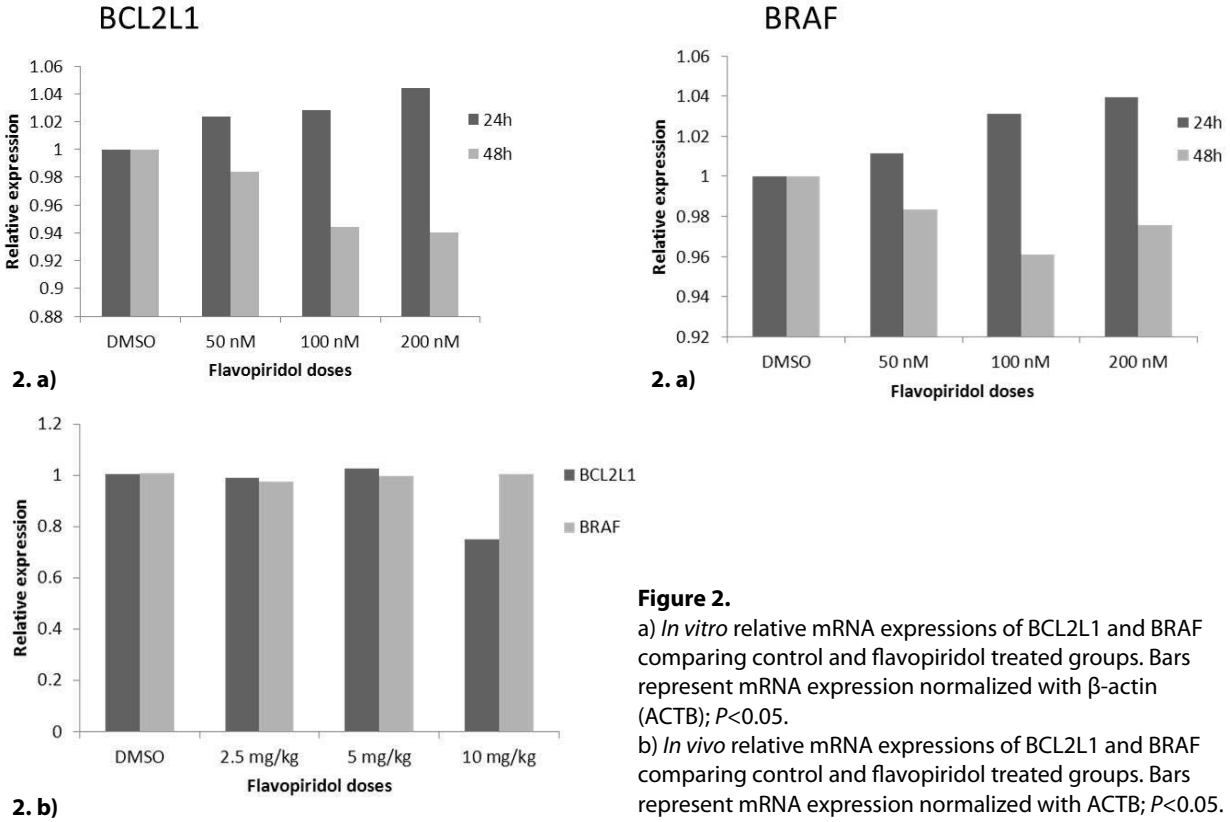
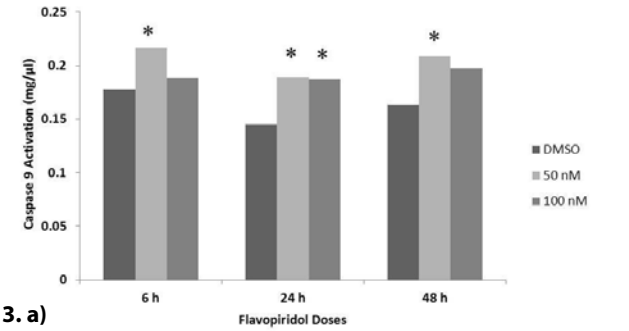
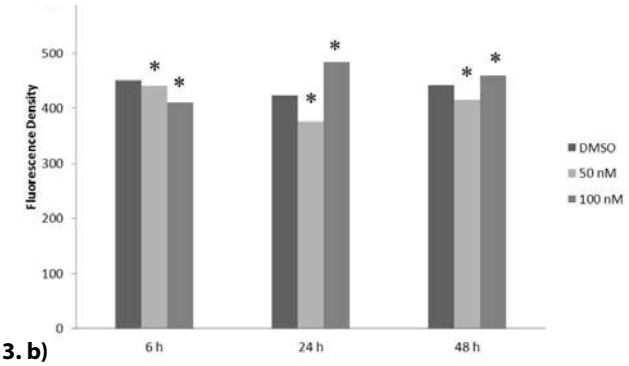


