Flavopiridol Targets c-KIT Transcription and Induces Apoptosis in Gastrointestinal Stromal Tumor Cells

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Abstract
Gastrointestinal stromal tumors (GIST) are characterized by activating mutations in the c-KIT gene which confers ligand-independent activation of the KIT receptor. Imatinib mesylate has been shown to effectively block constitutively active KIT and delay tumor growth. However, resistance to imatinib mesylate is emerging as a major clinical problem and novel therapies are needed. We report that treatment of GIST cells with the transcriptional inhibitor flavopiridol, initially down-regulates the antiapoptotic proteins bcl-2, mcl-1, and X-linked inhibitor of apoptosis protein which occurs as early as 4 hours after exposure. This is followed at 24 hours by the transcriptional suppression of KIT resulting in poly(ADP-ribose) polymerase cleavage and apoptosis. To separate the apoptotic effect of KIT suppression relative to the down-regulation of antiapoptotic proteins, we used small interfering RNA–directed knockdown of KIT. Results show that focused suppression of KIT alone is sufficient to induce apoptosis in GIST cells, but not to the same extent as flavopiridol. In contrast, imatinib mesylate, which inhibits KIT kinase activity but does not suppress total KIT expression, fails to cause apoptosis. We also show that flavopiridol suppresses KIT mRNA expression through positive transcriptional elongation factor inhibition and decreases KIT promoter activity. This causes a global decrease in the level of functionally mature KIT at the cell surface, resulting in a decrease in autophosphorylation at tyrosine residues 703 and 721, which characterizes activated KIT. Our results indicate that targeting KIT expression and these antiapoptotic proteins with flavopiridol represents a novel means to disrupt GIST cell dependence on KIT signaling and collectively renders these cells sensitive to apoptosis. (Cancer Res 2006; 66(11): 5858-66)

Introduction
The c-KIT gene product is a 145 kDa transmembrane protein belonging to the type III receptor tyrosine kinase family. It is an integral cellular component required for normal hematopoiesis, melanogenesis, gametogenesis, and the growth and differentiation of mast cells and the interstitial cells of Cajal (1). Mutations in the c-KIT gene resulting in its constitutive activation have been implicated in various cancers including germ cell tumors (2), mastocytosis (3), small cell lung cancers (4), breast cancers (5), and gastrointestinal stromal tumors (GIST; ref. 6). The KIT receptor has become a model for targeted therapeutics with the advent of imatinib mesylate (Gleevec, Novartis Pharma AG, Basel, Switzerland). Imatinib mesylate is a tyrosine kinase inhibitor that preferentially blocks the constitutively active mutated forms of the KIT (7), bcr-abl (8), and platelet-derived growth factor receptors (9). KIT inhibition by imatinib mesylate abrogates receptor dimerization and resultant autophosphorylation suppressing downstream activity. In KIT-dependent GIST cell lines, imatinib mesylate has been shown to be highly active as an antiproliferative agent (10, 11) and is now used clinically for the treatment of advanced GIST.

Despite the effectiveness of imatinib mesylate in ~80% of patients with metastatic GIST (12), resistance is emerging as a major clinical problem. Some 20% of GIST patients show overt disease progression within 3 months of exposure indicating primary resistance to imatinib mesylate. In contrast, ~50% of patients who initially respond to imatinib mesylate, relapse after 2 years (13). The mechanism for this acquired resistance is postulated to occur because of: (a) secondary mutations, (b) amplification of KIT, and (c) alternative signaling kinases driving cell growth (14). For these reasons, it has become essential to identify novel therapeutics for GIST that, although targeting the KIT receptor, work through different pathways.

One potential agent that has gained interest is flavopiridol, a semisynthetic flavone, and one of the few cyclin-dependent kinase inhibitors that has been extensively studied in multiple phase I and II clinical trials (15). It primarily functions by blocking CDK activity by competitively interfering with the docking of ATP in the adenine-rich binding pockets of these and other enzymes (16). Despite having a high affinity for the CDKs, flavopiridol is not specific to only these enzymes. It has also been shown to inhibit the src family of kinases (17), as well as inhibit phosphorylation of survivin (18). In addition, by suppressing CDK9-cyclin T function (19), also known as the positive transcriptional elongation factor (P-TEFb), flavopiridol becomes a global transcriptional repressor of numerous genes (20). Studies have shown that flavopiridol as a single agent has antiproliferative activity in non-small cell lung, breast, prostate, glioma, and head and neck cancer cell lines (21). To date, however, it has not been studied in GIST, nor has it been shown to modulate the KIT pathway.

