Flavopiridol-Induced Apoptosis during S Phase Requires E2F-1 and Inhibition of Cyclin A-Dependent Kinase Activity

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INTRODUCTION

Transformed cells are selectively sensitized to apoptosis induced by the cyclin-dependent kinase inhibitor flavopiridol after their recruitment to S phase. During S phase, cyclin A-dependent kinase activity neutralizes E2F-1 allowing orderly S phase progression. Inhibition of cyclin A-dependent kinase by flavopiridol could cause inappropriately persistent E2F-1 activity during S phase traversal and exit. Transformed cells, with high baseline levels of E2F-1 activity, may be particularly sensitive to cyclin A-dependent kinase inhibition, as the residual level of E2F-1 activity that persists may be sufficient to induce apoptosis. Here, we demonstrate that flavopiridol treatment during S phase traversal results in persistent expression of E2F-1. The phosphorylation of E2F-1 is markedly diminished, whereas that of the retinoblastoma protein is minimally affected, so that E2F-1/DP-1 heterodimers remain bound to DNA. In addition, manipulation of E2F-1 levels leads to predictable outcomes when cells are exposed to flavopiridol during S phase. Tumor cells expressing high levels of ectopic E2F-1 are more sensitive to flavopiridol-induced apoptosis during S phase compared with parental counterparts, and high levels of ectopic E2F-1 expression are sufficient to sensitize nontransformed cells to flavopiridol. Furthermore, E2F-1 activity is required for flavopiridol-induced apoptosis during S phase, which is severely compromised in cells homozgyous for a nonfunctional E2F-1 allele. Finally, the response to flavopiridol during S phase is blunted in cells expressing a nonphosphorylatable E2F-1 mutant incapable of binding cyclin A, suggesting that the modulation of E2F-1 activity produced by flavopiridol-mediated cyclin A-dependent kinase inhibition is critical for the apoptotic response of S phase cells.

ABSTRACT

Transformed cells are selectively sensitized to apoptosis induced by the cyclin-dependent kinase inhibitor flavopiridol after their recruitment to S phase. During S phase, cyclin A-dependent kinase activity neutralizes E2F-1 allowing orderly S phase progression. Inhibition of cyclin A-dependent kinase by flavopiridol could cause inappropriately persistent E2F-1 activity during S phase traversal and exit. Transformed cells, with high baseline levels of E2F-1 activity, may be particularly sensitive to cyclin A-dependent kinase inhibition, as the residual level of E2F-1 activity that persists may be sufficient to induce apoptosis. Here, we demonstrate that flavopiridol treatment during S phase traversal results in persistent expression of E2F-1. The phosphorylation of E2F-1 is markedly diminished, whereas that of the retinoblastoma protein is minimally affected, so that E2F-1/DP-1 heterodimers remain bound to DNA. In addition, manipulation of E2F-1 levels leads to predictable outcomes when cells are exposed to flavopiridol during S phase. Tumor cells expressing high levels of ectopic E2F-1 are more sensitive to flavopiridol-induced apoptosis during S phase compared with parental counterparts, and high levels of ectopic E2F-1 expression are sufficient to sensitize nontransformed cells to flavopiridol. Furthermore, E2F-1 activity is required for flavopiridol-induced apoptosis during S phase, which is severely compromised in cells homozygous for a nonfunctional E2F-1 allele. Finally, the response to flavopiridol during S phase is blunted in cells expressing a nonphosphorylatable E2F-1 mutant incapable of binding cyclin A, suggesting that the modulation of E2F-1 activity produced by flavopiridol-mediated cyclin A-dependent kinase inhibition is critical for the apoptotic response of S phase cells.

INTRODUCTION

The Rb tumor suppressor pathway is universally disrupted in human cancer. Rb is the prototype member of a family of proteins that govern cell cycle progression, and prevents S phase entry in its active, hypophosphorylated form. The G1→S transition requires phosphorylation of Rb by cdks (1), which are regulated by the state of their own phosphorylation, as well as their association with activating cyclins and endogenous cdk inhibitors (2, 3). Whereas some tumors lack Rb itself, most overexpress cyclins or lose expression of cdk inhibitors, resulting in deregulated cdk activity and functional inactivation of Rb (4, 5). Inhibition of cdk activity in exponentially growing transformed cells frequently results in cell cycle arrest, with cytostatic effects on tumor growth (reviewed in Ref. 6).

Rb prevents cell cycle progression by binding to E2F transcription factor family members; Rb-E2F complexes are potent transcriptional repressors (7). The sequential phosphorylation of Rb during G1 by cyclin D- and E-dependent kinases results in derepression and eventual release of free, transcriptionally active E2F, which, along with a heterodimeric DP family member partner, directs the expression of genes required for S phase entry and advance (8). However, following S phase entry, E2F is only activated transiently. Orderly S phase progression requires the timely inactivation of E2F, in part accomplished by cyclin A-dependent kinase activity. Cyclin A-cdk2 stably associates with E2F-1, and directs phosphorylation of the E2F/DP heterodimer, neutralizing its DNA binding capacity (9–12). Cyclin A-cdk1 holoenzymes also contribute to E2F-1 phosphorylation (13).

Inappropriately persistent E2F-1 activity during S phase traversal and exit has been shown to result in apoptosis. For example, the ectopic expression of an E2F-1 mutant incapable of binding to cyclin A or of a nonphosphorylatable DP-1 mutant causes S phase delay and apoptosis (14). Both p53-dependent and p53-independent apoptotic pathways may be activated in response to E2F-1 (15).

Disruption of Rb or its upstream regulators in tumor cells would be expected to result in high levels of free E2F-1 compared with levels found in their normal counterparts. Transformed cells may be particularly dependent on cyclin A-dependent kinase activity during S phase to limit E2F-1 activity and prevent apoptosis. A reduction in cyclin A-dependent kinase activity may result in the persistence of residual E2F activity that may cause the death of cells with high enough E2F-1 activity to surpass the threshold required to induce apoptosis and may therefore be lethal to transformed cells yet tolerable to normal cells. This concept was tested recently using short peptides that block the interaction of cyclin A-cdk2 with substrates such as E2F-1. Introduction of these peptides induced S phase arrest and apoptosis; cell death occurred selectively in transformed cells (16).

