Flavopiridol, a Cyclin-dependent Kinase Inhibitor, Prevents Spindle Inhibitor-induced Endoreduplication in Human Cancer Cells[1]

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ABSTRACT

Defects in cell cycle checkpoints can lead to chromosome abnormality, aneuploidy, and genomic instability, all of which can contribute to tumorigenesis. Recent studies and data presented in this study indicate that cells with compromised G_1 checkpoint endoreduplicate and become polyploid in response to microtubule inhibitors. Previous studies have shown that polyploid cells are unstable and lose chromosomes randomly to give aneuploidy. In this study, we show that endoreduplication and polyploidation can be prevented by inhibiting the cyclin-dependent kinases (Cdks) by flavopiridol, a synthetic flavone presently undergoing phase II clinical trials. In our initial studies, we treated MCF-7 cells with paclitaxel, which results in the arrest of cells in G_1 with 4n DNA content (pseudo G_1). This was coincident with increased p53 and p21 protein expression and decreased cyclin E/Cdk2 kinase activity. In contrast, G_1 checkpoint-compromised MDA-MB-468 (p53−/− and pRb−/−) and p21−/− HCT116 do not arrest in the pseudo G_1 state after exposure to microtubule inhibitors and enter in the S phase with 4n DNA content. More than 60% of MDA-MB-468 cells accumulate with >4n DNA content after 72 h of nocodazole treatment. The MPM-2 labeling showed that 8n cells also undergo mitosis. These cells display deregulated and persistent activation of cyclin E/Cdk2 and cyclin B1/cdc2 kinase activity. Administration of flavopiridol after mitotic block results in the arrest of cells in the pseudo G_1 state and the dramatic decrease in cells containing >4n DNA content in MDA-MB-468 cells. The cyclin E/Cdk2 and cyclin B1/cdc2 kinase activities remained low after exit from mitosis. Furthermore, pRb was hypophosphorylated after the addition of flavopiridol in p21-deficient HCT116 cells, indicating the arrest of cells at the pseudo G_1 state. Based on these studies, we propose that flavopiridol preserves the genomic stability by preventing endoreduplication and polyploidy and thus has the potential to be used as a chemopreventive agent to prevent the occurrence of neoplasia.

INTRODUCTION

Aneuploidy, a common phenomenon in the development of neoplasia, can originate due to multiple factors, including genomic instability and defects in cell cycle checkpoints (1, 2). The cell cycle checkpoints safeguard faithful cell division by ensuring that some events (such as DNA replication) are completed before others begin (such as chromosome segregation; Ref. 3). The mitotic spindle checkpoints delay the onset of chromosome segregation during anaphase if the spindle is not formed properly (4). Loss of mitotic checkpoints results in aberrant exit from mitosis, and cells enter G_1 with 4n DNA content (tetraploid or pseudo G_1 cells; Ref. 5). Checkpoints at G_1 delay the onset of DNA synthesis if previous mitosis has been defective or DNA is damaged (6). If the G_1 checkpoint is not intact, the tetraploid cells have the potential to enter the S phase with 4n DNA content; this process is known as endoreduplication (7).

Endoreduplication has been observed in response to MTIs[3] in cells lacking p53, pRb, and CDKIs p21 or p16 (7–11). In response to MTIs, p53 is induced after the cells exit mitosis following "mitotic slippage" and enter G_1 with 4n DNA content. The p53 then transactivates p21, which binds and inactivates the cyclin E/Cdk2 complex (11). The inactivation of the cyclin E/Cdk2 complex prohibits the phosphorylation of pRb (12, 13) and prevents the release of E2F-1, a transcription factor essential to activate genes required for S-phase entry (14–16) and arrest cells in G_1. In contrast, p53−/− cells do not arrest at the pseudo G_1 state, and they endoreduplicate, resulting in the accumulation of cells with >4n DNA content (polyploidy; Refs. 7–11). p16, a member of the INK family of CDKIs, inhibits cyclin D/Cdk 4 and 6 kinase and prevents the pRb phosphorylation (17). Therefore, loss of p16 also initiates MTI-induced endoreduplication. Therefore, inactivation of Cdks in a situation where the previous cell cycle phase has been defective is a crucial step in preserving the genomic integrity because polyploid cells are genetically unstable and lose chromosomes randomly and generate aneuploidy (18, 19). Because some of the routinely used chemotherapy drugs interfere with microtubule dynamics and many of human cancers have a compromised G_1 checkpoint, it is essential to explore ways to reduce the risks of development of polyploidy in response to these drugs. One of the possible ways can be to combine the MTI treatment with agents that can inhibit Cdks directly, thus preventing the polyploidy and hence pre-

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[3] The abbreviations used are: MTI, microtubule inhibitor; CDKI, cyclin-dependent kinase inhibitor; Cdk, cyclin-dependent kinase; PI, propidium iodide; DAPI, 4′,6′-diamidino-2-phenylindole.

serving the genomic stability. Additionally, neoplastic progression in Barrett's esophagus and pancreatic tumors in transgenic mice expressing SV40 TAg has been shown to arise through the development of the tetraploid→polyploid→aneuploid sequence (20–22). In that respect, MTI-induced polyploidy may provide a model to test the utility of Cdk inhibitors for the prevention of neoplasia.

In the National Cancer Institute drug screen, flavopiridol, a synthetic flavone presently undergoing clinical trial (23), has been identified as an agent that inhibits Cdks 1, 2, 4, and 6 in nanomolar concentrations (24, 25). On account of this, flavopiridol has been shown *in vitro* to inhibit tumor cell growth through the blockade of cell cycle progression at G_1 or G_2 (24, 26). We have previously reported that flavopiridol at noncytotoxic, nanomolar concentrations significantly enhances the induction of apoptosis by the chemotherapeutic agent mitomycin C and paclitaxel in gastric and breast cancer cell lines (27). Synergism between flavopiridol and paclitaxel has also been observed against A549 non-small cell lung cancer cells (28). We have recently reported that flavopiridol enhances paclitaxel-induced apoptosis in a sequential-dependent manner such that paclitaxel should precede flavopiridol to achieve this synergistic effect (29). This observation has been translated into a Phase I clinical trial of sequential paclitaxel and flavopiridol at our center, and promising clinical activity has been reported (30).

In the present study, we show that in response to MTIs, human breast and colon cancer cells with intact G_1-S checkpoints arrest in G_1 with 4n DNA content (pseudo G_1), whereas cells with defective G_1-S checkpoints endoreduplicate and become polyploid. Flavopiridol at nanomolar concentrations, when administered after MTIs, prevents the endoreduplication and development of polyploidy.

MATERIALS AND METHODS

Cell Culture and Drug Treatments for Cell Lines. The human breast cancer cell lines MCF-7 and MDA-MB-468 were purchased from American Type Culture Collection. The HCT116 p21−/− human colon cancer cell line in which both alleles of $p21^{Waf1/Cip1}$ deleted through homologous recombination were kindly provided by Dr. Bert Vogelstein (Johns Hopkins Oncology Center). The cell lines were maintained in RPMI supplemented with 10% heat-inactivated fetal bovine serum, penicillin, and streptomycin at 37°C in 5% carbon dioxide. All cultures were tested as *Mycoplasma*-free. When indicated, cells were treated with 100 nM paclitaxel (Bristol-Myers Squibb), 100 nM (for MDA-MB-468) or 33 nM (for HCT116 p21−/−) nocodazole (Sigma) diluted in DMSO, and 300 nM (for MDA-MB-468) or 150 nM (for HCT116 p21−/−) flavopiridol (graciously supplied by Dr. Edward Sausville, National Cancer Institute, Bethesda, MD). The cells were incubated for indicated time periods in drug media.