Considering the requirement for constitutive KIT signaling in GIST, we questioned whether flavopiridol could be an effective alternative to imatinib mesylate by inducing transcriptional suppression of the KIT receptor. We hypothesized that the cellular loss of total KIT protein would result in a reduction in active...
autophosphorylated KIT leading to subsequent downstream silencing and eventually apoptosis. Furthermore, the global transcriptional effect induced by flavopiridol may further enhance GIST cell apoptosis by altering the milieu of antiapoptotic signals. Using the GIST cell line, GIST882 (10), we show that flavopiridol blocks KIT promoter activity and inhibits RNA polymerase II (RNA pol II) activation, thereby reducing transcribable mRNA. This causes a time-dependent cellular depletion in mcl-1, bcl-2, X-linked inhibitor of apoptosis protein (XIAP), and total KIT protein, with a reduction in KIT surface expression that potently induces an apoptotic response in these cells that is superior to imatinib mesylate. We show that targeting KIT and these antiapoptotic proteins at the transcriptional level could be more effective than direct receptor inhibition.

Materials and Methods

Cell lines and drug preparation. GIST882 cells (kindly provided by Dr. J. Fletcher, Department of Pathology, Harvard Medical School, Boston, MA) harbor a homozygous missense mutation in c-KIT exon 13 (K642E) confirmed by standard DNA sequencing (10). GIST882 was maintained in standard RPMI 1640 supplemented with 2% glutamate and either 15% heat-inactivated fetal bovine serum (FBS) or 10% CLEC human serum (Fisher, Fair Lawn, NJ). HCT 116 cells (American Type Culture Collection, Rockville, MD) were maintained in RPMI supplemented with 10% FBS. All cells were cultured in media supplemented with 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) and kept at 37°C and humidified in 5% CO2.

Flavopiridol (National Cancer Institute, Bethesda, MD) and imatinib (Pharmingen, San Diego, CA) stock solutions were prepared in water. 5,6-Dichloro-1-D-ribofuranosylbenzimidazole (DRB; Sigma, St. Louis, MO) was resuspended in DMSO.

Proliferation assays. To evaluate the antiproliferative effect of drug exposure on GIST cells, DNA content was estimated using the CyQuant Cell Proliferation Kit (Molecular Probes, Eugene, OR) as previously described (22). In brief, cells were plated in 96-well plates at a density of 2 x 10^3 cells per well and allowed to adhere overnight at 37°C. After treatment, cells were incubated with CyQuant dye and lysed. This dye exhibits strong fluorescent signal is proportional to the DNA content. Plates were read using the Spectramax M2 fluorescence microplate reader (Molecular Devices, Sunnyvale, CA) at 480/520 nm excitation/emission.

Assessment of apoptosis. Evaluation of apoptosis was carried out using the Guava Nexin Kit (Guava Technologies, Hayward, CA). Briefly, cells were treated with varying concentrations of flavopiridol or imatinib mesylate for 12 to 48 hours in 10 cm culture plates. Both floating and adherent cells were collected and stained with Annexin V and 7-AAD. The Annexin V– and propidium iodide–positive population was counted using the Guava Nexcelus Kit (Guava Technologies, Hayward, CA). The sequence of the resulting construct (pGL2-KIT) was verified by automated DNA sequencing.

Luciferase reporter assay. An engineered reporter plasmid containing KIT promoter (23) containing 720 bp of the 5′-flanking region was prepared by PCR amplification of human genomic DNA (Clontech, Mountain View, CA). The following primers were used: 5′-CCGGATTACCCTCTCCACAAGTCCCGGC-3′ (forward) and 5′-ATTACGAGCTTCTCTGCCACCTCGACTACAGCTCAAGCTTGCTCTGGTC-3′ (reverse) containing 5′-overhang restriction sites 5′-GCGGCCGC (HindIII) and 5′-GCGGATCC (BamHI), respectively (underlined). A standard PCR reaction was conducted for 30 cycles using a PTC-200 Peltier Thermal Cycler (MJ Research, Watertown, MA). The purified fragment was subcloned upstream of the firefly luciferase reporter gene in a pGL2-Basic vector (Promega, Madison, WI). The sequence of the resulting construct (pGL2-KIT) was verified by automated DNA sequencing.