Flavopiridol is a small molecule cdk inhibitor currently in clinical development (17–19). As flavopiridol inhibits multiple cyclin-cdk holoenzymes (including cyclin D-cdk4, cyclin A/E-cdk2, and cyclin B-cdk1), it induces GI and G2 arrest in a wide variety of exponentially growing cells (18). In many solid tumor cell lines, death of exponentially growing cells only occurs after prolonged exposures to concentrations of drug greater than the range necessary to inhibit cdks, suggesting that cytotoxicity is related to the effects of high flavopiridol concentrations on other cellular targets (20).

However, we have recently shown that cells can be sensitized to flavopiridol if they are first recruited to S phase. Instead of an initial cytostatic cell cycle arrest, treatment of cells during S phase results in inhibition of S phase progression and apoptosis at concentrations of drug that correspond to cdk inhibition (21). The result is similar whether cells are recruited to S phase by synchronization or by prior exposure to noncytotoxic concentrations of chemotherapy drugs that cause S phase accumulation, including cisplatin and gemcitabine. In
addition, flavopiridol-induced apoptosis during S phase occurs selectively in transformed cells (21).

The mechanism of S phase sensitization to flavopiridol may be related to the known consequences of cdk inhibition during S phase. Flavopiridol-mediated inhibition of cyclin A-dependent kinase activity during S phase would be expected to result in decreased phosphorylation of the E2F/DP heterodimer, permitting inappropriately persistent E2F-1 activity that induces an apoptotic response. In addition, cyclin A-dependent kinase inhibition may also account for the selective killing of transformed cells when they are exposed to flavopiridol during S phase (16).

In this report, we have tested several predictions of this model. First, we show that following synchronization at the G1-S boundary, flavopiridol treatment results in persistent expression of E2F-1 during S phase traversal. Consistent with the inhibition of cyclin A-cdk2 by flavopiridol, the phosphorylation of E2F-1 is markedly diminished, so that it remains capable of binding to DNA. The phosphorylation of Rb is only minimally affected, and increased complex formation between Rb and E2F-1 is not detected during S phase in the presence of flavopiridol. Moreover, we show that manipulation of the cellular level of E2F-1 activity affects the apoptotic response induced by flavopiridol during S phase. For example, ectopic expression of high levels of E2F-1 renders transformed cells more sensitive to flavopiridol during S phase than parental controls expressing lower levels.

In addition, nontransformed cells engineered to express high levels of E2F-1 are similarly sensitized, suggesting that it is indeed the high baseline levels of E2F-1 in transformed cells that accounts for their selective sensitivity to flavopiridol during S phase. Furthermore, disruption of E2F-1 activity prevents the apoptotic response to flavopiridol during S phase. We have investigated this question using immortalized cells homozygous for a nonfunctional E2F-1 allele. Finally, we show that expression of an E2F-1 mutant that cannot be regulated by cyclin A-dependent kinase activity diminishes the degree of sensitization to flavopiridol during S phase, indicating that the modulation of E2F-1 activity resulting from flavopiridol-mediated cdk inhibition contributes to induction of the apoptotic response. These data explain the sensitivity of transformed cells to flavopiridol during S phase, as well as the synergism observed when flavopiridol follows chemotherapy agents that retard S phase progression.

**MATERIALS AND METHODS**

**Tumor Cell Lines.** NCI-H661 and A549 non-small cell lung cancer cell lines as well as U2OS osteosarcoma cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in DMEM supplemented with 10% BCS.

**Generation of Cell Lines Expressing Ectopic E2F-1.** Stable cell lines were generated by retroviral infection. Fugene 6 reagent was used to transfect Bing packaging cells (22) with pBABE-puro or pBABE-puro into which a cDNA containing hemagglutinin-tagged wild-type E2F-1 or [E2F-1 (Δ24)] (9), encoding an E2F-1 species incapable of cyclin A binding. In these experiments, cells were cotransfected with 4 μg of a construct encoding DP-2 (pCMV- DP-2) to permit nuclear localization of the E2F-1 mutant species (24).

**Ratla Fibroblasts.** Ratla fibroblasts with stably integrated p1093 or p1093-E2F-1 (in which E2F-1 cDNA is under the control of the metallothio-

**Drug Treatment.** Stock solutions of flavopiridol (provided by the Drug Synthesis & Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute), hydroxurea (Sigma-Aldrich Co., St. Louis, MO), and gemcitabine (Eli Lilly and Co., Indianapolis, IN) were prepared as described previously (21, 28). Flavopiridol was used at concentrations ranging from 150 to 300 nM. Cells were treated with 1 μM hydroxyurea to achieve synchronization at the G1-S boundary. Gemcitabine concentrations ranging from 10 nM to 10 μM were used. The concentration and protocol for gemcitabine treatment varied with each cell line and were designed to allow accumulation of cells in early-mid S phase or at the G1-S boundary before flavopiridol treatment. Cells were plated 24–30 h before drug treatment and were at subconfluent density at the time of drug exposure. For sequential drug treatments, cells were washed in PBS before application of the second drug.

**Fluorescence-Activated Cell-Sorting Analysis.** Cell cycle analysis was performed as described previously (21, 28). Nonadherent and adherent cells were combined. After fixation and treatment in 500 μg/ml RNase A, cells were resuspended in 1 ml 69 μM propidium iodide in 30 mM sodium citrate. Cells were analyzed for DNA content by flow cytometry using the ModFit program (Verity Software House, Topsham, ME).