MPM-2/PI Bivariate Flow Cytometry. In a 100-mm dish, 1.4×10^6 cells were cultured for 48 h and treated with paclitaxel, nocodazole, and flavopiridol as a single agent or sequentially. The cells were harvested at specific time points by trypsinization, pooled with floating cells, and fixed overnight with ice-cold 70% ethanol. After washing with PBS containing 0.05% Tween 20 and 1% fetal bovine serum, cells were labeled with MPM-2 antibody (final concentration of 1 μg MPM-2 antibody/ml; Upstate Biotechnology) for 1 h at 4°C. Cells were washed twice with PBS and incubated with goat antimouse FITC (Boehringer Mannheim) for 1 h at room temperature in the dark. After washing twice with PBS, cells were resuspended in 5 μg/ml PI containing 50 μg/ml RNase A. Samples were analyzed on a Becton Dickinson FACScan, and data of 20,000 events for each sample were plotted with CellQuest software (Becton Dickinson). The MPM-2-positive (mitotic) cells will show increased green fluorescence and shift above the baseline of the dot plot (shown in the box in Figs. 1 and 2).

Kinase Activity Assay. After completion of drug treatment, cells were lysed in lysis buffer [50 mM HEPES-KOH (pH 7.5), 150 mM NaCl, 1 mM of each EDTA, NaF and DTT, 2.5 mM EGTA, 0.1% Tween 20, 10% glycerol, 10 mM β-glycerophosphate, 0.1 mM Na3VO4, 0.2 mM phenylmethylsulfonyl fluoride, and 10 μg/ml of each aprotinin and leupeptin] and disrupted by passing through a 21-gauge syringe 10 times, and lysates were clarified by centrifugation (10 min at $10,000 \times g$). Two hundred μg of soluble protein were incubated with 1 μg of either anticyclin E (sc-248) or anticyclin B1 antibody (sc-245; Santa Cruz Biotechnology Inc.) at 4°C for 2 h. Immune complexes were then precipitated with 40 μl of immobilized rProtein A (RepliGen) overnight at 4°C and washed three times with lysis buffer and twice with kinase assay buffer [50 mM HEPES-KOH (pH 7.5), 10 mM MgCl2 and β-glycerophosphate, 1 mM DTT, 2.5 mM EGTA, 0.1 mM Na_3VO4, 1 μM NaF]. The kinase assay was carried out by combining the washed protein beads with 20 μl of kinase buffer plus 10 μCi of [(γ^{32}p) ATP], 15 μM ATP, and 50 μg/ml Histone-H1 (Boehringer Mannheim). The reaction was allowed to proceed for 20 min at 30°C and was terminated by adding 10 μl of Laemmli sample buffer and boiling for 5 min. Products were resolved by 10% SDS-PAGE. The activity levels on autoradiographs were quantified using a densitometric scanning system.

Western Analysis. Protein lysates prepared for kinase assays were used for Western analysis. Fifty μg of soluble protein were resolved by SDS-PAGE and transferred to Immobilon-P membranes (Millipore). The equal loading of proteins was confirmed by amido black staining (Sigma). The membranes were probed with mouse monoclonal antibodies specific to pRb, p53, p21, cyclin E (Santa Cruz Biotechnology), and mouse monoclonal cyclin B1 (kindly provided by Dr. Tim Hunt, Imperial Cancer Research Fund Clare Hall Laboratories, United Kingdom). The primary antibodies were detected with sheep antimouse horse radish peroxide secondary antibody (Amersham Life Sciences) and subjected to enhanced chemiluminescence reagents (DuPont NEN Life Science Products, Boston, MA). The levels of expression were quantified using a densitometric scanning system.

RESULTS

Cells with Intact G_1 Checkpoint Arrest in Pseudo G_1 Phase in Response to MTIs. MCF-7 cells treated with 100 nM paclitaxel for 18 h arrest in mitosis as evident by the accumulation of cells with 4n DNA content and increased labeling of 4n cells with MPM-2, an antibody that recognizes the phosphoproteins that appear during mitosis (Ref. 31; Fig. 1).