Luciferase reporter assay. Transient transfections on HCT 116 cells were done in six-well plates using FuGene 6 transfection reagent (Roche). Plasmid DNA consisting of the promoter construct, pGL2-KIT (1 μg/well) and pHRL-TK, a Renilla luciferase vector (0.05 μg/well) was then added to the

Figure 1. Dose- and time-dependent cytotoxicity profile of GIST882 cells following exposure to flavopiridol or imatinib. A and B, exponentially growing GIST882 cells were exposed to varying concentrations of either imatinib mesylate (A) or flavopiridol (B) in RPMI supplemented with 10% FBS, and cell proliferation was determined at 1 to 5 days. Experiments were done in 96-well plates using the CyQuant cell proliferation kit according to the manufacturer’s instructions. Points, mean fluorescence of six replicate wells relative to untreated controls; bars, ± SD; bottom left, concentrations used. The experiments were repeated on three separate occasions with similar results.
diluted transfection reagent in a 3:1 reagent/DNA ratio. Twenty-four hours after transfection, cells were treated with drug for an additional 24 hours, then harvested in 200 μL of 1× passive lysis buffer (Dual-Luciferase Reporter Assay, Promega). Both firefly and Renilla luciferase activity was analyzed using a TD 20/20 Luminometer (Turner Designs, Sunnyvale, CA). pGL-2-KIT luciferase activity was normalized to Renilla luciferase to control for variations in transfection efficiency.

Analysis of KIT surface expression. After drug treatment, both floating and adherent cells were collected for fluorescence-activated cell sorting (FACS) analysis. Approximately 3 × 10⁶ cells were stained with phycoerythrin-conjugated KIT monoclonal antibody (BD PharMingen). To control for nonspecificity, cells were similarly stained with a phycoerythrin-conjugated isotype IgG1 monoclonal antibody. Following staining, surface expression was determined using a FACS Calibur flow cytometer (BD Immunocytometry Systems, San Jose, CA) and analyzed using FloJo software.

Small interfering RNA experiments. c-KIT and control small interfering RNAs (siRNA; 800 ng/mL) were obtained from the SureSilencing human c-KIT siRNA kit (SuperArray Biosciences, Frederick, MD) and used according to the recommendations of the manufacturer. Subconfluent GIST882 cells in six-well plates were transiently transfected with the siRNAs in OptiMEM 1 medium (Invitrogen) using OligofectAMINE transfection reagent (Invitrogen). After 4 hours of incubation, the medium was changed to a complete culture medium. Apoptotic effects were evaluated 72 hours after transfection using Annexin V/7-AAD as described above.

Statistical analysis. Student’s t test was used to determine significant differences between two means (P < 0.05).

Results

Flavopiridol has potent antiproliferative effects in GIST882 cells. In agreement with previous studies (10), GIST cell proliferation, as measured by nucleic acid content, was inhibited by imatinib mesylate exposure in a dose- and time-dependent fashion, with up to 60% inhibition by day 5 (Fig. 1A). The maximal effect was achieved at 500 nmol/L with no substantial difference even at 20 times (10 μmol/L) that dose. This plateau of activity is important because it has been shown that imatinib mesylate binds α1 acid glycoprotein in human plasma, which decreases the level of unbound active imatinib mesylate by ~90% (24). By testing a considerably higher dose (10 μmol/L) in RPMI which has lower acid glycoprotein levels compared with human plasma, we failed to increase cytotoxicity. Therefore, we used 1 μmol/L for all subsequent experiments. With flavopiridol exposure, GIST proliferation relative to control cells was markedly reduced, achieving >80% inhibition by day 5 at 500 nmol/L (Fig. 1B). These viability assays reveal that both drugs are capable of suppressing GIST cell growth. However, in comparison, 300 nmol/L of flavopiridol decreased viability by 77 ± 0.8% compared with 63 ± 2.7% for 1 μmol/L imatinib mesylate at 5 days (P < 0.001). The combination of flavopiridol and imatinib mesylate was essentially equivalent to flavopiridol alone (data not shown).