**Detection of Apoptosis by Flow Cytometry.** A fluoroein apoptosis detection kit was used (Promega, Madison, WI), as described previously (21, 28). Nonadherent and adherent cells were combined. After formaldehyde and ethanol fixation, cells were incubated with fluoroein-12 dUTP in the absence or presence of TdT. After washes prescribed in the manufacturer’s instructions, cells were resuspended in PBS containing 5 μg/ml propidium iodide and 500 μg/ml RNase A. Cells were analyzed for DNA content and apoptosis using two-color flow cytometry. Apoptosis was quantitated as the percentage of cells shifting to fluorescence-positivity in the presence of TdT.

**Western Blot Analysis.** Whole cell lysates were prepared in NP40 lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP40, and 1 mM phenylmethylsulfonyl fluoride], and clarified by centrifugation. Nuclear extracts were prepared using NE-PER Nuclear Extraction Reagents (Pierce, Rockford, IL), according to the manufacturer’s instructions. The nuclear lysates were prepared in buffer supplemented with a protease inhibitor mixture (Calbiochem-Novabiochem, San Diego, CA) and 1 mM phenylmethylsulfonyl fluoride. Protein concentrations were determined using the Bradford assay (Bio-Rad, Richmond, CA), and equivalent amounts (10–100 μg) were subjected to SDS-PAGE. Western blotting was performed as described previously (28).

Following the antibodies were used: 1:500 dilution of anti-E2F-1 (C-20; Santa Cruz Biotechnology, Santa Cruz, CA); 1:1000 dilution of anti-E2F-1 (NB100-189; Novus Biologicals); 1:500 anti-HA, clone 12CA5 (Roche Molecular Biochemicals, Indianapolis, IN); 1:1000 dilution of anti-p16INK4A (M-156; Santa Cruz Biotechnology); 1:1000 anti-pan phospho-ERK (Cell Signaling Technology, Beverly, MA); 1:1000 dilution of anti-β-tubulin (Sigma-Aldrich Co.). For analysis of Rb, the following antibodies were used at 1:1000 dilution: anti-Rb, clone G3–245 (PharMingen, San Diego, CA); anti-COOH-terminal Rb, anti-Rb (pS795), anti-Rb (pS780), anti-Rb (pS807/811), and anti-Rb...
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RESULTS

Flavopiridol Treatment During S Phase Inhibits Cell Cycle Progression and Results in Persistent Expression of E2F-1. In many exponentially growing solid tumor cell lines, treatment with flavopiridol causes arrest at the G1 and G2 boundaries. G1 arrest is associated with direct inhibition of G1 cdk activity and transcriptional repression of D-cyclins, leading to decreased E2F-1 expression and a diminution in cellular S phase DNA content. To examine the effects of flavopiridol treatment during S phase, NCI H661, A549, and U2OS cells were synchronized at the G1-S boundary with hydroxyurea, and released into DMSO or 300 nm flavopiridol (Figs. 1 and 2). Fig. 1 demonstrates that S phase progression is slowed after flavopiridol

A.

B.

C.

Fig. 1. Flavopiridol treatment during S phase slows cell cycle progression and causes persistent expression of E2F-1 in NCI-H661 cells. A, cells were treated with 1 mM hydroxyurea for 24 h, achieving synchronization at the G1-S boundary. Cells were then released into DMSO or 300 mM flavopiridol for 2, 4, 6, 8, or 10 h. Adherent and nonadherent cells were pooled, fixed, and analyzed by flow cytometry. B, nuclear lysates were extracted directly after release into DMSO or flavopiridol, and subjected to Western blotting. Results demonstrate the persistent expression of E2F-1 as cells traverse S/G2, in the presence of flavopiridol. Levels of cyclin A and cdk2 are stable, whereas levels of p27Kip1 fall in flavopiridol. Rb was analyzed with an antibody recognizing phosphorylated and unphosphorylated forms (G3-245, designated Rb), a COOH-terminal antibody, as well as with phosphorylating antibodies. Minimal dephosphorylation of Rb occurs over this time course, as indicated by results with phosphorylating Rb antibodies. C, cells were treated with 1 mM hydroxyurea alone or followed by release into DMSO for 2 h (D2) or flavopiridol for 4 or 6 h (F4 and F6). One mg of nuclear lysate was subjected to immunoprecipitation with anti-E2F-1 (C-20) or control rabbit IgG antibodies, followed by Western blotting with anti-Rb (G3-245) or anti-E2F-1 (KH95) antibodies (top blot, first four lanes; and bottom blot). In addition, 50 µg of each extract (D2, F4, and F6) was subjected to Western blotting with the anti-Rb (G3-245) antibody (top blot, last 3 lanes). Exposure of the top blot was ~1 h; exposure of the bottom blot was ~1 min. The data demonstrate that only a small amount of E2F-1 is in complex with Rb after release from the hydroxyurea-induced block and that E2F-1 does not accumulate in a complex with Rb as cells traverse S phase in the presence of flavopiridol.
A549

1H 2 4 6 8 10
E2F-1
cyclin A
cdk2
p27 [pT187]
p27
p21
Rb [pS780]
Rb [pS795]
Rb [pS807/811]
Rb [pT821]

U2OS

1H 2 4 6 8 10
E2F-1
cyclin A
cdk2
p27
p21
Rb
Rb [pS780]
Rb [pS795]
Rb [pS807/811]
Rb [pT821]

Fig. 2. Flavopiridol treatment during S phase causes persistent expression of E2F-1 in A549 and U2OS cells without evidence of Rb dephosphorylation. Cells were treated with 1 mM hydroxyurea for 24 h, followed by release into DMSO or 300 nM flavopiridol for 2, 4, 6, 8, or 10 h. In both of these cell lines, E2F-1 expression persists in the presence of flavopiridol, without detectable effects on the phosphorylation of Rb. During S phase traversal in flavopiridol, levels of cyclin A and cdk2 are stable, whereas levels of p21WAF1/Cip1 and p27Kip1 fall.

exposure. In addition, whereas E2F-1 levels are diminished in DMSO-treated cells as they approach the G2 boundary, they remain persistently high in the presence of flavopiridol. In contrast, levels of cyclin A and cdk2 are not affected and remain constant over the first 10 h of drug treatment, whereas levels of Cip/Kip proteins are decreased by flavopiridol during S phase traversal. Our previous studies demonstrated that in NCI-H661 cells treated with flavopiridol in this manner, a small amount of apoptosis is detected at 6 h, but becomes more substantial after 12 h of drug exposure (21). In A549 and U2OS cells, the slowing of S phase progression is not as marked, and the onset of apoptosis of the S/G2 population is more delayed, occurring between 18 and 24 h. Nonetheless, in all of these cell lines, persistent expression of E2F-1 in the presence of flavopiridol precedes the onset of apoptosis as cells traverse S and G2.