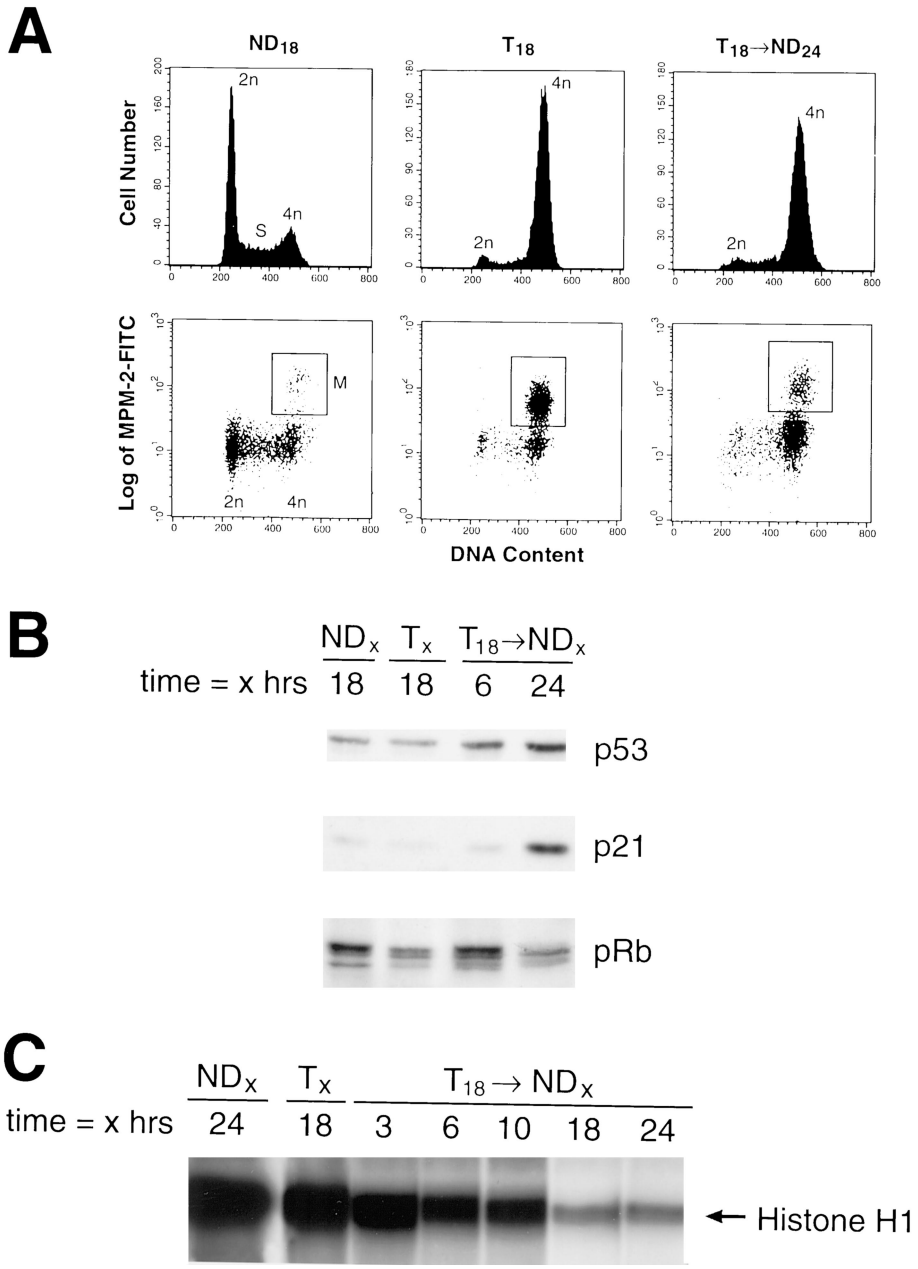

Fig. 1 MCF-7 cells treated with paclitaxel enter, transiently arrest, and exit mitosis. *A*, flow cytometric analysis of asynchronous MCF-7 cells either untreated (ND_{18}), treated with 100 nM paclitaxel for 18 h (T_{18}), and 24 h after paclitaxel removal ($T_{18}\rightarrow ND_{24}$). Top panel, cell cycle profile of MCF-7 cells. DNA content is represented on the *X* axis, and the number of cells counted is represented on the *Y* axis. *Bottom panel*, dot plots of MCF-7 cells labeled with the MPM-2 antibody. DNA content is represented on the *X* axis, and the log of fluorescence exhibited by MPM-2-FITC conjugate is plotted on the *Y* axis. The cells staining positive for MPM-2 show increased fluorescence and shift above the baseline. These cells are captured in the box (shown as *M*) and represented in the histogram. Data are representative of three independent experiments. *B*, Western analysis of p53, p21, and pRb proteins from MCF-7 cells. Asynchronous cells were either untreated (ND_{18}) or treated with 100 nM paclitaxel for 18 h (T_{18}) and 6 and 24 h after paclitaxel removal ($T_{18}\rightarrow ND_{6-24}$), and the protein was harvested. The equal loading of proteins was estimated by Amido Black staining. *C*, cyclin E/Cdk2 kinase activity in MCF-7 cells. Anticyclin E antibody was used to immunoprecipitate kinase complexes; histone H1 was used as a substrate. Data are representative of three independent experiments.

Twenty-four h after the removal of paclitaxel, cells exit mitosis as MPM-2 labeling decreases; however, the 4n peak remains constant. The exited cells are multinucleated and have a decondensed nuclear morphology as assessed by DAPI staining (29), suggesting that cells enter the interphase without undergoing cytokinesis, a state we refer to as "pseudo G_1." The expression of p53 and its direct transcriptional target p21 was induced 3- and 4-fold, respectively in pseudo G_1 cells compared to untreated controls (ND_{24}). At the similar time period, most of the pRb was in its faster migrating hypophosphorylated form (Fig. 1*B*). The p21 induction also corresponds to the decrease in cyclin E/Cdk2 kinase activity after the removal of paclitaxel from the MCF-7 cells (Fig. 1*C*).

Next, we examined whether p53 and p21 induction was associated with prevention of the next round of DNA synthesis. The percentages of cells with >4n DNA content did not increase 24 and 48 h after the removal of paclitaxel (data not shown). Similar results were observed with cells treated with nocodazole.

Cells with Compromised G_1 Checkpoint Endoreduplicate and Become Polyploid in Response to MTIs, and Flavopiridol Suppresses the Polyploidy. The MDA-MB-468 breast cancer cells are p53- and pRb-negative and posses little or no Cdk4 kinase activity. To determine the endoreduplication in these cells in response to MTIs, cells were first synchronized at the G_1-S boundary by a thymidine double block. This resulted in