Figure 2.
a) *In vitro* relative mRNA expressions of BCL2L1 and BRAF comparing control and flavopiridol treated groups. Bars represent mRNA expression normalized with β-actin (ACTB); $P<0.05$.
b) *In vivo* relative mRNA expressions of BCL2L1 and BRAF comparing control and flavopiridol treated groups. Bars represent mRNA expression normalized with ACTB; $P<0.05$.



3. a)



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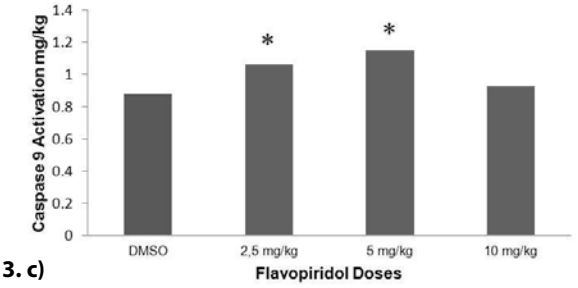

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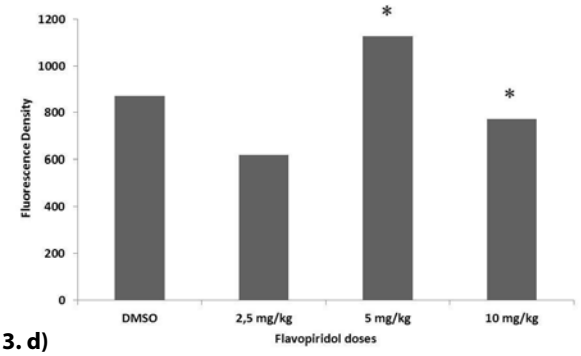


3. d)

Figure 3. a) Increased *in vitro* caspase 9 protein activity in B16F10 cells in a time- and dose-dependent manner after flavopiridol administration. *P<0.05
b) *In vitro* caspase 3/7 protein activity in B16F10 cells in a time- and dose-dependent manner after flavopiridol administration. *P<0.05
c) Increased *in vivo* caspase 9 protein activity of B16F10 allograft tumors in C57BL6 mice after flavopiridol treatment. *P<0.05
d) *In vivo* caspase 3/7 protein activity of B16F10 allograft tumors in C57BL6 mice after flavopiridol treatment. *P<0.05

at 50 nM flavopiridol concentration at all hours. On the other hand, 100 nM of flavopiridol increased caspase 3/7 activity significantly at 24 and 48 hours (Figure 3, b).

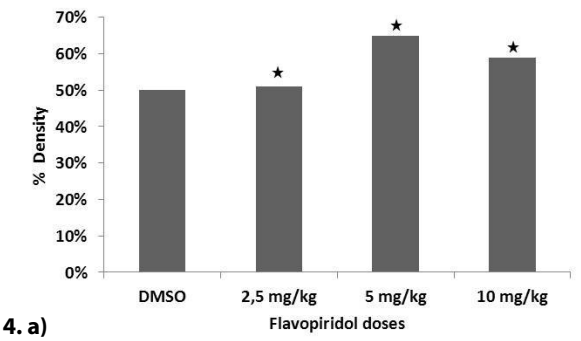
When C57BL6 mice were injected with B16F10 cells, tumors were formed after 7 days. Flavopiridol was applied two times intraperitoneally at the concentrations of 2.5, 5.0, and 10.0 mg/kg. All RNA and protein analysis was done from the same tissues. *In vivo* caspase 9 protein activity was significantly increased at 2.5 and 5.0 mg/kg of flavopiridol concentrations (Figure 3, c). At the same time, caspase 3/7 activity was significantly increased at 5.0 and 10.0 mg/kg flavopiridol (Figure 3, d).