These experiments were performed after release from synchronization at the G1-S boundary, past the peak of cyclin E-cdk2 activity, a time when Rb is phosphorylated and incapable of E2F-1 binding. Nonetheless, it is possible that flavopiridol-mediated cdk inhibition could result in Rb dephosphorylation during S phase, resulting in E2F-1 inactivation. Therefore, we examined Rb phosphorylation in response to flavopiridol after release from the hydroxyurea-induced G1-S block. In A549 and U2OS cells, dephosphorylation of Rb during S phase traversal in the presence of flavopiridol is not detected, either with an antibody recognizing phosphorylated and unphosphorylated forms or with phosphospecific antibodies (Fig. 2). In NCI-H661 cells, a slightly faster migrating form of Rb is present at 8 and 10 h after exposure to flavopiridol during S phase. This band is present, but not as apparent when an antibody recognizing the COOH terminus of Rb is used. Therefore, a low level of either dephosphorylation or caspase cleavage (30), or both could generate the faster migrating band. If Rb dephosphorylation occurs, it is only to a small degree, as the phosphorylation at many of the cdk4- and cdk2-specific sites is largely preserved (Refs. 31–34; Fig. 1B), with no change in the phosphorylation of Rb at S780 and S795, and only a slight decrease at S807/811, T821, and S249/252. In addition, as cells pass through S phase, an increase in complex formation between E2F-1 and Rb is not observed (Fig. 1C). After release from hydroxyurea into DMSO for a short period (2 h) or flavopiridol for longer periods (4 or 6 h), nuclear lysates were subjected to immunoprecipitation with an anti-E2F-1 antibody, followed by Western blotting with either anti-Rb or anti-E2F-1 antibodies. Complexes containing E2F-1 and Rb are only detected after prolonged exposures of the blots, indicating that E2F-1 remains largely free of Rb during S phase in the presence of flavopiridol. Similar results were obtained after release into flavopiridol for 8 h and with reciprocal immunoprecipitations (data not shown).

E2F-1 Phosphorylation Is Inhibited by Flavopiridol during S Phase, Facilitating Persistent DNA Binding of the E2F-1/DP-1 Heterodimer. Following activation of E2F-1 during early S phase, cyclin A-cdk2 phosphorylates both E2F-1 and DP-1, neutralizing the DNA binding capacity of the heterodimer (9–12). Appropriately timed deactivation of E2F-1 by cyclin A-cdk2 is critical for proper S phase progression. Cyclin A-cdk2 inhibition by flavopiridol should result in decreased phosphorylation of E2F-1, which would result in persistent DNA binding of the transcription factor. Fig. 3 demonstrates that phosphorylation of E2F-1 is reduced over the course of S phase traversal in the presence of flavopiridol in U2OS cells. Similar data were obtained in NCI-H661 cells (data not shown). Consequently, both E2F-1 and DP-1 remain capable of binding to their consensus sequence throughout the course of S phase (Fig. 4), demonstrated by an ELISA assay designed to detect DNA binding by either E2F-1 or DP-1.

High Levels of E2F-1 Additionally Sensitize Transformed Cells to Flavopiridol-Induced Apoptosis during S Phase. To determine whether the amount of E2F-1 expressed can affect sensitivity to flavopiridol-mediated apoptosis during S phase, NCI-H661, A549, and U2OS cells were engineered to express high levels of ectopic E2F-1 by retroviral transfer. Fig. 5A demonstrates that E2F-1 expression could be increased several fold with this strategy. Examination of nuclear and cytoplasmic lysates indicated that in pBABE-E2F-1-infected cells, ~80% of the ectopic E2F-1 is nuclear (data not shown). Under conditions of normal exponential growth in 10% serum, these cell lines were able to tolerate increased expression of E2F-1 without a significant change in cell cycle pattern (Fig. 5B). Control and E2F-1 overexpressing cells were then treated with hydroxyurea to achieve a
The concentration of flavopiridol was lowered to 150 nM so that fewer cells die from flavopiridol treatment after release from hydroxyurea, and 7 experiments performed at 8 h. At 8 h, the difference in the amount of E2F-1 capable of DNA binding was statistically greater at 8 h compared with 2 h (P = 0.00005). In flavopiridol-treated cells, the amount of E2F-1 capable of DNA binding was statistically greater at 8 h compared with 2 h (P = 0.02). Bars, ±SD.

