Flavopiridol Inhibits Smooth Muscle Cell Proliferation In Vitro and Neointimal Formation In Vivo After Carotid Injury in the Rat

Johannes Ruef, MD; Adam S. Meshel, BS; Zhaoyong Hu, MD; Chris Horaist, MEd; Carol A. Ballinger, PhD; Larry J. Thompson, PhD; Vijay D. Subbarao, MD; Jennifer A. Dumont, PhD; Cam Patterson, MD

Background—Smooth muscle cell (SMC) proliferation is a critical component of neointimal formation in many models of vascular injury and in human lesions as well. Cell-cycle inhibition by gene transfer techniques can block SMC proliferation and lesion formation in animal models, although these methods are not yet applicable to the treatment of human disease. Flavopiridol is a recently identified, potent, orally available cyclin-dependent kinase inhibitor.

Methods and Results—Using human aortic SMCs, we found that flavopiridol in concentrations as low as 75 nmol/L resulted in nearly complete inhibition of basic fibroblast growth factor–induced and thrombin-induced proliferation. At this dose, flavopiridol inhibited cyclin-dependent kinase activity, as measured by histone H1 phosphorylation, but had no effect on mitogen-activated protein kinase activation. Induction of the cell cycle–related proteins cyclin D1, proliferating cell nuclear antigen, and phosphorylated retinoblastoma protein was also blocked by flavopiridol. Flavopiridol had no effect on cellular viability. To test whether flavopiridol had a similar activity in vivo when administered orally, we examined neointimal formation in rat carotid arteries after balloon injury. Flavopiridol 5 mg/kg reduced neointimal area by 35% and 39% at 7 and 14 days, respectively, after injury.


Key Words: muscle, smooth growth substances angioplasty carotid arteries
Cdk2, cell division cycle 2 (Cdc2), and Cdk4. In contrast to other pharmacological inhibitors of Cdns, flavopiridol is remarkable for its kinase selectivity, its oral availability, and its potency, being effective in nanomolar concentrations. These features result in a favorable side effect profile that has led to testing of flavopiridol in phase 1 clinical trials for the treatment of refractory neoplasms. Given these properties, we have examined the ability of flavopiridol to inhibit SMC proliferation in vitro and after balloon injury to the rat carotid artery.

Methods

Materials
Flavopiridol ([L68-8275, (-)-cis-5,7-dihydroxy-2-(2-chlorophenyl)-8-[4-(3-hydroxy-1-methyl)piperidinyl]-4H-benzopyran-4-one] was provided by Hoechst Marion Roussel, Inc. Basic fibroblast growth factor (bFGF) was purchased from Collaborative Biochemical and thrombin from Sigma.

Cell Culture
Human aortic smooth muscle cells (HASMCs) were obtained from Clonetics and were cultured as previously described. Before experiments were performed, cells were growth-arrested at 80% confluence for 48 hours with medium containing 0.2% FBS.

Cell-Proliferation ELISA
Cell proliferation was measured by ELISA (Amersham Life Science). HASMCs were grown in 96-well plates and made quiescent. Cells were treated with 10 ng/mL bFGF, 2 U/mL thrombin, or vehicle for 24 hours. Flavopiridol 75 nmol/L was administered 1 hour before growth factor treatment. 5-Bromo-2-deoxyuridine (BrdU) was added to a final concentration of 10 µmol/L during the last 2 hours of treatment. BrdU incorporation was measured as described. Results are expressed as mean±SEM for 12 samples per condition.

Cell Counts
Growth-arrested HASMCs grown to 50% confluence in 6-well plates were treated with or without flavopiridol 75 nmol/L or bFGF 10 ng/mL. At intervals after treatment, cells were trypsinized and cell numbers determined with a hemocytometer.

Western Blot Analysis
Western blot analysis was performed as previously described. The primary antibodies were anti–cyclin D1 antibody (M-20, Santa Cruz), anti–proliferating cell nuclear antigen (PCNA) antibody (PC10, Sigma), a phosphorylation-specific p44/42 (Erk1/Erk2) mitogen-activated protein (MAP) kinase antibody (New England Biolabs), and anti-Rb antibody (G3-245, Pharmingen), which recognizes the phosphorylated (pRb) and highly phosphorylated (ppRb) Rb species.