Flavopiridol mediates its antiproliferative effect through a potent induction of apoptosis in GIST cells that is superior to imatinib mesylate. To further characterize the antiproliferative effect of flavopiridol in GIST cells, we evaluated the apoptotic response by Annexin V labeling. As shown in Fig. 2A, 12 hours of exposure was insufficient to induce any measurable increase in apoptosis above the baseline (<10%) across all drug conditions tested. At 24 and 48 hours, 150 nmol/L of flavopiridol and 1.0 μmol/L of imatinib mesylate still failed to cause a significant apoptotic response with essentially no increase in Annexin V labeling above the baseline. With 300 nmol/L of flavopiridol, however, there was a profound increase in the level of apoptosis with ~42% and 58% Annexin V staining at 24 and 48 hours, respectively. This represents a 3.0- and 4.5-fold higher level of apoptosis induction following 300 nmol/L of flavopiridol exposure compared with 1.0 μmol/L of imatinib mesylate (P < 0.005). In addition, the induction of apoptosis did not seem to appreciably increase with imatinib mesylate concentrations up to 10 μmol/L (data not shown). The effect of flavopiridol was dependent on caspase-3 activity (25), as evidenced by the significant increase in cleavage product measured using a fluorescence-based assay (Fig. 2B). Flavopiridol treatment at 300 nmol/L resulted in a 4.4- and 3.9-fold higher level of activity at 24 and 48 hours, respectively, compared with imatinib mesylate (P < 0.001). However, there was no significant increase in caspase...
activity above the baseline at 12 hours with 300 nmol/L of flavopiridol, in concert with the lack of early Annexin V labeling. Considering that flavopiridol, like imatinib mesylate, is also highly bound to plasma proteins (26), we sought to test the effects of human plasma on the capacity of flavopiridol to induce apoptosis. As such, Annexin V labeling done in cells cultured in 10% human serum exhibited an almost complete loss of apoptotic effect at 300 nmol/L (Fig. 2C). However, by increasing the flavopiridol concentration to 3.0 μmol/L, a clinically achievable dose, 65% of the cells underwent apoptosis. Thus, under conditions which recapitulate those achieved in human plasma, we can still induce high levels of apoptosis with flavopiridol in GIST cells.

**Flavopiridol-mediated down-regulation of KIT induces apoptosis.** Based on the apoptotic response observed with flavopiridol exposure, we sought to examine the molecular changes associated with this effect. The ability of flavopiridol to down-regulate antiapoptotic proteins such as XIAP, mcl-1, and bcl-2, which occurs in other systems (27), was verified in the GIST model (Fig. 3A). As evidenced by Western blot analysis, these three antiapoptotic proteins were almost completely suppressed after 24 hours of exposure to 300 nmol/L of flavopiridol. Concomitant with this loss was PARP cleavage; the 85 kDa cleaved product of PARP is identified as an 85 kDa band. Membranes were probed for α-tubulin to confirm equal protein loading. These blots were done on three independent occasions with similar results.

In examining the time dependence for down-regulation of the antiapoptotic milieu (Fig. 3B), 300 nmol/L of flavopiridol caused an early reduction in bcl-2, mcl-1, and XIAP. Compared with untreated cells, bcl-2 and mcl-1 were reduced as early as 4 hours after exposure, and by 24 hours, were undetectable. XIAP down-regulation began by 12 hours and also progressively decreased to undetectable levels. KIT protein, however, remained relatively stable until 24 hours, at which point, we observed significant reductions compared with earlier time points. However, PARP cleavage and apoptosis only occurred following 24 hours of flavopiridol exposure, concomitant with KIT down-regulation.

The observation that KIT down-regulation occurred in conjunction with apoptosis, whereas the early decrease in XIAP, mcl-1, and bcl-2 was not associated with an observable apoptotic response, suggested that KIT down-regulation may in itself be required for cell death. In order to separate the effect of KIT suppression from that of mcl-1, bcl-2, and XIAP in these cells, we used a focused siRNA-based approach to substantially disrupt KIT protein levels after a 72-hour transfection in GIST cells. As shown in Fig. 3C, compared with the control siRNA, KIT siRNA reduced KIT protein expression by ~80%, demonstrating a high efficiency of knockdown. Staining with Annexin V (Fig. 3D) revealed substantial apoptosis in siRNA-treated cells (29 ± 2.3%) compared with the control siRNA (9.7 ± 0.5%; P < 0.01). This data supports the notion that a reduction in KIT protein, by itself, induces an apoptotic response in GIST cells. However, this still remains less than that observed with flavopiridol, suggesting that the maximal induction of apoptosis with flavopiridol reflects a collective suppression of both KIT, as well as the antiapoptotic proteins.