Interestingly, we did not observe a decrease at Bcl-2 staining with flavopiridol treatment upon immunohistochemical analysis (Figure 4, a and b). Bcl-2 was significantly increased with flavopiridol treatment in each treatment group. However, PCNA levels significantly decreased in coordination with caspase levels in the flavopiridol treated groups (Figure 4, c and d). This data was also correlated with decreased *in vitro* proliferation rates.

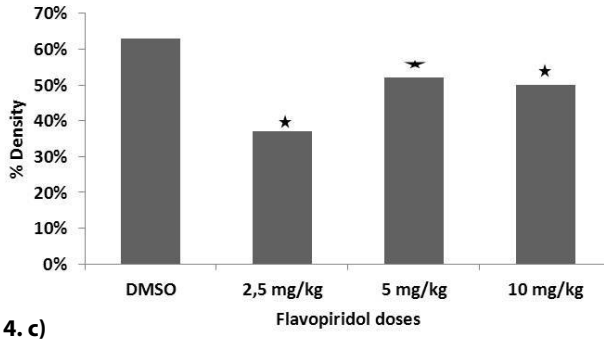
DISCUSSION

Malignant melanoma has 10-year survival rates less than 10% and has been difficult to treat for clinicians (1). However, several molecular approaches have helped understand the biology of malignant melanoma and lead to progress in treatment strategies. Driver BRAF and MEK gene mutations are the most famous molecular targets. To date, vemurafenib and dabrafenib (mutant BRAF inhibitors), (34) trametinib (MEK1/2 inhibitor) (35), and their combinations (36) have been used and approved by the FDA for metastatic malignant melanoma. However, resistance mechanisms such as the alternative pathway or CDK4/6 activations affect response rates even for combination therapies (37). The small molecule inhibitors those target the antiapoptotic BCL2 family members or IAPs are important to understand the apoptosis resistance mechanisms. Although melanoma cells are generally insensitive to these single agents, combination therapies have yielded promising results (38). CTLA-4, PD-1, and PDL-1 targets have also been tried in clinical trials (39,40) with promising results as combination therapies (41). Thus, even though new molecular targets and their inhibitors have been determined, blocking the cell cycle is still a rational option for inhibiting tumor cell proliferation.

Flavopiridol, the first approved CDK inhibitor, has been shown to mimic p16, which is frequently lost or mutated in malignant melanoma. In this study,



4. a)



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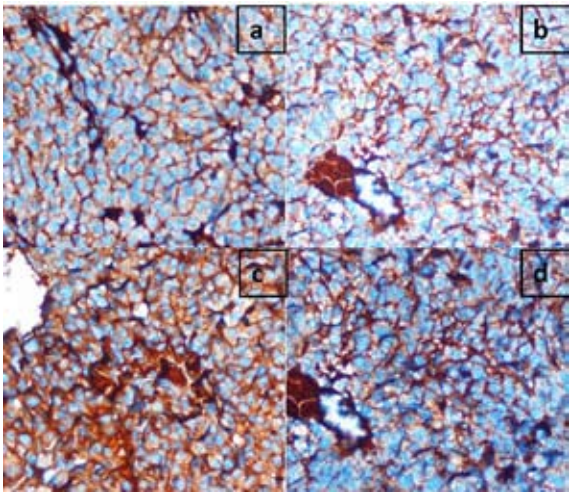