Overexpression of E2F-1 Sensitizes Nontransformed Cells to Flavopiridol-Mediated Apoptosis. In our previous work, recruitment to S phase selectively sensitized transformed cell populations to flavopiridol (21). Because of Rb pathway disruption, transformed cells are expected to have higher levels of free E2F-1 activity than their normal counterparts. To examine whether high levels of E2F-1 activity could account for the differential sensitivity of transformed cells to flavopiridol-induced apoptosis during S phase, we used a rat fibroblast cell line stably transfected with an E2F-1 cDNA under control of the metallothionein promoter (16, 25). After induction with zinc, Rat 1a cells transfected with p1093-E2F-1 express high levels of E2F-1, whereas cells transfected with the parent vector maintain expression of baseline levels of E2F-1 (Fig. 7A). Under conditions of exponential growth in 10% serum, induction of E2F-1 did not significantly affect the cell cycle pattern of transfected cells (Fig. 7B, left panels). Treatment of Rat 1a fibroblasts with either 250 or 300 nM flavopiridol for 24 h slows cell cycle progression but does not result in tight cell cycle arrest. Because these cells do not undergo pro-
nounced G₁ arrest after flavopiridol exposure, recruitment to S phase first was not necessary, and we were able to examine the response of control and E2F-1 overexpressing cells to flavopiridol alone. As shown by the fluorescence-activated cell sorter analyses in Fig. 7B, induction of E2F-1 sensitized these cells to flavopiridol-induced apoptosis, evidenced by the large sub-G₁ peak in p1093-E2F-1 cells maintained in zinc and flavopiridol. This was confirmed when cell death was measured by TUNEL assay or by PARP cleavage (Fig. 7, C and D). Viable cell number over 48 h of flavopiridol treatment is shown in Fig. 7E. Cells expressing parent vector grow sluggishly in the presence or absence of zinc, consistent with flavopiridol-induced slowing of cell cycle progression. In the subclone expressing the E2F-1 inducible vector, viable cell count is relatively stable over the 48 h of flavopiridol treatment in the absence of zinc. However, after induction of E2F-1 in the presence of zinc, viable cell number decreases by 24 h, and the cell population is almost entirely dead by 48 h. Therefore, high levels of E2F-1 can confer flavopiridol sensitivity on a nontransformed cell line otherwise resistant to flavopiridol-induced apoptosis.

Confirmatory experiments were performed in these cells after S phase recruitment with a noncytotoxic concentration of gemcitabine (Fig. 8A). The sequential gemcitabine/flavopiridol combination resulted in the greatest amount of apoptosis after induction of ectopic E2F-1 in the presence of zinc (Fig. 8, B and C).

**Disruption of E2F-1 Activity Inhibits the Apoptotic Response to Flavopiridol during S Phase.** To confirm the critical role of E2F-1 in flavopiridol-induced apoptosis during S phase, we examined 3T3 cells derived from E2F-1 +/-, +/-, and --/-- MEFs. Fig. 9A demonstrates that E2F-1 was only detectable in nuclear extracts from
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Fig. 7. High levels of ectopic E2F-1 are sufficient to sensitize nontransformed cells to flavopiridol. A, characterization of Rat1a fibroblasts engineered to express control vector (p1093) or ectopic E2F-1 under the control of the metallothionein promoter (p1093-E2F-1). Subconfluent cells were left untreated or treated with 100 μM ZnCl₂ for 24 h, after which protein was extracted and subjected to Western blotting with an anti-E2F-1 antibody (clone KH95). High levels of E2F-1 are induced in p1093-E2F-1 cells in the presence of zinc. B, cell cycle profiles of control (p1093) and p1093-E2F-1 cells. Cells were fixed and stained with propidium iodide for flow cytometry. Left panels demonstrate that after growth in 100 μM ZnCl₂ for 24 h, a high level of ectopic E2F-1 expression does not significantly alter the cell cycle distribution of these cells. Treatment with 250 nM flavopiridol for 24 or 48 h results in cell death primarily in E2F-1 overexpressing cells. C, p1093 or p1093-E2F-1 cells were grown in the absence or presence of ZnCl₂ for 24 h, and then exposed to DMSO, 250 nM, or 300 nM flavopiridol in the absence or presence of ZnCl₂, for an additional 48 h. At the end of 48 h, adherent and nonadherent cells were pooled, fixed, and subjected to the flow cytometry-based TUNEL assay in which apoptosis was quantitated. Only Rat1a fibroblasts expressing high levels of ectopic E2F-1 undergo significant apoptosis after 48 h of flavopiridol exposure. Data represent a compilation of a minimum of three and a maximum of six experiments for each condition. In p1093-E2F-1 cells, differences in the amount of apoptosis in the absence and presence of ZnCl₂ were statistically significant at both 250 nM flavopiridol (P = 0.048) and 300 nM flavopiridol (P = 0.008). Bars, ±SD. D, cells were grown in the absence or presence of ZnCl₂ for 24 h and then treated with DMSO or 300 nM flavopiridol in the absence or presence of ZnCl₂, for an additional 48 h. Nonadherent and adherent cells were pooled, and whole cell lysates were subjected to Western blotting using antibodies detecting cleaved PARP and tubulin. E, left, subconfluent p1093 cells were grown in the absence or presence of zinc for 24 h, and then treated with 300 nM flavopiridol for an additional 48 h in the absence (○) or presence (●) of zinc. Time 0 represents the initiation of flavopiridol exposure. At the indicated times, adherent and nonadherent cells were collected, and viable cell count determined by trypan blue exclusion. Right, the same experiment performed with p1093-E2F-1 cells.

+/+ and +/− cells. Several sets of cell lines were characterized, and growth curves and cell cycle distribution patterns were comparable (Fig. 9B). During immortalization, these cell lines retained p16INK4A expression and displayed markedly diminished expression of the Rb protein (Fig. 9A). After release from a hydroxyurea-induced block into flavopiridol, cells expressing E2F-1 underwent cell death. In contrast, apoptosis was diminished in cells lacking E2F-1 (Fig. 9, C and D). When E2F-1 expression was restored to E2F-1 −/− cells,
apoptosis after release from a hydroxyurea-induced block into flavopiridol occurred at a degree comparable with that seen in E2F-1 +/+ cells (Fig. 10A). Similarly, following S phase recruitment with noncytotoxic concentrations of gemcitabine, release into flavopiridol resulted in cell death in cell lines expressing E2F-1 and was markedly compromised in cells in which E2F-1 expression was genetically disrupted, when measured by either TUNEL assay (Fig. 10B) or PARP cleavage (data not shown).

**Inhibition of Cyclin A-Dependent Kinase Activity by Flavopiridol Likely Contributes to the Induction of Apoptosis during S Phase.** To validate the importance of inhibition of cyclin A-dependent kinase activity by flavopiridol in the induction of apoptosis during S phase, an E2F-1 mutant incapable of binding to cyclin A [E2F-1 (Δ24)] was examined. Because this mutant is not phosphorylatable by cyclin A-cdk2, its expression is expected to result in constitutive deregulated E2F-1 activity, which itself can induce apoptosis (14, 23). Effects of flavopiridol in the presence of this mutant are expected to be diminished, because inhibition of cyclin A-cdk2 will not affect the activity of this E2F-1 species.