**Suppression of total KIT by flavopiridol causes a loss in autophosphorylated active KIT.** The constitutive activity of mutant KIT is identified by autophosphorylation of the receptor at the cell surface. Considering that apoptosis induction by flavopiridol occurs concomitant with KIT down-regulation, we sought to examine the changes in KIT autophosphorylation following flavopiridol treatment. Phospho-KIT at Y721 is the association site for phosphoinositide-3-kinase, which is one of the first proteins to be phosphorylated in the KIT cascade (28). Phospho-KIT at Y703 has been shown to be the binding partner for the adapter protein GRB2 which activates the ras pathway leading to cell growth (29). As shown in Fig. 4A, GIST882 cells in drug-free medium have constitutively active KIT with no change in total KIT protein over time. Flavopiridol (150 nmol/L) had no effect on inhibiting KIT autophosphorylation at either Y721 or Y703, and there were only minor reductions in total KIT protein expression observed over time. As anticipated, 1.0 μmol/L of imatinib mesylate completely abolished KIT phosphorylation at both residues within 2 hours of GIST cell death. In order to separate the effect of KIT suppression from that of mcl-1, bcl-2, and XIAP in these cells, we used a focused siRNA-based approach to substantially disrupt KIT protein levels after a 72-hour transfection in GIST cells. As shown in Fig. 3C, compared with the control siRNA, KIT siRNA reduced KIT protein expression by ~80%, demonstrating a high efficiency of knockdown. Staining with Annexin V (Fig. 3D) revealed substantial apoptosis in siRNA-treated cells (29 ± 2.3%) compared with the control siRNA (9.7 ± 0.5%; P < 0.01). This data supports the notion that a reduction in KIT protein, by itself, induces an apoptotic response in GIST cells. However, this still remains less than that observed with flavopiridol, suggesting that the maximal induction of apoptosis with flavopiridol reflects a collective suppression of both KIT, as well as the antiapoptotic proteins.

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exposure to the drug. This is a result of direct binding of imatinib mesylate into the juxtamembrane pocket of the receptor with subsequent inhibition of kinase activity (30). There was no change in total KIT across time points. In contrast, with 300 nmol/L of flavopiridol, there was no immediate effect on the suppression of KIT autophosphorylation at 2 or 4 hours. However, after 24 hours of flavopiridol exposure, phospho-KIT Y721 was almost completely abolished and Y703 was drastically reduced. Thus, the concomitant decrease in total KIT protein levels observed at 24 to 48 hours depletes the pool of available receptor to undergo autophosphorylation.

Based on the observed loss in total KIT protein with 300 nmol/L of flavopiridol exposure, we elected to determine the extent to which mature surface receptor expression is reduced. This is important because it is the mature form of the receptor that propagates constitutive survival signals. FACS analysis of KIT surface expression was done on GIST882 cells after 24 hours of drug exposure (Fig. 4B). The untreated population stained uniformly for the KIT receptor (shaded histogram, A). Exposure to 300 nmol/L of flavopiridol (solid line, D), however, resulted in a drastic reduction in KIT surface expression by ~75% compared with the untreated cells as well as the other drug conditions.

Abrogation of KIT autophosphorylation by flavopiridol reduces activation of downstream signals. The ability of flavopiridol to suppress GIST cell growth in a KIT-dependent manner would require a reduction in the activity of downstream signaling molecules including Ras/Erk, phosphoinositide-3-kinase, mitogen-activated protein kinase, and STAT pathways (31, 32). We therefore evaluated the expression and activity of several of these signaling proteins over a time course by probing for the phosphorylated active forms by Western blotting (Fig. 5A). We compared the differences in expression after treatment with 300 nmol/L of flavopiridol and 1.0 μmol/L of imatinib mesylate. The data reveals that phosphorylated active STAT1 (p-STAT1 Y701), STAT3 (p-STAT3 Y705), and ERK (p-ERK Y204) were all reduced in both treatment groups. However, with imatinib mesylate treatment, there was an earlier loss of these activated forms which parallels the early abolishment of KIT autophosphorylation (Fig. 4A). All activated proteins were significantly reduced as early as 4 hours after imatinib mesylate exposure and were undetectable by 24 hours. With flavopiridol treatment, the decrease occurred later. To confirm that the down-regulation of these downstream mediators was not related to generalized inhibition by flavopiridol, we evaluated the amount of total protein. As shown in Fig. 5B, total protein for ERK1/2, STAT1, and STAT3 were unchanged in the flavopiridol and imatinib mesylate treatment groups relative to untreated cells at 24 hours. This suggests that the loss of the phosphorylated active downstream mediators was likely related to reduced constitutive KIT signaling.