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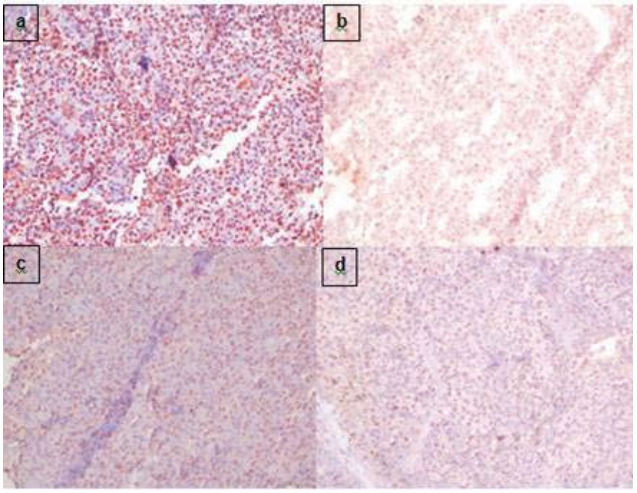

4. d)

Figure 4. a) Quantification of immunohistochemistry for *in vivo* B-cell lymphoma 2 (BCL-2) protein expression of B16F10 allograft tumors in C57BL6 mice after flavopiridol treatment. Columns represent mean percentage of tumor cells with positive staining. **P*<005

b) Representative images of immunohistochemistry for BCL-2 staining of B16F10 allograft tumors in C57BL6 mice after dimethyl sulfoxide (DMSO) (a), 2.5 mg/kg flavopiridol (b), 5.0 mg/kg flavopiridol (c), and 10.0 mg/kg flavopiridol treatment (×200)

c) Quantification of immunohistochemistry for *in vivo* proliferating cell nuclear antigen (PCNA) protein expression of B16F10 allograft tumors in C57BL6 mice after flavopiridol treatment. Columns represent mean percentage of tumor cells with positive staining. **P*<0.05

d) Representative images of immunohistochemistry for PCNA staining of B16F10 allograft tumors in C57BL6 mice after DMSO (a), 2.5 mg/kg flavopiridol (b), 5.0 mg/kg flavopiridol (c), and 10.0 mg/kg flavopiridol (d) treatment (×200)

we investigated the effects of flavopiridol at various concentrations in anti-proliferative and apoptotic events and gene expression profiles in a p16 deficient B16F10 mouse melanoma cell line and an allograft tumor model in C57BL6 mice. In our study, IC_{50} value was determined as 100 nM for 24 hours in flavopiridol applied B16F10 cells. In agreement with our study, Robinson *et al*. reported that cell cycle was inhibited even for low doses of flavopiridol (12.5 nM – 25.0 nM) and caused apoptosis at 100 nM. Although higher concentrations decreased IC_{50} concentration they did not affect expression of anti-apoptotic BCL2 expression (29). Jackman *et al*. showed flavopiridol induced cell cycle arrest and apoptosis at lower concentrations. However, reduced CDK activity was observed at higher flavopiridol doses in acute lymphoblastic leukemia cells (42).

The antiproliferative effect of flavopiridol has been shown to increase when applied with various agents in clinical and preclinical studies (30-32). In our study, oxaliplatin decreased proliferation at 80 μM (IC_{50} concentration) at 48 hours. However, combination of flavopiridol and oxaliplatin did not have as much antiproliferative effects as single agents alone. In a phase I study, a flavopiridol and oxaliplatin/fluorouracil (5-FU)/folinic acid combination was safe and tolerable for advanced solid tumors (31).