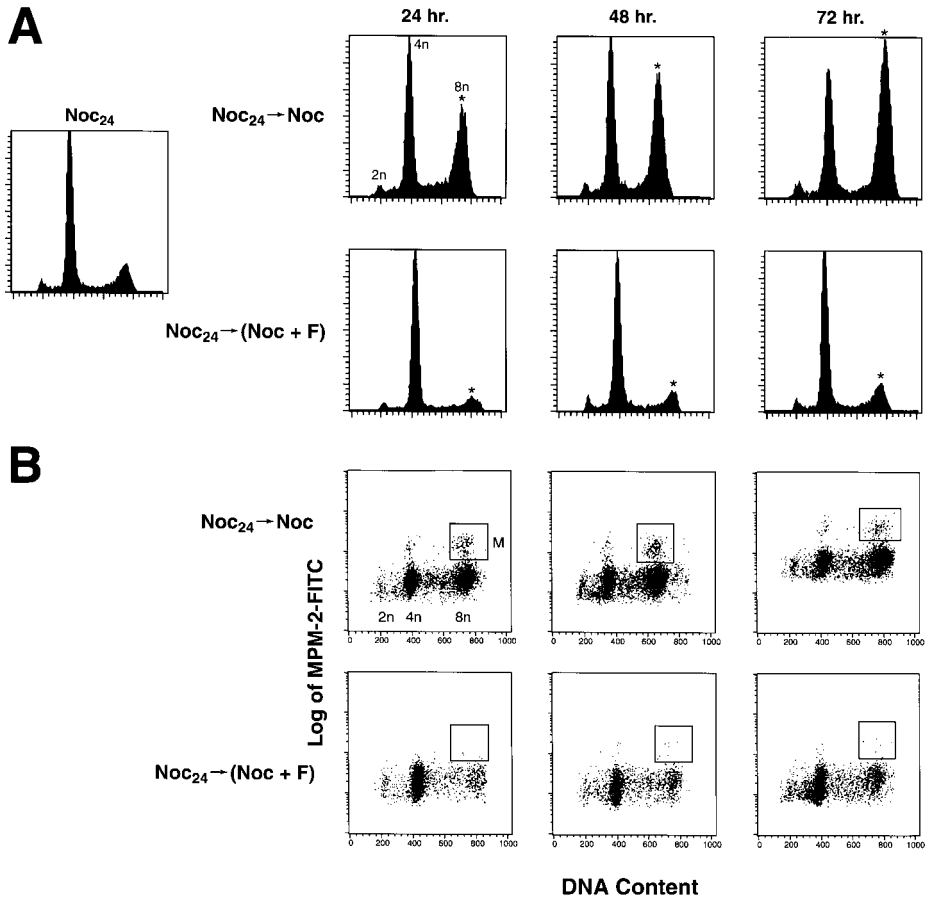

Fig. 2 MDA-MB-468 cells endoreduplicate after MTI treatment. *A*, flow cytometric analysis of synchronized (with thymidine double block) MDA-MB-468 cells treated with nocodazole (100 nM) for 24 h (Noc_{24}) or nocodazole for 24 h followed by either nocodazole ($Noc_{24}\rightarrow Noc_{24-72}$) or nocodazole plus flavopiridol (300 nM; $Noc_{24}\rightarrow[Noc + F]_{24-72}$). The cells were harvested at indicated time points, and 20,000 gated events were analyzed for each sample. DNA content is represented on the *X* axis, and the number of cells counted is on the *Y* axis. The subdiploid populations have been excluded. Data are representative of three independent experiments. *B*, the endoreduplicated MDA-MB-468 cells (8n DNA content cells) also undergo mitosis. Displayed are dot plots of MDA-MB-468 cells labeled with MPM-2 antibody. The MDA-MB-468 cells were treated as above, and fixed cells were labeled with primary MPM-2 monoclonal antibody followed by secondary goat antimouse-FITC antibody. The cells were harvested at indicated time points, and 20,000 gated events were analyzed for each sample. DNA content is represented on the *X* axis, and the log of fluorescence exhibited by MPM-2-FITC conjugate is plotted on the *Y* axis. The cells staining positive for MPM-2 show increased fluorescence and shift above the baseline. These cells are captured in the box (shown as *M*) and represented in the histogram. Data are representative of three independent experiments.

>90% cells in the G_1-S phase of the cell cycle. As shown in Fig. 2*A*, after 24 h of nocodazole treatment (Noc_{24}), the majority of cells accumulated with 4n DNA content. A small 8n population was also observed, which increased steadily after 24, 48, and 72 h of additional nocodazole treatment ($Noc_{24}\rightarrow Noc_{24-72}$). More than 65% of cells contained >4n DNA content after 96 h of total nocodazole treatment ($Noc_{24}\rightarrow Noc_{72}$). We did not observe a 16n or 32n population. Addition of 300 nM flavopiridol for 24, 48, and 72 h after 24 h of initial nocodazole treatment ($Noc_{24}\rightarrow[Noc + F]_{24-72}$) prevented the cells from synthesizing additional DNA because the number of cells containing >4n DNA content dropped by >80% compared to nocodazole treatment ($Noc_{24}\rightarrow Noc_{24-72}$; Fig. 2*A*). The MPM-2 labeling shows that 8n cells also undergo mitosis by nocodazole treatment, and the addition of flavopiridol to nocodazole-treated cells prevents the entry of 8n cells to undergo mitosis (Fig. 2*B*).

The p21-deficient HCT116, a human colon cancer cell line, also undergoes MTI-induced endoreduplication (11). Flavopiridol at concentrations of 150 nM prevented MTI-induced endoreduplication in these cells, with a dramatic decrease in the number of cells containing >4n DNA content (data not shown). Taken together, these data suggest that treatment with MTIs induce endoreduplication in human tumor cells with compromised G_1 checkpoint, and flavopiridol prevents the endoreduplication.

Inhibition of Cyclin E/Cdk2 Kinase Activity by Flavopiridol Prevents Endoreduplication. Cyclin D/Cdk4 (6) and cyclin E/Cdk2 (32, 33) carry out the transition from G_1 to S and progression in the S phase. These kinases phosphorylate pRb sequentially, rendering the pRb inactive. Previous reports indicate that inhibition of cyclin E/Cdk2 and not cyclin D/Cdk4 kinase activity prevents endoreduplication in HCT116 cells

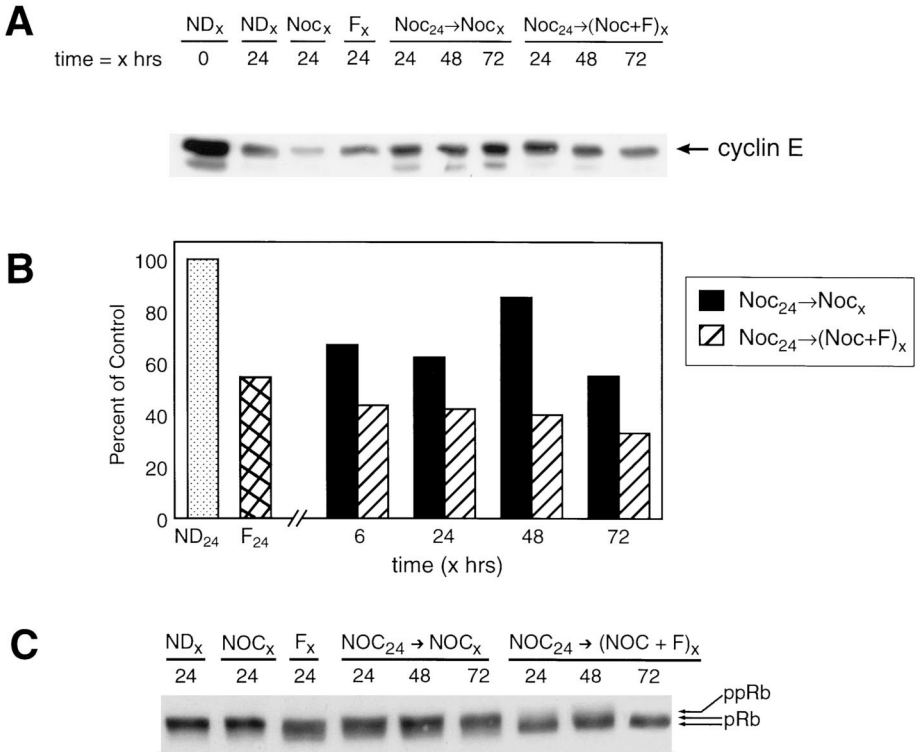

Fig. 3 Cyclin E/Cdk2 kinase activity is deregulated during MTI-induced endoreduplication; addition of flavopiridol inhibits cyclin E/Cdk2 kinase activity, hypophosphorylates pRb, and prevents endoreduplication in MDA-MB-468 and HCT116 p21−/− cells. *A*, Western analysis of cyclin E. Synchronized MDA-MB-468 cells were treated with nocodazole (100 nM) for 24 h (Noc_{24}) or nocodazole for 24 h followed by either nocodazole (Noc_{24}→Noc_x) or nocodazole plus flavopiridol (300 nM; Noc_{24} 253 $[Noc + F]_x$), and protein was harvested. The equal loading of proteins was estimated by Amido Black staining. x, the indicated time of treatment. *B*, quantitation of cyclin E/Cdk2 kinase activity in MDA-MB-468 cells. Anticyclin E antibody was used to immunoprecipitate kinase complexes; histone H1 was used as a substrate. The kinase activity of untreated cells was taken as a control. Results are representative of three independent experiments. *C*, Western analysis of pRb in p21-deficient HCT116 cells. Asynchronous cells were treated with either nocodazole (33 nM) for 24 h (Noc_{24}), flavopiridol (150 nM) for 24 h (F_{24}), or nocodazole for 24 h followed by either nocodazole (Noc_{24} 253 Noc_x) or nocodazole plus flavopiridol (Noc_{24} 253 $[Noc + F]_x$), and protein was harvested. x, the indicated time of treatment. The equal loading of proteins was estimated by Amido Black staining. *ppRb*, the hyperphosphorylated pRb; *pRb*, the hypophosphorylated pRb.