Flavopiridol inhibits RNA pol II phosphorylation and blocks KIT promoter activity causing a reduction in KIT transcription. Based on our findings of reduced expression of total KIT protein, we sought to explore the mechanism responsible. As a general transcriptional inhibitor which suppresses elongation control by RNA pol II (19), we hypothesized that flavopiridol activity functioned through potent reductions in KIT mRNA. As shown in Fig. 6A, GIST882 cells were treated with either flavopiridol or imatinib mesylate for 4, 6, 12, and 24 hours, whereas KIT mRNA levels were determined by qPCR. Flavopiridol at 300 nmol/L induced significant reductions in KIT mRNA relative to the untreated cells with a 68% and 81% down-regulation at 12 and 24 hours, respectively (P < 0.01). The initial decrease in KIT mRNA was observed at 12 hours, whereas reductions in KIT protein were not observed until 24 hours (Fig. 3B). This time lag can be explained by the prolonged half-life of KIT mRNA, which was
shown to be ~10 to 12 hours, especially in cells with high levels of KIT protein (33).

We then showed that 300 nmol/L of flavopiridol was capable of inhibiting phosphorylation of the CTD at Ser\(^2\) on RNA pol II. This site is preferentially phosphorylated by the CDK9/cyclin T complex (P-TEFb) and is required for effective elongation control of transcription (34). Western blotting in Fig. 6B shows a significant reduction in the level of phospho-CTD (Ser\(^2\)) RNA pol II as early as 4 hours after exposure to 300 nmol/L of flavopiridol, and by 24 hours, the protein is undetectable. In contrast, 1.0 μmol/L of imatinib mesylate and 150 nmol/L of flavopiridol did not have an appreciable effect on phospho-CTD RNA pol II compared with the untreated control (data not shown). Total RNA pol II did not significantly change and remains constant across all sampling time points. This data indicates that flavopiridol-mediated inhibition of transcription happens early and is dependent on direct inhibition of RNA pol II activation rather than loss of RNA pol II protein.

We further examined this transcriptional effect of flavopiridol on KIT by assessing promoter activity. We used HCT116 colon cancer cells because of an inability to adequately transfect the reporter vector into the nucleus of the GIST cell line despite optimization of the transfection conditions. Treatment of vector-transfected cells for 24 hours produced significant reductions in KIT promoter activity with 300 nmol/L of flavopiridol (Fig. 6C). There was an 11.5-fold decrease in luciferase activity at 300 nmol/L compared with untreated cells (P < 0.02) after normalization to the Renilla vector. The promoter effect also seemed to be dependent on a threshold concentration of 300 nmol/L, because half the dose resulted in only a modest decrease in promoter activity, 1.5-fold lower than the untreated cells (P = 0.10). Imatinib mesylate, which binds directly to the KIT receptor, did not have an appreciable effect on the promoter activity in these cells. These results support the observation that the suppression of KIT expression by flavopiridol occurs at the transcriptional level, likely through P-TEFb.

Finally, considering that this is the first time flavopiridol has been shown to transcriptionally suppress KIT, we wanted to confirm our findings with another established inhibitor of P-TEFb. The compound DRB is a nucleoside analogue that also potently inhibits P-TEFb, thus, inhibiting CTD phosphorylation (35). As shown in Fig. 6D, following exposure of GIST cells to 50 μmol/L of DRB for up to 24 hours, both KIT autophosphorylation sites at Y703 and Y721 were almost completely suppressed. Total KIT protein expression was significantly down-regulated to a similar level as that observed with flavopiridol after 24 hours. In addition, examination of mcl-1 and bcl-2 showed an early reduction by at least 4 hours that preceded KIT suppression. Annexin V labeling (Fig. 6E) revealed that DRB exposure for 24 hours was required for significant apoptosis induction compared with control (30 ± 1.6% versus 8 ± 0.72%; P < 0.01). Again, earlier sampling did not show measurable apoptosis (data not shown). Thus, with DRB, in transcriptionally down-regulated KIT, we observed an apoptotic response in these cells that approximates the effect mediated by flavopiridol.