The apoptosis mechanism triggered by flavopiri-

dol has still not been well-established. It has however been shown that it may be BCL2 dependent or independent. Ma *et al*. showed flavopiridol treatment can enhance apoptosis through stabilizing E2F1 and transcriptionally repressing MCL-1 in H1299 lung carcinoma cells (43). Lucas *et al*. also showed lower doses of flavopiridol increased apoptotic NOXA protein levels and sensitizes MM200 and Me4405 melanoma cells to ABT-737, a BH-3 mimetic agent in 2D and 3D cultures [44). According to our findings, BCL2L1 mRNA expression did not change significantly in control and flavopiridol treated groups in both *in vitro* and *in vivo* studies. We also did not observe any decrease in BCL2 protein levels in flavopiridol treated allograft tumors. In agreement with our study, it has been shown that flavopiridol induced apoptosis independently from BCL2 expression (45). On the other hand, flavopiridol has downregulated BCL2 mRNA and protein expression and induced apoptosis within 24 hours in B cell leukemia cell lines (46). It has been shown that BCL2 expression was induced by flavopiridol in leukemic blasts in adult acute leukemia patients (47). The studies examining flavopiridol's apoptotic effects do not clearly explain the role of BCL2 protein expression in apoptosis. Although Konig *et al*., Sato *et al*., and Newcomb *et al*. showed BCL2 decreased as a result of the apoptotic effects of flavopiridol in different cell lines, we could not find any difference between flavopiridol treated and untreated groups in our study (46,48,49). In line with our study, flavopiridol induced apoptosis through caspase 3 activation independently from BCL2 or p53 function in chronic lymphocytic leukemia cells (50).

It is also known that flavopiridol can induce apoptosis through caspase-dependent or independent mechanisms according to the mutational status and histologic type of the cell. In our study, flavopiridol induced caspase 9 activation both *in vivo* and *in vitro.* Also, caspase 3/7 was upregulated at higher doses of flavopiridol in the cells and tumor model. In glioma cell lines, flavopiridol did not activate caspase 3, poly ADP ribose polymerase (PARP), or caspase 8 independently from tumor suppressor pathway changes such as retinoblastoma and p53. Mitochondrial damage and cytochrome c release were also not observed in those cells (47). However, Li *et al*. showed flavopiridol caused the release of cytochrome c from mitochondria, activated caspase 9, 8, and 3, increased proapoptotic BAX, and decreased BCL2 protein levels in drug resistant osteosarcoma and Ewing's family tumor cells (51). Additionally, Puppo *et al*. determined that flavopiridol increased caspase 3 activation, however it had no effect in stimulating caspase 9 and led to apoptosis through mitochondrial pathway activation in neuroblastoma cells (52). G2 arrest and cyclin B1 downregulation were also observed in low concentrations of flavopiridol application in rhabdoid cells. When flavopiridol was combined with 4OH-tamoxifen it did not affect flavopiridol induced G2 arrest but led to caspase-3/7 activation. p53 inhibition by siRNA removed flavopiridol-induced G2 arrest but enhanced apoptosis by activating caspase 2 and 3 (53).

On the other hand, Mahoney *et al*. have shown that flavopiridol protects chronic lymphocytic leukemia cells from autophagy, which causes resistance to cyclin-dependent kinase targeted therapies [(54,55)]. However, Xiao *et al*. showed a HSP90 inhibitor 17-AAG can sensitize mantle cell lymphoma cells to flavopiridol induced authophagy and also enhanced apoptosis (56). Immunochemical evaluation of PCNA protein expression is one of the methods used to determine cellular proliferation level in tumor tissue. PCNA is synthesized in the S phase of cell cycle that is an indicator of DNA synthesis; it is important in determining mitosis number and mitotic index (57). Verdaguer *et al*. observed decreased PCNA expression and apoptosis after flavopiridol application in primary cultures of rat cerebellar granule cells (58). In our *in vivo* study, the decrease in PCNA protein expression and increase in caspase 3/7 and caspase 9 activities may show that flavopiridol stopped the cell cycle and led to apoptosis through the mitochondrial pathway.

CONCLUSION

Apoptosis was induced by flavopiridol independently from BCL2L1 through the mitochondrial pathway in which caspase 3/7 and caspase 9 activated in p16INK4A and p14ARF mutant cell line B16F10 and B16F10 injected C57BL6 mice. PCNA expression was decreased by flavopiridol, which caused cell cycle arrest in the melanoma model. Nonetheless, while there are still many mechanisms and unclear results to investigate, flavopiridol can be used as a cell cycle inhibitor and apoptosis inducer in malignant melanoma.

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References:

1. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. CA Cancer J Clin 2014;64:9-29.
2. Ko JM, Velez NF, Tsao H. Pathways to melanoma. Semin Cutan Med Surg 2010;29: 210-7.

3. Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, *et al*. Mutations of the BRAF gene in human cancer. Nature 2002;417:949-54.
4. Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, *et al*. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. N Engl J Med 2011;364:2507-16.
5. Flaherty KT, Robert C, Hersey P, Nathan P, Garbe C, Milhem M, *et al*. Improved survival with MEK inhibition in BRAF-mutated melanoma. N Engl J Med 2012;367:107-14.
6. Hauschild A, Grob JJ, Demidov LV, Jouary T, Gutzmer R, Millward M, *et al*. Dabrafenib in BRAF-mutated metastatic melanoma: a multicentre, open-label, phase 3 randomised controlled trial. Lancet 2012;380:358-65.
7. Ugurel S, Hildenbrand R, Zimpfer A, La Rosee P, Paschka P, Sucker A, *et al*. Lack of clinical efficacy of imatinib in metastatic melanoma. Br J Cancer 2005;92:1398-405.
8. Cannon-Albright LA, Goldgar DE, Meyer LJ, Lewis CM, Anderson DE, Fountain JW, *et al*. Assignment of a locus for familial melanoma, MLM, to chromosome 9p13-p22. Science 1992;258:1148-52.
9. Gruis NA, van der Velden PA, Sandkuijl LA, Prins DE, Weaver-Feldhaus J, Kamb A, *et al*. Homozygotes for CDKN2 (p16) germline mutation in Dutch familial melanoma kindreds. Nat Genet 1995;10:351-3.
10. Peters G. Tumor suppression for ARFicionados: the relative contributions of p16INK4a and p14ARF in melanoma. J Natl Cancer Inst 2008;100:757-9.
11. Fecher LA, Cummings SD, Keefe MJ, Alani RM. Toward a molecular classification of melanoma. J Clin Oncol 2007;25:1606-20.
12. Serrano M, Hannon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature 1993;366:704-7.
13. Pomerantz J, Schreiber-Agus N, Liegeois NJ, Silverman A, Alland L, Chin L, *et al*. The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. Cell 1998;92:713-23.
14. Taniguchi T, Chikatsu N, Takahashi S, Fujita A, Uchimaru K, Asano S, *et al*. Expression of p16INK4A and p14ARF in hematological malignancies. Leukemia 1999;13:1760-9.
15. Walczak H, Bouchon A, Stahl H, and Krammer PH. Tumor necrosis factor-related apoptosis-inducing ligand retains its apoptosis-inducing capacity on Bcl-2- or Bcl-xL-overexpressing chemotherapy-resistant tumor cells. Cancer Res 2000;60:3051-7.
16. Green DR, Reed JC. Mitochondria and apoptosis. Science 1998;281:1309-12.
17. Desagher S, Osen-Sand A, Nichols A, Eskes R, Montessuit S, Lauper S, *et al*. Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. J Cell Biol 1999;144:891-901.
18. Griffiths GJ, Dubrez L, Morgan CP, Jones NA, Whitehouse J, Corfe BM, *et al*. Cell damage-induced conformational changes of the pro-apoptotic protein Bak in vivo precede the onset of apoptosis. J Cell Biol 1999;144:903-14.
19. Wolter KG, Hsu YT, Smith CL, Nechushtan A, Xi XG, Youle RJ. Movement of Bax from the cytosol to mitochondria during apoptosis. J Cell Biol 1997;139:1281-92.
20. Saelens X, Festjens N, Vande Walle L, van Gurp M, van Loo G, Vandenabeele P. Toxic proteins released from mitochondria in cell death. Oncogene 2004;23:2861-74.
21. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, *et al*. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 1997;91:479-89.
22. Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. Genes Dev 1999;13:1899-911.
23. Shimizu S, Narita M, Tsujimoto Y. Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature 1999;399:483-7.
24. Serrano M, Lee H, Chin L, Cordon-Cardo C, Beach D, DePinho RA. Role of the INK4a locus in tumor suppression and cell mortality. Cell 1996;85:27-37.
25. Bartkova J, Lukas J, Guldberg P, Alsner J, Kirkin AF, Zeuthen J, *et al*. The p16-cyclin D/Cdk4-pRb pathway as a functional unit frequently altered in melanoma pathogenesis. Cancer Res 1996;56:5475-83.
26. Kaur G, Stetler-Stevenson M, Sebers S, Worland P, Sedlacek H, Myers C, *et al*. Growth inhibition with reversible cell cycle arrest of carcinoma cells by flavone L86-8275. J Natl Cancer Inst 1992;84:1736-40.
27. Carlson BA, Dubay MM, Sausville EA, Brizuela L, Worland PJ. Flavopiridol induces G1 arrest with inhibition of cyclin-dependent kinase (CDK) 2 and CDK4 in human breast carcinoma cells. Cancer Res 1996;56:2973-8.