Cdk Activity
Quiescent HASMCs were treated for 24 hours, and total cell lysates were prepared. The kinase assay was performed with a histone H1 kinase assay kit (Upstate Biotechnology) according to the manufacturer’s instructions. Results are expressed as the mean±SEM for 3 samples and are representative of 3 independent experiments.

In-Gel Kinase Assay
Quiescent HASMCs were treated with growth factors for 30 minutes, and total cell lysates were prepared. In some experiments, HASMCs were pretreated for 60 minutes with 30 µmol/L PD98059, flavopiridol, or vehicle. Proteins were resolved on a polyacrylamide gel that was copolymerized with myelin basic protein. The gel was treated with [γ-32P]ATP, and autoradiography was performed as described.

Trypan Blue Exclusion
HASMCs were grown in 5-cm dishes and growth-arrested. Cells were treated with flavopiridol 75 nmol/L or tumor necrosis factor-α (TNF-α) 50 ng/mL for the indicated times. After the removal of the medium, 0.4% trypan blue was added to the dishes. After 5 minutes, the cells in the dishes were counted. Blue cells were counted as nonviable cells.

Rat Carotid Injury Model
Injury to the rat carotid artery was performed essentially as described. Adult male Sprague-Dawley rats (400 to 500 g, Zivic-Miller, Zelienople, Pa) were anesthetized with ketamine 2 mg/kg and xylazine 4 mg/kg. The left internal carotid artery was then cannulated with a 2F embolectomy catheter. The balloon was inflated and withdrawn across the artery to produce a distending and denuding injury. Immediately after surgery and for 4 days thereafter, rats were given flavopiridol 5 mg/kg in water, or water alone, by gavage. At specified times after carotid injury, rats were anesthetized and perfusion-fixed. Right and left carotid arteries were removed and distended by injection of 4% paraformaldehyde through the lumen, after which they were dehydrated and stored at 4°C. Immunohistochemistry was performed as previously described with the PCNA antibody and an anti-Cdk2 antibody (M2-G, Santa Cruz).

Image Analysis
The extreme distal and proximal regions of each artery were removed. Ten intermediate cross sections (8 µm each) taken 500 µm apart were analyzed from each artery. Slides were fixed and stained with hematoxylin and eosin as previously described. Under a Nikon Diaphot 300 microscope, each cross section was captured as a digital image with a Hamamatsu video camera. Medial and neointimal areas were determined by use of NIH Image software. Lesion size was expressed as the neointima/media ratio. Results for each group were expressed as the mean±SEM.

Statistical Analysis
When appropriate, data from quantitative studies were expressed as the mean±SEM. For multiple treatment groups, a factorial ANOVA followed by Fisher’s least significant difference test was applied. Statistical significance was accepted at P<0.05.

Results
Flavopiridol Inhibits HASMC Proliferation
Because flavopiridol can inhibit proliferation in multiple tumor cell lines, we tested the hypothesis that its use would block the growth of human SMCs. Growth-arrested HASMCs were treated with bFGF 10 ng/mL for 24 hours in the presence of increasing concentrations of flavopiridol, and proliferation was measured by an ELISA-based assay. Compared with untreated cells, proliferation of bFGF-treated cells was increased 5.4-fold (Figure 1A). Pretreatment for 1 hour with as little as 50 nmol/L flavopiridol significantly decreased HASMC proliferation (to 3.9-fold, P<0.05), an effect that was nearly maximal at concentrations of 75 nmol/L.

To test the generality of the effects of flavopiridol on SMC proliferation, we examined its effect on mitogenesis elicited by thrombin 2 U/mL. Flavopiridol 75 nmol/L significantly and potently inhibited both bFGF- and thrombin-induced HASMC proliferation (5.4-fold versus 1.8-fold and 2.4-fold versus 0.7-fold, respectively, P<0.05, Figure 1B). We performed cell counts to confirm that the effect of flavopiridol on cell-cycle progression in HASMCs truly reflected changes in proliferation. bFGF 10 ng/mL induced a 3-fold increase in cell number after 3 days of treatment (Figure 2). As in the
ELISA-based assays, flavopiridol 75 nmol/L efficiently blocked bFGF-induced proliferation.