**Discussion**

In the present study, we have shown for the first time that flavopiridol inhibits receptor autophosphorylation by depleting KIT through transcriptional suppression. In doing so, we have taken an alternative approach to blocking KIT dependency. The loss of critical levels of KIT protein, especially at the cell surface, produced an absolute decrease in phosphorylated KIT. This, in turn, inhibited the downstream events that would normally propagate unchecked proliferation signals in GIST cells that have constitutively active KIT. Although flavopiridol initially down-regulated the antiapoptotic proteins bcl-2, mcl-1, and XIAP, demonstrable PARP cleavage and apoptosis was only induced when KIT protein levels were significantly reduced. Exposure of GIST882 cells to siRNA directed at KIT further indicates that suppression of KIT alone results in apoptosis. Whereas KIT siRNA resulted in ~30% apoptosis, 48 hours of exposure to flavopiridol resulted in almost 60% apoptosis. This increased induction is likely related to the shift in the antiapoptotic milieu to include mcl-1, bcl-2, and XIAP, but still requires KIT suppression. Other factors may also contribute to lethality, including the inhibition of other CDKs, as well as nuclear factor κB inhibition (36). The role these other factors play remains the subject of future investigations.

The concept of using transcriptional suppression to block receptor tyrosine kinase signaling has been explored in other studies. Nahta and colleagues showed that the transcriptional suppression of epidermal growth factor receptor by flavopiridol, independent of its effect on the cell cycle, caused apoptotic synergy with trastuzumab in breast cancer cells (37). They confirmed their findings by substituting flavopiridol with DRB, a known transcriptional suppressor and P-TEFb inhibitor. We similarly treated our cells with DRB and recapitulated the same time-dependent reduction in KIT autophosphorylation and overall KIT expression observed with flavopiridol, whereas inducing an apoptotic response. Our data, showing inhibition of RNA pol II phosphorylation by flavopiridol, coupled with the results after DRB exposure, indicate for the first time that KIT expression is regulated, at least...
in part, by P-TEFb. As P-TEFb has the capacity to be recruited to gene promoters to enhance transcription, the observed reduction in luciferase activity with flavopiridol exposure also implicates P-TEFb in c-KIT promoter control (38, 39).

An interesting finding of this study was that imatinib mesylate at 1.0 μmol/L, despite completely inhibiting KIT activation at both Y703 and Y721, produced minimal apoptosis over a 48-hour period. The antiproliferative effects of imatinib mesylate were abated by a G1 cell cycle arrest (data not shown). We are not the first to show that imatinib mesylate fails to induce apoptosis despite blocking the KIT receptor. In neuroblastoma cells, Beppu et al. has shown that imatinib mesylate concentrations of 0.5 to 1.0 μmol/L were capable of inhibiting KIT autophosphorylation but could not induce apoptosis after 48 hours (40). Other investigators have shown that imatinib mesylate can induce apoptosis in GIST cells, but only with protracted drug exposure of 4 to 7 days, despite showing a reduction in KIT phosphorylation as early as 90 minutes after imatinib mesylate treatment (10). In addition, clinically, a paucity of apoptosis has also been observed with imatinib mesylate therapy, and to date, there have been only rare complete responses in GIST patients.

Collectively, this data would indicate that GIST882 cells are not completely dependent on KIT signaling for cell survival. Although we show that targeted disruption of KIT with siRNA results in cell death, the enhanced induction of apoptosis by flavopiridol indicates a co-dependency on antiapoptotic proteins including XIAP, mcl-1, and bcl-2. In addition, by transcriptionally silencing KIT expression with either siRNA or flavopiridol, we seem to induce a more complete interruption of this signaling cascade, leading to cell death that is not achievable with direct receptor inhibition by imatinib mesylate alone.

In evaluating the dose dependency of flavopiridol activity, 150 nmol/L was relatively ineffective in the GIST882 cells, having minimal antiproliferative and apoptotic effects, and failing to induce significant reductions in KIT mRNA or protein. Other cancer cell lines have been shown to be inhibited at these lower concentrations of flavopiridol, mostly through CDK modulation. The threshold dependency of 300 nmol/L in these cells, however, confirms the likelihood that transcriptional suppression of KIT is required for activity. Lam et al. did a microarray analysis on flavopiridol-treated lymphoma cells and found that gene expression profiles were unaffected by concentrations <300 nmol/L (41).