28. Losiewicz MD, Carlson BA, Kaur G, Sausville EA, Worland PJ. Potent inhibition of CDC2 kinase activity by the flavonoid L86-8275. Biochem Biophys Res Commun 1994;201:589-95.
29. Robinson WA, Miller TL, Harrold EA, Bemis LT, Brady BM, Nelson RP. The effect of flavopiridol on the growth of p16+ and p16- melanoma cell lines. Melanoma Res 2003;13:231-8.
30. Bible KC, Lensing JL, Nelson SA, Lee YK, Reid JM, Ames MM, *et al*. Phase 1 trial of flavopiridol combined with cisplatin or carboplatin in patients with advanced malignancies with the assessment of pharmacokinetic and pharmacodynamic end points. Clin Cancer Res 2005;11:5935-41.
31. Rathkopf D, Dickson MA, Feldman DR, Carvajal RD, Shah MA, Wu N, *et al*. Phase I study of flavopiridol with oxaliplatin and fluorouracil/leucovorin in advanced solid tumors. Clin Cancer Res 2009;15:7405-11.
32. Guo J, Zhou AW, Fu YC, Verma UN, Tripathy D, Frenkel EP, *et al*. Efficacy of sequential treatment of HCT116 colon cancer monolayers and xenografts with docetaxel, flavopiridol, and 5-fluorouracil. Acta Pharmacol Sin 2006;27:1375-81.
33. Naryzhny SN. Proliferating cell nuclear antigen: a proteomics view. Cell Mol Life Sci 2008;65:3789-808.
34. Luke JJ, Hodi FS. Ipilimumab, vemurafenib, dabrafenib, and trametinib: synergistic competitors in the clinical management of BRAF mutant malignant melanoma. Oncologist 2013;18:717-25.
35. Infante JR, Fecher LA, Falchook GS, Nallapareddy S, Gordon MS, Becerra C, *et al*. Safety, pharmacokinetic, pharmacodynamic, and efficacy data for the oral MEK inhibitor trametinib: a phase 1 dose-escalation trial. Lancet Oncology 2012;13:773-81.
36. Flaherty KT, Infante JR, Daud A, Gonzalez R, Kefford RF, Sosman J, *et al*. Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. N Engl J Med 2012;367:1694-703.
37. Robert C, Karaszewska B, Schachter J, Rutkowski P, Mackiewicz A, Stroiakovski D, *et al*. Improved overall survival in melanoma with combined dabrafenib and trametinib. N Engl J Med 2015;372:30-9.
38. Mohana-Kumaran N, Hill DS, Allen JD, Haass NK. Targeting the intrinsic apoptosis pathway as a strategy for melanoma therapy. Pigment Cell Melanoma Res 2014;27:525-39.
39. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, *et al*. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 2010;363:711-23.
40. Hamid O, Robert C, Daud A, Hodi FS, Hwu WJ, Kefford R, *et al*. Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. N Engl J Med 2013;369:134-44.
41. Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, *et al*. Nivolumab plus ipilimumab in advanced melanoma. N Engl J Med 2013;369:122-33.
42. Jackman KM, Frye CB, Hunger SP. Flavopiridol displays preclinical activity in acute lymphoblastic leukemia. Pediatr Blood Cancer 2008;50:772-8.
43. Ma Y, Cress WD, Haura EB. Flavopiridol-induced apoptosis is mediated through up-regulation of E2F1 and repression of Mcl-1. Mol Cancer Ther 2003;2:73-81.
44. Lucas KM, Mohana-Kumaran N, Lau D, Zhang XD, Hersey P, Huang DC, *et al*. Modulation of NOXA and MCL-1 as a strategy for sensitizing melanoma cells to the BH3-mimetic ABT-737. Clin Cancer Res 2012;18:783-95.
45. Sedlacek HH. Mechanisms of action of flavopiridol. Crit Rev Oncol Hematol 2001;38:139-70.
46. Konig A, Schwartz GK, Mohammad RM, Al-Katib A, Gabrilove JL. The novel cyclin-dependent kinase inhibitor flavopiridol downregulates Bcl-2 and induces growth arrest and apoptosis in chronic B-cell leukemia lines. Blood 1997;90:4307-12.
47. Nelson DM, Joseph B, Hillion J, Segal J, Karp JE, Resar LM. Flavopiridol induces BCL-2 expression and represses oncogenic transcription factors in leukemic blasts from adults with refractory acute myeloid leukemia. Leuk Lymphoma 2011;52:1999-2006.
48. Newcomb EW, Tamasdan C, Entzminger Y, Alonso J, Friedlander D, Crisan D, *et al*. Flavopiridol induces mitochondrial-mediated apoptosis in murine glioma GL261 cells via release of cytochrome c and apoptosis inducing factor. Cell Cycle 2003;2:243-50.
49. Sato S, Kajiyama Y, Sugano M, Iwanuma Y, Tsurumaru M. Flavopiridol as a radio-sensitizer for esophageal cancer cell lines. Dis Esophagus 2004;17:338-44.
50. Byrd JC, Shinn C, Waselenko JK, Fuchs EJ, Lehman TA, Nguyen PL, *et al*. Flavopiridol induces apoptosis in chronic lymphocytic leukemia cells via activation of caspase-3 without evidence of bcl-2 modulation or dependence on functional p53. Blood 1998;92:3804-16.
51. Li Y, Tanaka K, Li X, Okada T, Nakamura T, Takasaki M, *et al*. Cyclin-dependent kinase inhibitor, flavopiridol, induces apoptosis and inhibits tumor growth