Flavopiridol Inhibits Cdk Activity and Cell Cycle–Related Gene Expression in HASMCs

To assess the specific effect of flavopiridol on the cell-cycle machinery, we measured histone H1 kinase activity in cellular lysates from growth factor–stimulated HASMCs. Phosphorylation of histone H1 reflects the activities of Cdc2 and Cdk2.16 Treatment of HASMCs with bFGF and thrombin resulted in 4.4-fold and 3.6-fold increases, respectively, in histone H1 kinase activity (Figure 3). These increases in cyclin-dependent kinase activity were totally blocked by pretreatment with flavopiridol 75 nmol/L.

By Western blot analysis, we also addressed whether flavopiridol influenced growth factor–induced regulation of cell cycle–related proteins. Cyclin D1 protein levels were upregulated 6.3-fold and 3.2-fold, respectively, in response to bFGF and thrombin treatment (Figure 4), an effect that could be blocked by pretreatment with flavopiridol. Similarly, increased expression of PCNA was also blocked by flavopiridol pretreatment. As a final measure of cell cycle–related proteins, we examined Rb phosphorylation in response to growth factor expression using an antibody that recognizes pRb. Phosphorylation inactivates Rb and allows progression through the S phase to proceed. Analysis of Rb phosphorylation is particularly relevant because Rb is a target of Cdk2 and Cdk4 in vivo. Both thrombin and bFGF induced hyperphosphorylation of Rb, an effect that was inhibited by flavopiridol. Taken together, these results indicate that flavopiridol influences the expression and activity of G1- and S phase–related cell-cycle control elements in HASMCs in association with its growth-inhibitory effects.

Figure 1. Effect of flavopiridol on HASMC DNA synthesis. A, HASMCs were treated in absence (−) or presence (+) of bFGF 10 ng/mL and with indicated concentrations of flavopiridol (in nmol/L) for 24 hours. BrdU incorporation as a measure of proliferation was determined by an ELISA-based assay and expressed as percentage of incorporation in absence of bFGF treatment. *P<0.05 vs untreated cells. †P<0.05 vs treatment with bFGF in absence of flavopiridol. B, HASMCs were treated with bFGF, thrombin, or vehicle in presence or absence of flavopiridol, and BrdU incorporation was measured. †P<0.05 vs untreated cells. **P<0.05 vs treatment with bFGF alone. ‡P<0.05 vs treatment with thrombin alone.

Figure 2. Effect of flavopiridol on HASMC proliferation. HASMCs were treated with bFGF alone (●), bFGF and flavopiridol (●), or vehicle (■) for times indicated, and cell numbers after treatment were determined. Results are expressed as cell counts per well ×10⁴.

Figure 3. Effect of flavopiridol on Cdk activity in HASMCs. HASMCs were treated with bFGF, thrombin, or vehicle in presence or absence of flavopiridol, and phosphorylation of histone H1 was quantified as a measure of Cdk activity and expressed as a percentage of Cdk activity in absence of bFGF treatment. *P<0.05 vs untreated cells. **P<0.05 vs treatment with bFGF alone. †P<0.05 vs treatment with thrombin alone.