(11). Loss of p21 in these cells by homologous recombination results in persistent activation of cyclin E/Cdk2 kinase activity and endoreduplication. We examined the expression of cyclin E, an essential cyclin partner for activating Cdk2 kinase and cyclin E-associated Cdk2 kinase activity in nocodazole and nocodazole-plus-flavopiridol-treated MDA-MB-468 cells.

As shown in Fig. 3*A*, after the thymidine double block (ND_0), the expression of cyclin E was 3-fold higher compared to 24 h after release from the thymidine double block (ND_{24}). This is in consistent with the cell cycle data indicating an accumulation of cells at the G_1-S transition by thymidine double block (Refs. 34 and 35; data not shown). Twenty four h after the flavopiridol addition, there was a slight decrease in cyclin E expression compared to the untreated control (ND_{24}), indicating a loss of some G_1 cells. Similarly, treatment of thymidine blocked cells with nocodazole for 24 h (Noc_{24}) resulted in a 2-fold decrease in cyclin E expression compared to the untreated control (ND_{24}), indicating a loss of G_1 cells and an accumulation of cells in the M phase. As the cells entered into the pseudo G_1 phase after 30 h (Noc_{24}→Noc_6), the cyclin E levels increased again and remained unchanged thereafter in nocodazole-treated cells (Noc_{24}→Noc_{24-72}). Addition of flavopiridol after 24 h of initial nocodazole treatment resulted in a time-dependent decrease in cyclin E protein expression (Noc_{24}→$[Noc + F]_{24-72}$).

To determine the activity of cyclin E/Cdk2 kinase, cyclin E was immunoprecipitated from the lysates obtained from either nocodazole or nocodazole-plus-flavopiridol-treated MDA-MB-468 cells, and the kinase activity was measured by determining the ability of the immunoprecipitates to phosphorylate the histone H1 substrate (Fig. 3*B*). In cells treated with nocodazole only, cyclin E/Cdk2 kinase activity was deregulated and persistently active, suggesting that cells were cycling. Treatment with flavopiridol alone resulted in an inhibition of cyclin E/Cdk2 kinase activity by 40% compared to the untreated control. Addition of flavopiridol after 24 h of initial nocodazole treatment resulted in a time-dependent decrease in kinase activity. At 72 h after the addition of flavopiridol (Noc_{24}→$[Noc + F]_{72}$), ~30% kinase activity was remaining compared to the untreated control (ND_{24}). In view of the prevention of endoreduplication by flavopiridol on nocodazole-treated cells, we anticipated a greater degree of inhibition in cyclin E/Cdk2 kinase activity. However, flavopiridol is a competitive inhibitor of Cdks with

respect to ATP and inhibits the kinase activities of Cdks 1, 2, 4, and 6 by directly binding to the ATP-binding site of Cdks (24, 25). As previously reported, during immunoprecipitation, flavopiridol gets displaced from the ATP-binding site of Cdk2 and competes with the ATP added during the *in vitro* assays, rendering this complex an active kinase during *in vitro* kinase assays (24). Thus, the actual Cdk2 kinase activity in intact cells should be substantially less than that observed from immunoprecipitates of flavopiridol-treated cells. We confirmed this observation by adding the flavopiridol directly to the Cdk2 kinase reaction. This resulted in a 500–600% inhibition of kinase activity (data not shown). This would indicate that the dramatic loss of >4n DNA content cells and decrease in cyclin E/Cdk2 kinase activity after the addition of flavopiridol to nocodazole-treated MDA-MB-468 cells could be by direct binding of flavopiridol to Cdk2 or indirectly due to decreased availability of cyclin E.

Flavopiridol Treatment Results in Hypophosphorylation of pRb in Nocodazole-treated HCT116 p21−/− Cells. Phosphorylation status of pRb dictates the cell cycle clock (36). pRb is found in the hypophosphorylated form in the preceding hours of G_1 and needs to undergo phosphorylation to initiate the G_1-to-S-phase transition. Previous reports have indicated that MTI-induced endoreduplication in p21-deficient HCT116 cells is due to persistent activation of cyclin E/Cdk2 kinase activity. This is associated with hyperphosphorylated pRb in nocodazole-treated p21-deficient HCT116 cells (11). To demonstrate further that flavopiridol prevents endoreduplication by inhibiting Cdks, the phosphorylation status of pRb was examined in nocodazole and nocodazole-plus-flavopiridol-treated p21-deficient HCT116 cells (Fig. 3*C*). In untreated (ND_{24}) and nocodazole-treated (Noc_{24} and $Noc_{24}\rightarrow Noc_{6-72}$) HCT116 p21−/− cells, pRb remained in the slower migrating hyperphosphorylated form. Treatment of cells with flavopiridol alone results in the loss of the hyperphosphorylated band of pRb. After 24–72 h of the addition of flavopiridol to nocodazole-treated cells ($Noc_{24}\rightarrow[Noc + F]_{24-72}$), pRb was mostly present in its faster migrating hypophosphorylated form. The appearance of the hypophosphorylated form of pRb in nocodazole-plus-flavopiridol-treated cells also coincides with arrest or maintenance of cells with 4n DNA content. This data supports the fact that inhibition of Cdks by flavopiridol prevents the endoreduplication and polyploidy in G_1 checkpoint-compromised cells.

Flavopiridol Potently Inhibits Cyclin B1/cdc2 Kinase Activity on Nocodazole-treated MDA-MB-468 Cells. The activation of cyclin B1/cdc2 kinase is required for the G_2-M transition and progress in mitosis (37). As discussed earlier, the MTI-induced endoreduplicated cells underwent mitosis, whereas flavopiridol completely prohibited the entry of 8n cells to mitosis (Fig. 2*B*). This would suggest that cyclin B1/cdc2 kinase activity is persistently active in nocodazole-treated cells, and flavopiridol inhibits its activity. To evaluate this, MDA-MB-468 cells were either treated with nocodazole alone or nocodazole plus flavopiridol. In keeping with the cell cycle data, after 24 h of nocodazole treatment (Noc_{24}), cyclin B1 protein was increased 2–3 fold compared to untreated cells (ND_{24}; Fig. 4*A*). At subsequent time points for nocodazole or nocodazole-plus-flavopiridol-treated cells, the protein levels of cyclin B1 remained constant (Fig. 4*A*). Flavopiridol alone did not alter cyclin B1 expression substantially. To evaluate the cyclin B1/cdc2 kinase activity under these conditions in MDA-MB-468 cells, cyclin B1 was immunoprecipitated, and associated kinase activity was measured by determining the ability of immunoprecipitate to phosphorylate histone H1 (Fig. 4*B*). After 24 h of nocodazole treatment (Noc_{24}), cyclin B1/cdc2 kinase activity increased by 35-fold compared to control untreated cells (ND_{24}), which is consistent with the arrest of cells in mitosis (29). Thereafter, with additional 6 h of nocodazole treatment ($Noc_{24}\rightarrow Noc_6$), the kinase activity decreased to 50% of the 24-h nocodazole treatment (Noc_{24}) as cells exited mitosis and entered the pseudo G_1 state. The cyclin B1/cdc2 kinase remained active at subsequent time points ($Noc_{24}\rightarrow Noc_{24-72}$), although it decreased to 50–35% of the 24-h nocodazole treatment alone (Noc_{24}), indicating continuous recruitment of cells into mitosis. Addition of flavopiridol dramatically inhibited the kinase activity, and as early as 6 h after the addition of flavopiridol, a significant 700–800% drop in kinase activity was observed compared to nocodazole treatment alone at similar time points (*e.g.,*. $Noc_{24}\rightarrow[Noc + F]_6$ *versus* $Noc_{24}\rightarrow Noc_6$). After 72 h of adding flavopiridol ($Noc_{24}\rightarrow[Noc + F]_{72}$), only 1% of kinase activity was left compared to the 24-h nocodazole treatment alone (Noc_{24}), whereas at a similar time point with nocodazole alone ($Noc_{24}\rightarrow Noc_{72}$), 50% kinase activity was remaining (Fig. 4*B*). The decrease in cyclin B1/cdc2 kinase activity in nocodazole followed by flavopiridol-treated cells ($Noc_{24}\rightarrow[Noc + F]_{72}$) can be due to either direct binding of flavopiridol to cdc2 or indirectly due to the arrest of the majority of the cells in a pseudo G_1 state, thus limiting the number of cells entering into the M phase.