Figure 6. KIT transcription is reduced by flavopiridol-mediated inhibition of RNA pol II phosphorylation and down-regulation of KIT promoter activity. A, qPCR analysis of GIST882 cells treated for various time intervals with either flavopiridol (150 and 300 nmol/L) or imatinib mesylate (1.0 μmol/L). Columns, mean of relative KIT expression determined by normalizing the level of KIT mRNA to the 18s rRNA gene; bars, ± SD; *, P < 0.01 versus untreated and imatinib mesylate groups at 12 and 24 hours. Means were obtained from three independently performed experiments done in triplicate. B, GIST882 cells were treated with 300 nmol/L flavopiridol for varying time intervals. Whole cell lysates were subjected to immunoblotting probing for RNA pol II phosphorylated at Ser2 on the CTD (CTD Ser2) as well as total RNA pol II and tubulin. C, KIT promoter activity in GIST 882 cells. The KIT promoter was cloned upstream of a luciferase reporter gene in a pGL2-basic vector (pGL2-KIT) as described in Materials and Methods. After transient transfection in HCT116 cells, treatment with flavopiridol or imatinib mesylate was initiated for 24 hours and lysates were harvested for luciferase activity. Columns, mean of the relative luciferase activity in arbitrary units of light for each condition, by normalizing pGL2-KIT activity to Renilla luciferase (pRL-TK) activity; bars, ± SD; **, P < 0.05 versus untreated. D, GIST 882 cells were treated for increasing time intervals with 50 μmol/L DRB. KIT activation, total KIT, bcl-2, and mcl-1 were analyzed via Western blotting. E, Annexin V/7-AAD labeling of GIST882 cells after 24 hours of exposure to DRB (50 μmol/L). The percentage of cells is noted in the respective gates as in the previous experiments. These assays were repeated thrice with similar results.
In relating the gene expression changes to drug activity, the authors similarly found no viability changes in their cell lines at concentrations <250 nmol/L. This suggests that higher concentrations of flavopiridol are required to produce the transcriptional suppression that we observed.

The emergence of imatinib mesylate resistance in both GIST as well as CML patient populations has prompted the search for other molecularly targeted therapeutics. SU11248, a tyrosine kinase inhibitor with KIT-inhibitory properties has had some success in patients with imatinib mesylate–refractory GIST, and is currently undergoing clinical trials (42). There are other tyrosine kinase inhibitors that have also been evaluated preclinically and shown to inhibit KIT kinase activity including PKC-412 (43) and PP1 (44); however, the direct receptor binding properties of many of these tyrosine kinase inhibitors, including imatinib mesylate, likely contribute to the mutational capacity of the gene. Structural studies have proven that imatinib mesylate binds in the juxtamembrane region and other mutations in the gene, specifically in the kinase region, render imatinib mesylate ineffective (30). Other human malignancies such as human mastocytosis are characterized by KIT kinase domain mutations (codon 816) that are unequivocally resistant to inhibition by imatinib mesylate (45). The capacity of flavopiridol to transcriptionally down-regulate the KIT gene and induce apoptosis suggests that a mutation, unless located in the promoter region, would not be a factor in the activity of this drug.

Clinically, peak flavopiridol levels of 3.0 μmol/L have been reported (46). With 90% protein binding, this would indicate that peak plasma levels of 300 nmol/L could be achieved. With 3.0 μmol/L of flavopiridol, in the presence of human plasma proteins, we show that flavopiridol can still induce significant degrees of apoptosis in the GIST cells. In addition, recent pharmacologic models have indicated that these levels can be maintained for several hours if the drug is first administered as an i.v. bolus followed by a 4-hour infusion. This approach has been clinically applied to the treatment of chemotherapy-refractory CLL resulting in dramatic clinical responses in the setting of tumor lysis syndrome (47). Longer durations of exposure beyond 4 hours are under clinical investigation. Thus, flavopiridol could represent a new direction for the treatment of imatinib mesylate–refractory GIST. Rather than targeting mutated sites on the KIT receptor, flavopiridol can induce a selective suppression of KIT expression by inhibiting transcription at the KIT promoter through P-TEFb. This, in essence, would encompass all forms of imatinib mesylate resistance. Although imatinib mesylate should continue to be used as a first line agent in the treatment of GIST, our improved understanding of flavopiridol pharmacology and transcriptional suppression has solidified a new niche for this drug. We envision flavopiridol as a strategic alternative to the current receptor-targeted approaches in the treatment of imatinib mesylate–refractory GIST tumors and perhaps other KIT-dependent malignancies.

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