Figure 4. Regulation of cell cycle–related proteins by flavopiridol. HASMCs were treated in presence (+) or absence (−) of bFGF, thrombin, and/or flavopiridol for 24 hours. Immunoblotting of cellular lysates was performed with specific antibodies recognizing cyclin D1 (top), PCNA (middle), and pRb and ppRb (bottom).
Flavopiridol Has No Effect on MAP Kinase Phosphorylation or Activity
To ensure that flavopiridol was acting specifically at the level of the cell cycle, rather than nonspecifically on upstream kinase pathways, we measured phosphorylation and activity of Erk1 (p44 MAP kinase) and Erk2 (p42 MAP kinase). We chose these kinases because they are immediately upstream of transcriptional events occurring in response to growth stimuli and downstream of a number of critical mitogenic signaling pathways. An intact response by MAP kinases indicates that the upstream mitogenic pathways are also intact. We measured the phosphorylation status of Erk1 and Erk2 with an antibody that specifically recognizes the phosphorylated and, hence, activated forms. As a control in these experiments, we used PD98059, a selective inhibitor of MAP kinase activation. Increased amounts of phosphorylated Erk1 and Erk2, compared with untreated cells, were detected after treatment of HASMCs with thrombin and bFGF for 30 minutes (Figure 5, top). Phosphorylation of Erk1 and Erk2 by both thrombin and bFGF was blocked by pretreatment with PD98059 but not with flavopiridol. To confirm these findings, we measured Erk1 and Erk2 activity by an in-gel kinase assay (Figure 5, bottom). Again, we found that Erk1 and Erk2 activities were increased in response to thrombin and bFGF, an effect that was inhibitable by PD98059 but not by flavopiridol. These experiments, in conjunction with those presented in Figures 3 and 4, provide evidence that the effects of flavopiridol on HASMC proliferation are due to a specific arrest of the cell-cycle machinery by blocking Cdk activity without affecting upstream signaling events.

Flavopiridol Does Not Decrease HASMC Viability
Previous reports of flavopiridol activity in other cell types have demonstrated that, depending on the cell line, flavopiridol either may induce growth arrest without affecting viability or may cause apoptosis. We therefore assessed whether flavopiridol decreased the viability of HASMCs. Quiescent HASMCs were treated with flavopiridol 75 nmol/L, vehicle, or TNF-α 50 ng/mL, a cytokine known to induce apoptosis in this cell type. Although TNF-α potently decreased the viability of HASMCs, flavopiridol had no such effect (Figure 6). We have noted that with higher concentrations and longer incubations, some decreases in viability in the presence of flavopiridol may occur (not shown). However, under the conditions tested, flavopiridol primarily induces growth arrest, without affecting SMC viability.

Flavopiridol Inhibits SMC Proliferation and Neointimal Formation In Vivo in a Rat Carotid Injury Model of Vascular Injury
We used the well-established rat carotid injury model to examine whether flavopiridol induces growth arrest of SMCs in vivo, as it does in vitro. We administered flavopiridol orally at a dose of 5 mg/kg once daily, beginning on the day of injury and for 4 days thereafter, because this time period covers the initial induction of Cdk2 and the first wave of SMC proliferation in this model. Mean intimal and medial areas were quantified 7 and 14 days after injury, and neointimal lesion size was expressed as the ratio of the neointimal to the medial area. The treated and untreated groups included 12 animals each. The neointima/media ratio at 7 days was 1.00 ± 0.05 in arteries of vehicle-treated rats and 0.65 ± 0.04 in flavopiridol-treated rat arteries, a reduction of 35.0% (Figure 7). At 14 days, the neointima/media ratio was 1.08 ± 0.04 in vehicle-treated rats and 0.66 ± 0.03 in flavopiridol-treated rats, a reduction of 38.9%. These effects were statistically significant at both time points (P<0.05). Representative arterial sections are shown in Figure 8.

To demonstrate directly that flavopiridol inhibited SMC proliferation, we stained sections for PCNA expression in representative fields from each artery and determined the percentage of PCNA-positive nuclei in the neointima. At 7 days, 31.1 ± 7.2% of nuclei in injured arteries from untreated rats were PCNA-positive, whereas only 11.8 ± 1.5% of injured arteries in flavopiridol-treated rats were PCNA-positive (Figure 7; P<0.05). At 14 days, PCNA-positive nuclei were

Thrombin  bFGF  PD98059  Flavopiridol
Thrombin  bFGF  PD98059  Flavopiridol
Thrombin  bFGF  PD98059  Flavopiridol
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present in 10.4±2.0% of neointimal cells from untreated but in only 4.2±0.5% of neointimal cells from treated injured rat arteries (P<0.05). Similarly, Cdk2-positive cells were much less common in the neointima of flavopiridol-treated rats (Figure 9, A and C) than in arteries from untreated rats (B and D) at both 7 and 14 days after injury.