DISCUSSION

Aneuploidy can develop when the mechanisms that control coupling between mitosis and DNA synthesis are damaged. Recent studies have indicated that loss of p21 causes uncoupling between mitosis and DNA synthesis resulting in endoreduplication in response to DNA damage (38). Cross *et al.* (7) have indicated that loss of p53 leads to endoreduplication in response to MTIs in p53−/− murine embryo fibroblasts. Additional studies have shown that loss of pRb and CDKIs p21 and p16 also leads to MTI-induced endoreduplication (8–10). Stewart *et al.* (11) have recently reported an extensive study on the role of p21 in MTI-induced endoreduplication. The major finding in these studies indicates that compromise in the G_1 checkpoint results in continuous activation of cyclin E/Cdk2 kinase activity that leads to the endoreduplication. Thus, it would be reasonable to hypothesize that if CDKIs are administered after the MTI-induced mitotic arrest, the endoreduplication can be prevented in cells with a compromised G_1 checkpoint. Indeed, our results demonstrate that inhibition of Cdks by clinically achievable nanomolar concentrations of flavopiridol prevents endoreduplication in human cancer cells defective in G_1 checkpoint proteins. These results also significantly extend the previous findings that deregulated Cdk kinase activities are the felon in initiation of endoreduplication in response to MTIs.

In response to MTIs, cells enter, transiently arrest, and exit mitosis without undergoing cytokinesis. The protein expression of p53 and p21 is induced after exit from mitosis and entry in the

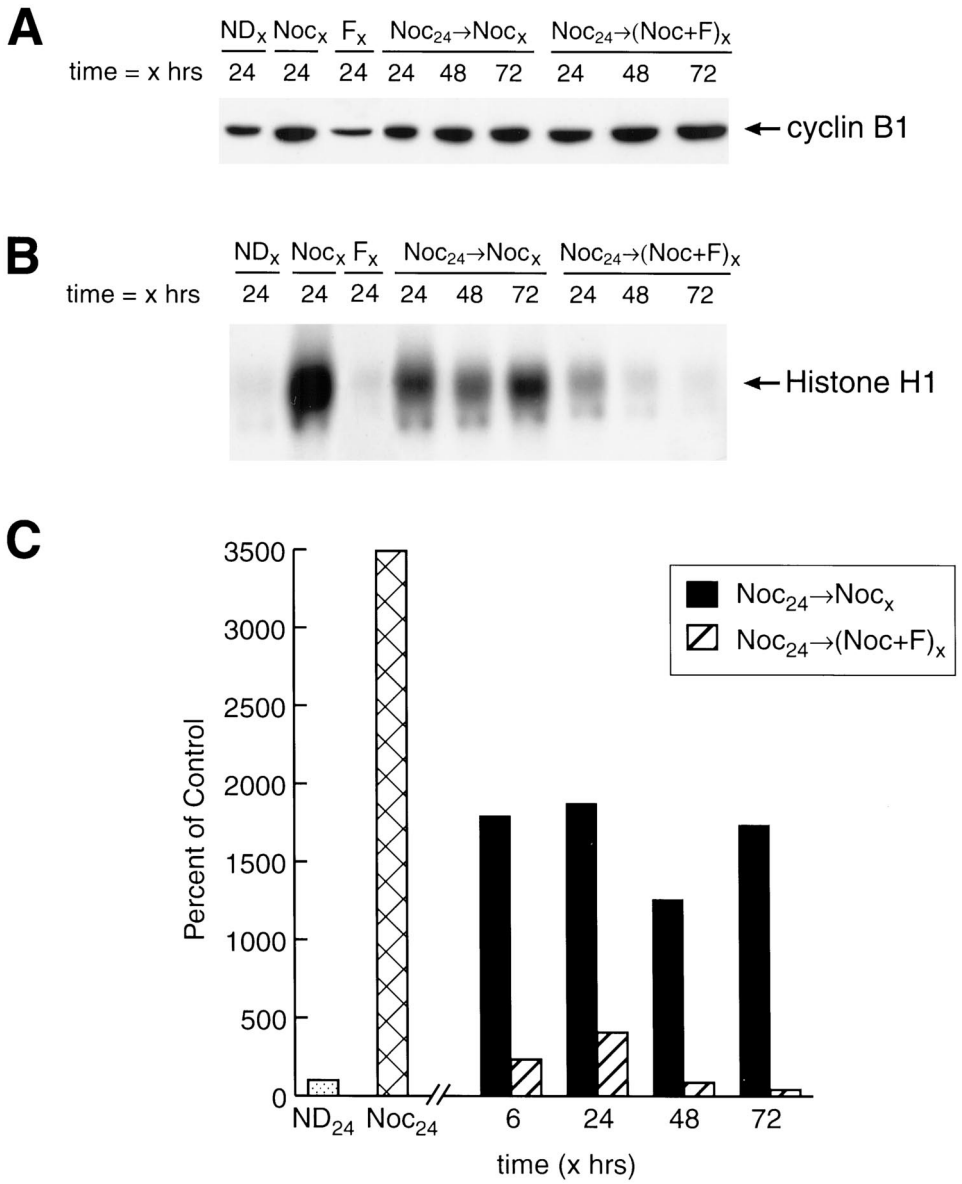

Fig. 4 Addition of flavopiridol to nocodazole-treated cells results in inhibition of cyclin B1/cdc2 kinase activity in MDA-MB-468 cells. *A*, Western analysis of cyclin B1. Asynchronous cells were treated with nocodazole (100 nM) for 24 h (Noc_{24}) or nocodazole for 24 h followed by either nocodazole (Noc_{24} 253 Noc_x) or nocodazole plus flavopiridol (300 nM) (Noc_{24}→$[Noc + F]_x$), and protein was harvested. The equal loading of proteins was estimated by Amido Black staining. x, the indicated time of treatment. *B*, cyclin B1-associated kinase activity. Anticyclin B1 antibody was used to immunoprecipitate kinase complexes, and histone H1 was used as a substrate. *C*, quantification of the autoradiogram signals represented in the bar graph.