Fig. 11A demonstrates the expression of wild-type E2F-1 and [E2F-1 (Δ24)] after transient transfection in NCI-H661 cells. Because cyclin A-binding mutants of E2F-1 also lack nuclear localization signals, these constructs were introduced along with DP-2, permitting similar levels of nuclear expression of wild-type and mutant E2F-1 (24). After transfection, cells were synchronized and released into DMSO or flavopiridol, again at reduced concentration (150 nM) to avoid a large amount of cell death in control populations. Fig. 11B demonstrates that expression of wild-type E2F-1 sensitized NCI-H661 cells to flavopiridol-induced apoptosis during S phase by 8-fold in the context of these experimental conditions. As expected, nuclear expression of the nonphosphorylatable [E2F-1 (Δ24)] resulted in baseline apoptosis in S phase cells exposed only to DMSO. However, when the cyclin A-binding mutant is expressed, treatment with flavopiridol modulated apoptosis only by 1.5–2-fold. These data suggest that inhibition of cyclin A-dependent kinase-mediated phosphorylation of E2F-1/DP-1 plays a major role in the induction of apoptosis by flavopiridol during S phase.

**DISCUSSION**

Flavopiridol is the first potent inhibitor of cdks to enter clinical trials. The response of many solid tumors, both in vitro and in vivo, is cell cycle arrest, with reversible, cytostatic effects on tumor growth. We have shown previously that recruitment of cells to S phase, accomplished by cell synchronization or by pretreatment with chemotherapy agents that impose S phase delay, markedly sensitizes them to flavopiridol (21). The mechanism of S phase sensitivity and selectivity may be related to inhibition of cdk2 activity by flavopiridol during S phase, preventing the properly timed deactivation of E2F (9–11, 16), resulting in S phase delay and apoptosis.

Here, we have examined the effect of flavopiridol treatment as cells pass through S phase on the expression of several cell cycle proteins. In flavopiridol-treated cells, S phase progression is slowed and E2F-1 levels persist as cells move toward the G2-M boundary. Consistent with flavopiridol-mediated inhibition of cyclin A-dependent kinase activity, the phosphorylation of E2F-1 during S phase is markedly diminished in the presence of drug, so that the E2F-1/DP-1 heterodimer remains capable of DNA-binding. These events either coincide with or precede the onset of apoptosis in the cell lines examined (21).

Importantly, in the presence of flavopiridol, E2F-1 likely remains bound to DNA free of Rb. This is because Rb dephosphorylation occurs at most to a small degree in response to flavopiridol over the course of S phase traversal. In these experiments, the cell cycle period during which cells are exposed to flavopiridol is after cyclin D-cdk4/6 and cyclin E-cdk2 holoenzymes have already phosphorylated Rb. After the G1-S boundary, Rb typically remains phosphorylated to
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Fig. 7. Characterization of 3T3 cells with E2F-1 +/-, +/-, and +/- genotypes. Western blot analysis for two sets of 3T3 cell lines. Each set contains lines of the designated genotype, derived from MEFs, which, in turn, were derived from embryos generated from a cross of E2F +/- mice. To identify E2F-1 in these cells, 100 μg of nuclear protein was subjected to Western blotting with a polyclonal anti-E2F-1 antibody (C20). Whole cell lysates were used for Western blotting to identify Rb and p16INK4A, respectively. The positive control for Rb expression was a lysate from pIpMP-/-MEFs. B, representative cell cycle profiles from E2F-1 +/- and E2F-1 +/- 3T3 cells. Exponentially growing cells were collected, fixed, and stained with propidium iodide for flow cytometry. The absence of E2F-1 did not alter the cell cycle profile. C, E2F-1 +/- and +/- 3T3 cells were treated with 1 μm hydroxyurea for 24 h. After synchronization at the G1/S boundary, cells were rinsed with PBS and released into 300 μM flavopiridol for an additional 24 h. Adherent and nonadherent cells were pooled, fixed, and subjected to the flow cytometry-based TUNEL assay in the absence or presence of Tdt. The primary data demonstrate that cell death is significantly diminished in E2F-1 +/- cells; numbers indicate the percentage of fluorescein-positive cells. D, E2F-1 +/- and +/- 3T3 cells were treated for 24 h with DMSO, 150 μM, or 300 μM flavopiridol for 24 h. Alternatively, after synchronization with 1 μm hydroxyurea for 24 h, cells were released into DMSO, 150 μM, or 300 μM flavopiridol, as in B. Adherent and nonadherent cells were pooled, fixed, and subjected to TUNEL assay to quantify apoptosis. After S phase recruitment, flavopiridol-induced apoptosis in E2F-1 +/- cells is severely compromised. Data are shown for +/- and +/- 3T3 cells from one set. A minimum of 3 and a maximum of 12 experiments were used to generate the graphs. Differences in the amount of apoptosis in E2F-1 +/- and +/- cells treated with hydroxyurea followed by flavopiridol were statistically significant at 150 μM flavopiridol (P = 0.00027) and 300 μM flavopiridol (P = 0.0015). Bars, ±SD.