Discussion

We have examined whether the novel Cdk inhibitor flavopiridol is a suitable candidate for inhibiting SMC proliferation in vivo, particularly in the setting of vascular injury. Previous attempts to target the cell-cycle machinery therapeutically for the treatment of vascular disease have provided a rationale for the present studies6,7; however, methods used to this end have relied on gene transfer technologies to inhibit cell-cycle progression. At present, formidable obstacles prevent the clinical application of these techniques.25 During the course of our studies, it was reported that CVT-313, a recently identified compound that also has Cdk-inhibitory properties but at micromolar concentrations, can also inhibit neointimal formation; however, it was required that CVT-313 be instilled into the carotid artery at the time of injury to produce this effect.26 In contrast, we show that flavopiridol, when administered orally, can potently inhibit neointimal formation, to a degree comparable to other clinically relevant agents.8,27,28

The oral activity of flavopiridol makes it virtually unique among agents shown to be active in animal models of vascular injury. Its selectivity, potency, and ease of administration make flavopiridol an excellent candidate for examining the therapeutic benefits of cell-cycle inhibition in vivo in human vascular lesions.

We chose to administer flavopiridol orally in a concentration half that which inhibits tumor growth in a nude mouse xenograft model.19 It is notable that flavopiridol concentrations of 75 nmol/L result in nearly complete inhibition of SMC proliferation in our studies, whereas median serum concentrations of 425 nmol/L were attained at doses below the toxic threshold in phase 1 human studies of refractory carcinoma.13 Our results suggest that much lower doses of cell-cycle inhibitors than those used for neoplasia may be effective in the setting of vascular diseases, such as restenosis, with the concomitant benefit of increased tolerability.

Although we have demonstrated that flavopiridol induces growth arrest without affecting the viability of HASMCs in culture and have shown decreased neointimal formation after flavopiridol treatment in vivo, we cannot be sure that cell-cycle arrest is the only factor reducing neointimal formation in carotid lesions. Flavopiridol can induce growth arrest with...
or without inducing apoptosis, depending on the cell type observed.\textsuperscript{21} Interestingly, flavopiridol inhibits apoptosis in PC12 cells that have been terminally differentiated, yet it induces apoptosis in undifferentiated PC12 cells that are proliferating.\textsuperscript{20} Although our in vitro experiments were performed under conditions that would mimic the phenotype of SMCs before injury, it is possible that SMCs may respond differently to flavopiridol after injury and may even undergo apoptosis. Although the role of apoptosis in vascular lesions is unclear, expression of the Fas ligand in SMCs induces apoptosis and blocks neointimal formation in rabbits after balloon injury,\textsuperscript{29} suggesting that if flavopiridol does indeed induce apoptosis of SMCs in vivo, as it does in proliferating PC12 cells, this may be a salutary phenomenon in the context of neointimal formation.

Our results indicate that flavopiridol can inhibit SMC proliferation and, hence, neointimal formation in a well-accepted small animal model of vascular disease. It must be pointed out that the relevance of inhibition of SMC proliferation is controversial in human vascular lesions and may differ depending on the nature of the lesion and the time at which observations of proliferation are made. The proliferative index of SMCs in human atherectomy specimens is remarkably low,\textsuperscript{30} although these specimens may not reflect proliferative changes at earlier, more critical stages in lesion development. In addition, arterial remodeling independent of neointimal growth may account for a significant proportion of luminal obstruction after angioplasty in humans.\textsuperscript{31} In contrast, indices of mitotic activity in SMCs are much higher (25\% PCNA-positive) in atherectomy specimens from human lesions with in-stent restenosis, consistent with the established role of SMC hyperplasia, but not remodeling, in this process.\textsuperscript{2} As stent placement and the clinical problem of in-stent restenosis increases, so will the need for an effective means to arrest SMC hyperplasia and neointimal formation. Because flavopiridol is a potent, orally available drug with specific Cdk-inhibitory activity and because safe doses of flavopiridol are known in humans, flavopiridol can be considered a pharmacological candidate for prevention of in-stent restenosis in humans.

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References


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