in drug-resistant osteosarcoma and Ewing's family tumor cells. Int J Cancer 2007;121:1212-8.

52. Puppo M, Pastorino S, Melillo G, Pezzolo A, Varesio L, Bosco MC. Induction of apoptosis by flavopiridol in human neuroblastoma cells is enhanced under hypoxia and associated with N-myc proto-oncogene down-regulation. Clin Cancer Res 2004;10:8704-19.
53. Cimica V, Smith ME, Zhang Z, Mathur D, Mani S, Kalpana GV. Potent inhibition of rhabdoid tumor cells by combination of flavopiridol and 4OH-tamoxifen. BMC Cancer 2010;10:634.
54. Mahoney E, Byrd JC, Johnson AJ. Autophagy and ER stress play an essential role in the mechanism of action and drug resistance of the cyclin-dependent kinase inhibitor flavopiridol. Autophagy 2013;9:434-5.
55. Mahoney E, Lucas DM, Gupta SV, Wagner AJ, Herman SE, Smith LL, *et al*. ER stress and autophagy: new discoveries in the mechanism of action and drug resistance of the cyclin-dependent kinase inhibitor flavopiridol. Blood 2012;120:1262-73.
56. Xiao Y, Guan J. 17-AAG enhances the cytotoxicity of flavopiridol in mantle cell lymphoma via autophagy suppression. Neoplasma 2015;62:391-7.
57. Rosa JC, Mendes R, Filipe MI, Morris RW. Measurement of cell proliferation in gastric carcinoma: comparative analysis of Ki-67 and proliferative cell nuclear antigen (PCNA). Histochem J 1992;24:93-101.
58. Verdaguer E, Jimenez A, Canudas AM, Jorda EG, Sureda FX, Pallas M, *et al*. Inhibition of cell cycle pathway by flavopiridol promotes survival of cerebellar granule cells after an excitotoxic treatment. J Pharmacol Exp Ther 2004;308:609-16.