Fig. 10. A, E2F-1 re-expression in E2F-1 +/- cells restores their ability to undergo flavopiridol-mediated apoptosis following release from a hydroxyurea-induced block at the G1-S boundary. E2F-1 +/- cells were infected with control (C, pBABE) or E2F-1 (pBABE-E2F-1) expressing retroviruses. The Western blot demonstrates the restoration of E2F-1 expression in pBABE-E2F-1-infected cells. Cells were treated with 1 μM hydroxyurea for 24 h, rinsed with PBS, and then exposed to DMSO (1H/D) or 300 μM flavopiridol (1H/F) for an additional 24 h. Adherent and nonadherent cells were pooled, fixed, and subject to TUNEL assay to quantify apoptosis. Data from one (1H/D) or two (1H/F) experiments were used to generate the graphs. In flavopiridol-treated cells, differences in the amount of apoptosis between pBABE and pBABE-E2F-1 infected cells were statistically significant (P = 0.01). Bars, ±SD. B, flavopiridol-induced apoptosis following recruitment to S phase by gemcitabine is compromised in cells lacking functional E2F-1 alleles. E2F-1 +/-, +/-, or +/- 3T3 cells were treated with 1 μM (1H/D) or 10 μM (1H/F) gemcitabine for 24 h, concentrations that result in accumulation in S phase or at the G1-S boundary in these cells. After a PBS rinse, cells were treated with DMSO or 300 μM flavopiridol for an additional 24 h. Adherent and nonadherent cells were pooled, fixed, and subjected to TUNEL assay to quantitate apoptosis. Data are shown for 1 set of +/-, +/-, and +/- cells; a minimum of 3 and maximum of 10 experiments were used to generate the graphs for each condition. The 1 μM gemcitabine/DMSO sequential treatment was performed only once. P values demonstrated statistically significant differences between +/- or +/- and +/- cells (for 1HG/F: +/- versus +/-, P = 0.000001; +/- versus +/-, P = 0.000095; for 1HG/F: +/- versus +/-, P = 0.0034; +/- versus +/-, P = 0.006). Bars, ±SD.

permit S phase completion; maintenance of Rb phosphorylation most likely does not require continued cdk activity, which is typically diminished during S phase by expression of endogenous inhibitors, such as p19INK4A (35), as well as reduction of levels of cyclin E,
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Fig. 11. The effect of flavopiridol during S phase is blunted in NCI-H661 cells expressing (E2F-1ΔΔ24), a cyclin A-binding defective mutant. A. subconfluent cells were cotransfected with vector alone or constructs encoding either hemagglutinin-tagged wild-type E2F-1 or (E2F-1ΔΔ24), along with a construct encoding DP-2 (pCMV-DP-2). Nuclear lysates extracted 48 h after transfection were subjected to Western blotting with anti-E2F-1, anti-HA, or anti-DP-2 antibodies. B. transfections were carried out as in A. Twenty-four h after transfection, cells were treated with 1 mM hydroxyurea for 24 h. After a PBS rinse, cells were treated with either DMSO or 150 nM flavopiridol for an additional 24 h. Nonadherent and adherent cells were pooled and subjected to TUNEL assay. Graphs represent compilation of data from three experiments in the case of cells transfected with vector alone or the construct encoding wild-type E2F-1 and six experiments for cells transfected with the construct encoding (E2F-1ΔΔ24). In the case of vector-expressing cells, differences in the amount of apoptosis after treatment with hydroxyurea/DMSO or hydroxyurea/flavopiridol were not statistically significant (P = 0.308). These treatments did induce significant differences in apoptosis in cells expressing wild-type E2F-1 (P = 0.0029). Expression of (E2F-1ΔΔ24) produce significantly more baseline apoptosis compared with expression of wild-type E2F-1 (P = 0.026). The degree of apoptosis induced by flavopiridol was blunted in mutant expressing cells compared with wild-type (P = 0.018), indicating that the ability of flavopiridol to modulate E2F-1 via inhibition of cyclin A-dependent kinase activity is required for a maximal apoptotic response. However, in (E2F-1ΔΔ24)-expressing cells, flavopiridol induced an increase in apoptosis over baseline (i.e., DMSO) that was statistically significant (P = 0.0006), suggesting that other flavopiridol targets in addition to cyclin A-dependent kinase may also contribute to E2F-1-dependent apoptosis.

Events preserved in transformed cell types (36). Therefore, any additional reduction in cdk activity by flavopiridol specifically during S phase is unlikely to have major impact on the degree of Rh phosphorylation already accomplished during cell cycle progression.

Flavopiridol treatment during S phase also resulted in diminished expression of both p21Waf1/Cip1 and p27Kip1. The effect on p27Kip1 expression was surprising, as decreased phosphorylation of this protein (as shown in Fig. 2) after cdk2 inhibition should result in its stabilization (37, 38). Decreased levels of Cip/Kip proteins during S phase in the presence of flavopiridol may possibly be explained by decreased transcription mediated by inhibition of cdk9-pTEFb (39–43) or by cleavage of these proteins by caspases early after the induction of apoptosis (44–46).

A model in which the apoptotic response of cells to flavopiridol during S phase is dependent on modulation of E2F-1 activity by cyclin A-dependent kinase inhibition leads to several testable predictions. First, cells with high baseline levels of E2F-1 activity should be more sensitive to cyclin A-dependent kinase inhibition than cells with lower levels, because the higher levels of E2F-1 that persist are more likely to achieve the threshold activity necessary to generate an apoptotic response during S phase traversal and exit. Here, we have tested this hypothesis in several tumor cell lines engineered to express high levels of ectopic E2F-1. The high levels of expression were tolerable to these cells, all grown in high serum, and presumably ectopic protein was subject to the same phosphorylation and ubiquitination events that regulate endogenous E2F-1 activity. Following recruitment to S phase by synchronization or with noncytotoxic doses of gemcitabine, cells expressing high levels of E2F-1 were more sensitive to flavopiridol than their parental counterparts. In general, these differences were most notable at a low flavopiridol concentration, i.e., 150 nM; at the 300 nM concentration, parental cells are also sensitive to hydroxyurea/ flavopiridol and gemcitabine/flavopiridol treatments, so that differences were more difficult to appreciate. The exception was U2OS cells, in which S phase retardation and apoptosis are more separable in time; less of a delay in apoptosis occurred in cells expressing high levels of ectopic E2F-1 (Fig. 6B).

In previous work, we demonstrated that transformed cell populations were selectively sensitized to flavopiridol treatment during S phase (21). The universal alteration in expression of cell cycle proteins in human tumors is expected to produce higher levels of free E2F-1 activity in tumor cells compared with their normal counterparts. In this report, we have demonstrated that overexpression of E2F-1 in nontransformed Rat1a fibroblasts is sufficient to sensitize these cells to flavopiridol. These data suggest that higher levels of E2F-1 activity could indeed account for the selective killing of transformed cells when flavopiridol treatment occurs during S phase.