pseudo G_1 phase. The cyclin E/Cdk2 kinase activity is inhibited presumably due to binding of p21 to the cyclin E/Cdk2 complex, which prevents the phosphorylation of pRb and arrests the cells in the pseudo G_1 phase. In contrast, cells with a defective G_1 checkpoint (MDA-MB-468 and p21-deficient HCT116) endoreduplicate in response to MTIs. This is associated with continuous activation of cyclin E/Cdk2 kinase. Furthermore, the 8n population also undergoes mitosis as determined by positive labeling of 8n cells with MPM-2. The cyclin B1/cdc2 kinase activity was also persistently activated after treatment with MTIs. Addition of flavopiridol to MTI-treated cells dramatically decreases the number of cells containing >4n cells. Although *in vitro* cyclin E/Cdk2 kinase activity shows a modest decrease in its activity after the flavopiridol treatment, we believe that in intact cells, the decrease in cyclin E/Cdk2 kinase activity is higher because after immunoprecipitation, flavopiridol dissociates from the ATP binding site of Cdk2 and gets washed out during *in vitro* kinase assays. This has been indicated by adding flavopiridol to the lysates of either untreated or nocodazole-treated MDA-MB-468 and HCT116 p21−/− cells during *in vitro* kinase assays. Furthermore, the pRb was hypophosphorylated in p21-deficient HCT116 cells after flavopiridol treatment, indicating the stronger inhibition of Cyclin E/Cdk2 kinase activity in intact cells than observed by *in vitro* kinase assays. The other possibility is that flavopiridol selectively induces apoptosis in 8n cells. This may be only partially true because cell death was 30–40% higher after flavopiridol addition to nocodazole-treated cells compared to nocodazole treatment alone, whereas the decrease in polyploidy was >80% in similar treatment conditions. Even if flavopiridol selectively kills the polyploid cells, it would not negate the importance of a chemical means by which we can selectively remove highly polyploid cells. Recent

studies have also shown that flavopiridol transcriptionally down-regulates cyclin D1 and arrests cells in G_1 with intact pRb (39). This would suggest that flavopiridol has the potential to arrest the cells in G_1 and prevent polyploidy, in addition to down-regulating cyclin E/Cdk2 kinase activity. The higher *in vitro* inhibition of cyclin B1/Cdc2 can be due to other cellular events, including alterations in cdc25C, Chk-1, or Wee1 besides direct inhibition. It is also possible that the decrease in cyclin B1/cdc2 kinase activity is due to a decrease in the number of cycling cells that enter into 8n because they are already arrested in 4n.

The formation of tetraploid cells has been indicated as the first step in the development of aneuploidy (21). These cells develop as a result of missing cell cycle checkpoints that usually control the coupling between completion of mitosis and DNA synthesis. The formation of tetraploid and polyploid cells in response to MTIs can also occur in physiological conditions *in vivo*. For example, Levine *et al.* (21) have shown that loss of p53 in transgenic mice expressing SV40 TAg under the control of the elastase I gene causes mice to develop pancreatic tumors through the diploid→tetraploid→polyploid→aneuploid sequence. Tetraploid cells have also been identified in the development of Barrette's esophagus as the preceding step to the aneuploidy (20). Our results demonstrate that experimentally (MTIs) induced polyploidy can be effectively inhibited by nanomolar concentrations of flavopiridol. These results provide the foundation for the use of flavopiridol as a chemopreventive agent. Because the progression to malignancy from normal cells requires a stepwise progression from polyploidy to aneuploidy, any intervention at this step may provide the basis to inhibit malignant transformation. There are presently high risk groups, including women with Brca1 mutations, who may develop breast cancer. Recent *in vitro* data indicate that Brca1 mutations can result in abnormal mitotic exit, and cells can endoreduplicate if the G_1 checkpoint is not intact due to absence of p53 (40, 41). Oral preparations of flavopiridol are now under development. Therefore, flavopiridol, by inducing G_1 arrest in cells with a compromised G_1 checkpoint after aberrant mitotic exit, may provide a convenient means to inhibit progression to malignancy in specific high risk populations.

REFERENCES

1. Hartwell, L. Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cell. Cell, *71:* 543–546, 1992.

2. Hartwell, L. H, and Kastan, M. B. Cell cycle control and cancer. Science (Washington DC), *266:* 1821–1828, 1994.

3. Hartwell, L. H, and Weinert, T. A. Checkpoints: controls that ensure the order of cell cycle events. Science (Washington DC), *246:* 629–634, 1989.

4. Gorbsky, G. J. Cell cycle checkpoints: arresting progress in mitosis. Bioessays, *19:* 193–197, 1997.

5. Hoyt, M. A., Totis, L, and Roberts, B. T. *S. cerevisiae* genes required for cell cycle arrest in response to loss of microtubule function. Cell, *66:* 507–517, 1991.

6. Murray, A. W. Creative blocks: cell cycle checkpoints and feedback controls. Nature (Lond.), *359:* 569–604, 1992.

7. Cross, S. M., Sanchez, C. A, Morgan, C. A., Schimke, M. K., Ramel, S., Idzerda, R. L., Raskind, W. H., and Reid, B. J. A p53-dependent mouse spindle checkpoint. Science (Washington DC), *267:* 1353–1356, 1995.

8. Khan, S., and Wahl, G. p53 and pRb prevent rereplication in response to microtubule inhibitors by mediating a reversible G_1 arrest. Cancer Res., *58:* 396–401, 1998.

9. Lanni, J. S., and Jacks, T. Characterization of the p53-dependent postmitotic checkpoint following spindle disruption. Mol. Cell. Biol., *18:* 1055–1064, 1998.

10. Minn, A. I., Boise, L. H., and Thompson, C. B. Expression of Bcl-xL and loss of p53 can cooperate a cell cycle checkpoint induced by mitotic spindle damage. Genes Dev., *10:* 2621–2631, 1996.

11. Stewart, Z. A., Leach, S. D., and Pietenpol, J. A. $p21^{Waf1/Cip1}$ inhibition of cyclin E/Cdk2 activity prevents endoreduplication after mitotic spindle disruption. Mol. Cell. Biol., *19:* 204–215, 1999.

12. Gu, Y., Turck, C. W., and Morgan, D. O. Inhibition of CDK2 activity *in vivo* by an associated 20K regulatory subunit. Nature (Lond.), *366:* 707–710, 1993.

13. Harper, J. W., Adami, R., Wei, N., Keyomarsi, K., and Elledge, S. J. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G_1 cyclin-dependent kinases. Cell, *75:* 805–816, 1993.

14. Chellappan, S. P., Hiebert, S., Mudryj, M., Horowitz, J. M., and Nevins, J. R. The E2F transcription factor is a cellular target for the RB protein. Cell, *65:* 1053–1061, 1991.

15. Heibert, S. W., Chellappan, S., Horowitz, J. M., and Nevins, J. R. The interaction of RB with E2F coincides with inhibition of the transcriptional activity of E2F. Genes Dev., *6:* 177–185, 1992.