The mechanism for the sensitivity of S phase cells also predicts that disruption of E2F activity will inhibit the apoptotic response to flavopiridol. We have tested this using 3T3 cells derived from E2F-1+/+, +/-, and −− MEFs. The ability of E2F-1 −− cells to undergo flavopiridol-induced apoptosis during S phase is markedly compromised, indicating that E2F-1 activity is essential for a maximal apoptotic response. In addition, E2F-1 +/- cells underwent apoptosis to a greater degree than E2F-1 ++ cells, suggesting a gene dosage effect.

While our data are in agreement with a previous analysis of flavopiridol in E2F-1 −− cells (47), it is noteworthy that our experimental conditions were very different. In the former experiments, nonimmortalized MEFs were used, and exponentially growing cells were exposed to flavopiridol. A difference in the degree of cell death at 72 h (−14 versus 4%) was reported. Presumably, this is the cell death occurring late after cell cycle arrest that occurs in both transformed and nontransformed cells. It is of interest that E2F-1 expression may affect the degree of late cell death as well, in addition to the earlier cell death that occurs when cells are treated during S phase.

Our data are also consistent with results in exponentially growing NCI-H1299 cells, in which the deletion of E2F-1 by RNA interference (RNAi) was reported to compromise flavopiridol-induced apoptosis (47). Our attempts to deplete E2F-1 activity in the cell lines examined here, via RNAi or expression of dominant-negative E2F-1 mutants, has resulted in derivatives with higher G1 and diminished S phase content compared with parental cells. Among transformed tumor cell lines, cycling cells are more sensitive to flavopiridol than arrested cells (28, 48), so this approach has not allowed us to definitively attribute decreased sensitivity of these cells to the absence of E2F-1. In the cell lines used in this study, the expression of ectopic E2F-1 or the absence of endogenous E2F-1 did not affect cell cycle patterns in exponentially growing cells. Therefore, it is likely that E2F-1 is playing a primary role in flavopiridol-induced apoptosis during S phase, and that the effects seen are not dependent on E2F-1-mediated cell cycle progression or secondary to altered cell cycle distribution in engineered cells.

It will next be important to determine the mechanism by which E2F-1 induces an apoptotic response after flavopiridol-mediated cdk
inhibition during S phase. E2F-1 has been shown to induce apoptosis in cells by both p53-dependent and p53-independent pathways (25, 49–54). For example, when induced in growth factor-deprived quiescent cells, E2F-1 promotes S phase entry and p53-dependent apoptosis, in part via induction of p14ARF (55, 56). However, the induction of apoptosis resulting from uninterrupted E2F-1 activity beyond G1-S occurs in cells cultured in serum-containing medium and is independent of p53 (14, 23). Whereas A549 and U2OS cells express wild-type p53, NCI-H661 cells are hemizygous for p53 (57) and do not have normal p53 function in response to DNA damage, making it likely that the effects we are observing are independent of p53.

Mechanisms of p53-independent E2F-1-induced apoptosis include pathways that are both dependent and independent of E2F-1 transcriptional transactivation activity. It will be of interest to determine whether E2F-1 transcriptional targets such as p73 (58–60), caspase 3, or Apaf-1 (61, 62) are essential for flavopiridol-mediated cytotoxicity during S phase. However, flavopiridol-mediated inhibition of cyclin T/cdk9 (pTEFb) results in a global decrease in efficient transcriptional elongation (39), suggesting that increased E2F-1 transcriptional activity may be difficult to detect in flavopiridol-treated cells. Furthermore, an E2F-1 mutant lacking transcriptional transactivation activity, E2F-1 (1–374), can induce apoptosis as efficiently as wild-type E2F-1 (51, 52), which may occur via interaction with nuclear factor κB-mediated survival pathways (63–65). Finally, it has also been proposed that E2F-1 may also mediate down-regulation of Mcl-1 in flavopiridol-treated cells, although down-regulation of Mcl-1 occurs in E2F-1-deficient cells as well, presumably via pTEFb inhibition (47). Full analysis of these issues will require direct measurement of E2F-1-mediated transcriptional activity in the presence of flavopiridol during S phase as well as an analysis of the effect of flavopiridol on E2F-1 transcriptional targets. In addition, expression of E2F-1 mutants lacking either transactivation or DNA-binding activity, or the ability to bind to nuclear factor κB during S phase may be instructive.

Our results in gemcitabine-treated cells contrast with those reported in gastrointestinal cancer cell lines treated with sequential gemcitabine and flavopiridol (66). In these cells, both reduced levels of E2F-1 and phosphorylated Rb were noted, contributing to reduced E2F-1 activity mediated by cdk9 inhibition could result in decreased ribonucleotide reductase even whereas there is still sufficient E2F-2 activity to induce apoptosis by other mechanisms. To assess the contribution of cdk2-dependent kinase inhibition to the apoptotic response during S phase, we expressed an E2F-1 mutant containing a deletion that abrogates cyclin A-binding. When expressed alone in NCI-H661 cells, this nonphosphorylatable mutant provides a constitutive deregulated E2F-1 activity that itself induces apoptosis. This is detected continuously during our experimental manipulation, including synchronization with hydroxyurea, followed by release into DMSO. Compared with cells engineered to express wild-type E2F-1, in mutant-expressing cells the effect of flavopiridol-mediated inhibition of cyclin A-dependent kinase activity on the apoptotic response was diminished by several-fold.

Cyclin A-cdk2 has been proposed to be responsible for the majority of E2F-1 phosphorylation in vivo, and complexes of E2F-1 with cyclin A-cdk2 have been described both in vitro and in vivo (11). However, E2F-1 is also phosphorylated by cyclin A-cdk1 at S375, which may promote the formation of Rb-E2F-1 complexes, contributing to the

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