16. Helin, K., Harlow, E., and Fattaey, A. Inhibition of E2F-1 transactivation by direct binding of the retinoblastoma gene. Mol. Cell Biol., *13:* 6501–6508, 1993.

17. Serrano, M., Hannon, G. J., and Beach, D. A new regulatory motif in cell cycle control causing specific inhibition of cyclin D/Cdk4. Nature (Lond.), *366:* 704–707, 1993.

18. Bischoff, F. Z., Yim, S. O., Pathak, S., Grant, S., Siciliano, M. J., Giovanella, B. C., Strong, L. C., and Tainsky, M. A. Spontaneous abnormalities in normal fibroblasts from patients with Li-Fraumeni cancer syndrome: aneuploidy and immortalization. Cancer Res., *50:* 7979–7984, 1990.

19. Shackney, S. E., Smith, C. A., Miller, B. W., Burholt, D. R., Murtha, K., Giles, H. R., Ketterer, D. M., and Pollice, A. A. Model for the genetic evolution of human solid tumors. Cancer Res., *49:* 3344–3354, 1989.

20. Galipeau, P. C., Cowan, D. S., Sanchez, C. A., Barrett, M. T., Emond, M. J., Levine, D. J., Rabinovitch, P. S., and Reid, B. J. 17p (p53) allelic losses, 4N (G2/tetraploid) populations, and progression to aneuploidy in Barrett's esophagus. Proc. Natl. Acad. Sci., *93:* 7081–7084, 1996.

21. Levine, D. J., Sanchez, C. A., Rabinovitch, P. S., and Reid, B. J. Formation of tetraploid intermediate is associated with the development of cells with more than four centrioles in the elastase-simian virus 40 tumor antigen transgenic mouse model of pancreatic cancer. Proc. Natl. Acad. Sci. USA, *88:* 6427–6431, 1991.

22. Ornitz, D. M., Hammer, R. E., Messing, A., Palmiter, R. D., and Brinster, R. L. Pancreatic neoplasia induced by SV40 T antigen expression in acinar cells of transgenic mice. Science (Washington DC), *238:* 188–193, 1987.

23. Senderowicz, A. M., Headlee, D., Stinson, S. F., Lush, R. M., Kalil, N., Villalba, L., Hill, K., Steinberg, S. M., Figg, W. D., Tompkins, A., Arbuck, S. G., and Sausville, E. A. Phase I trial of continuous infusion flavopiridol, a novel cyclin-dependent kinase inhibitor, in patients with refractory neoplasms. J. Clin. Oncol., *16:* 2986–2999, 1998.

24. Carlson, B. A., Dubay, M. M., Sausville, E. A., Brizuela, L., and Worland, P. J. Flavopiridol induces G_1 arrest with inhibition of cyclin-dependent kinase CDK2 and CDK4 in human breast carcinoma cells. Cancer Res., *56:* 2973–2978, 1996.

25. Losiewicz, M. D., Carlson, B. A., Kaur, G., Sauville, E. A., and Worland, P. J. Potent inhibition of cdc2 kinase activity by the flavonoid L86–8275. Biochem. Biophys. Res. Commun., *201:* 589–595, 1994.

26. Kaur, G., Stetler-Stevenson, M., Sebers, S., Worland, P., Sedlacek, H., Myers, C., Czech, J., Naik, R., and Sausville, E. A. Growth inhibi-

tion with reversible cell cycle arrest of carcinoma cells by flavone L86–8275. J. Natl. Cancer Inst., *84:* 1736–1740, 1992.

27. Schwartz, G. K., Farsi, K., Maslak, P., Kelsen, D. P., and Spriggs, D. Potentiation of apoptosis by flavopiridol in mitomycin-C-treated gastric and breast cancer cells. Clin. Cancer Res., *3:* 1467–1472, 1997.

28. Bible, K. C., and Kaufmann, S. H. Cytotoxic synergy between Flavopiridol (NSC 649890, L86–8275) and various antineoplastic agents: the importance of sequence of administration. Cancer Res., *57:* 3375–3380, 1997.

29. Motwani, M., Delhorey, T. M., and Schwartz, G. K. Sequential dependent enhancement of caspase activation and apoptosis by flavopiridol on paclitaxel treated human gastric and breast cancer cells. Clin. Cancer Res., *5:* 1876–1883, 1999.

30. Schwartz, G. K., Kaubisch, A., Saltz, L., Ilson, D., O'Reily, E., Barazzuol, J., Endres, S., Saltz, M., Tong, W., Spriggs, D., and Kelsen, D. P. Phase I trial of sequential paclitaxel and the cyclin dependent kinase inhibitor flavopiridol. Proc. Am. Soc. Clin. Oncol., *18:* 614, 1999.

31. Vandre, D. D., and Borisy, G. G. Anaphase onset and dephosphorylation of mitotic phosphoproteins occur concomitantly. J. Cell Sci., *94:* 245–258, 1989.

32. Koff, A., Giordano, A., Desai, D., Yamashita, K., Harper, J. W., Elledge, S., Nishimoto, T., Morgan, D. O., Franza, B. R., and Roberts, J. M. Formation and activation of a cyclin E-cdk2 complex during the G_1 phase of the human cell cycle. Science (Washington DC), *257:* 1689–1694, 1992.

33. Sherr, C. J. G_1 phase progression: cycling on cue. Cell, *79:* 551–555, 1994.

34. Dulic, V., Lees, E., and Reed, S. I. Association of human cyclin E with a periodic G_1-S phase protein kinase. Science (Washington DC), *257:* 1958–1961, 1992.

35. Ohtsubo, M., Theodoras, A. M., Schumacher, J., Roberts, J. M., and Pagano, M. Human cyclin E, a nuclear protein essential for the G_1-to-S phase transition. Mol. Cell. Biol., *15:* 2612–2624, 1995.

36. Weinberg, R. A. The retinoblastoma protein and cell cycle control. Cell, *81:* 323–330, 1995.

37. Nurse, P. Universal control mechanism regulating onset of M-phase. Nature (Lond.), *344:* 503–508, 1990.

38. Waldmann, T., Lengauer, C., Kinzler, K. W., and Vogelstein, B. Uncoupling of S phase and mitosis induced by anticancer agents in cells lacking p21. Nature (Lond.), *381:* 713–716, 1996.

39. Carlson, B., Lahusen, T., Singh, S., Loaiza-Perez, A., M., Worland, P. J., Pestell, P. J., Albanese, C., Sausville, E. A., and Senderowicz, A. M. Down-regulation of cyclin D1 by transcriptional repression in Mcf-7 human breast carcinoma cells induced by flavopiridol. Cancer Res., *15:* 4634–4641, 1999.

40. Larson, J. S., Tonkinson, J. L., and Mei, M. T. A BRCA1 mutant alters G_2-M cell cycle control in human mammary epithelial cells. Cancer Res., *57:* 3351–3355, 1997.

41. Xu, X., Weaver, Z., Linke, S. P., Li, C., Gotay, J., Wang, X-W., Harris, C. C., Reid, T., and Deng, C-X. Centrosome amplification and a defective G_2-M cell cycle checkpoint induce genetic instability in BRCA1 exon 11 isoform-deficient cells. Mol. Cell, *3:* 389–395, 1999.

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