Flavopiridol Induces G₁ Arrest with Inhibition of Cyclin-dependent Kinase (CDK) 2 and CDK4 in Human Breast Carcinoma Cells

Bradley A. Carlson, Marja M. Dubay, Edward A. Sausville, Leonardo Brizuela, and Peter J. Worland

Laboratory of Biological Chemistry, Division of Basic Science, National Cancer Institute, NIH, Bethesda, Maryland 20892 [B. A. C., E. A. S., P. J. W.]. and Mitotix Incorporated, Cambridge, Massachusetts 02139 [M. D. L. B.]

ABSTRACT

Flavopiridol (L86–8275), a N-methylpiperidinyl, chlorophenyl flavone, can inhibit cell cycle progression in either G₁ or G₂ and is a potent cyclin-dependent kinase (CDK) 1 inhibitor. In this study, we used MCF-7 breast carcinoma cells that are wild type for p53 and pRb positive and contain CDK4-cyclin D1 and MDA-MB-468 breast carcinoma cells that are mutant p53, pRb negative, and lack CDK4-cyclin D1 to investigate the G₁ arrest produced by Flavopiridol. Recombinant CDK4-cyclin D1 was inhibited potently by Flavopiridol (K_{i} 65 nm), competitive with respect to ATP. Surprisingly, CDK4 immunoprecipitates derived from Flavopiridol-treated MCF-7 cells (3 h, 300 nm Flavopiridol) had an approximately 3-fold increased kinase activity compared with untreated cells. Cyclin D and CDK4 levels were not different at 3 h, but cyclin D levels and CDK4 kinase activity decreased thereafter. The phosphorylation state of pRb was shifted from hypercoincident to hypooincident with the development of G₁ arrest. Asynchronous MDA-MB-468 cells were inhibited in cell cycle progression at both G₁ and G₂ by Flavopiridol. Flavopiridol inhibited the in vitro kinase activity of CDK2 using an immune complex kinase assay (IC_{50} 100 nm at 400 μM ATP). Immunoprecipitated CDK2 kinase activity from either MCF-7 or MDA-MB-468 cells exposed to Flavopiridol (300 nm) for increasing time showed an initial increased activity (approximately 1.5-fold at 3 h) compared with untreated cells, followed by a loss of kinase activity to immeasurable levels by 24 h. This increased immunoprecipitated kinase activity was dependent on the Flavopiridol concentration added to intact cells and was associated with a reduction of CDK2 tyrOsine phosphorylation. Cyclin E and A levels were not altered to the same extent as cyclin D, and neither CDK4 nor CDK2 levels were changed in response to Flavopiridol. Inhibition of the CDK4 and/or CDK2 kinase activity by Flavopiridol can therefore account for the G₁ arrest observed after exposure to Flavopiridol.

INTRODUCTION

Flavopiridol (L86–8275) is a flavone derivative with potent anti-proliferative activity in vitro and antitumor activity in vivo (1–3). This compound has previously been shown to produce a block in cell cycle progression at either G₁ or G₂ (4). The induction of G₂ arrest by Flavopiridol appears to be a consequence of both direct inhibition of the cdc2-cyclin B kinase activity and alteration of normal regulatory phosphorylation of the catalytic subunit (2, 4). The mechanism underlying the development of the G₁ block has not been defined.

A greater understanding of molecular events controlling the transition from one phase of the cell cycle to the next has developed following the discovery of the CDKs (5–8). A complex regulatory system produces periodic CDK activity that results in the correct order and timing of cellular replicative processes permitting transition from one phase of the cell cycle to the next. As cells enter G₁, kinase activity of CDK4 and CDK6 appears necessary for transition through early G₁ checkpoints (5, 9), and the activity of the CDK2-cyclin E complex is necessary for transition from G₁ into S-phase (10, 11). Regulation of CDK activity is achieved by both transcriptional and posttranslational processes. Phosphorylation at specific sites of the catalytic subunit of the CDKs can modulate kinase activity in either a positive or negative manner, dependent on the phosphorylation site (12–15). Negative regulation of CDK1 and CDK2 occurs by phosphorylation of the threonine-14 and tyrosine-15 residues (16–18). The phosphorylation and dephosphorylation of these residues in CDK1 is coupled with the completion of DNA synthesis and repair processes (19, 20). There also appears to be an analogous inhibitory role for the tyrosine phosphorylation of CDK2 (13) and CDK4 (21) kinase.

Accumulation of the cyclin regulatory subunits that pair with their respective CDKs results from the balance of transcription and translation with proteolytic degradation (22, 23). More recently, a number of proteins with inhibitory activity (p15, p16, p21^{cip}), and p27) directed against the CDK complexes have been described (24–32). The appearance of these inhibitory molecules is in some instances regulated by functional tumor suppressor gene products such as p53 (28, 33, 34) and pRb (35–37) and can also be modulated in response to stimuli such as transforming growth factor β (38–39) or elevated cAMP levels (40). Particular association of the endogenous inhibitors p15 and p16 with CDK4 has been established, whereas p21 and p27 are more general inhibitors of CDKs (27, 41, 42). The discovery of these endogenous inhibitors indicates another critical level of control over the activity of the CDK family that appears to be lost in a certain percentage of tumor cells in which these natural inhibitors of CDK activity may be absent or mutated (43, 44).

The development of potent inhibitors of CDK activity would represent a relatively novel approach for the inhibition of tumor cell growth. This would provide a pharmacological means of overcoming the loss of tumor suppressor genes or the presence of aberrant endogenous cell cycle regulation that contributes to the tumorigenic process. The hypothesis that Flavopiridol produces G₁ arrest consequent to the inhibition of the G₁ CDKs, thus supplanting the role of the endogenous inhibitors, is examined in this report.

MATERIALS AND METHODS

Tissue Culture. MDA-MB-468 breast carcinoma cells were generously provided by Dr. Ruth Lepo (Georgetown University); MCF-7 cells were obtained from the American Type Culture Collection; and both were maintained in Iscove’s modified Dulbecco’s medium supplemented with 5% FCS. When required, cells were synchronized with 0.4 μg/ml nocodazole for 12 h. Cells were trypsinized, washed in PBS, and fixed with 80% ethanol prior to determination of cell cycle distribution by fluorescence-activated cell sorting analysis following propidium iodide staining by Fast Systems, Inc. (Rockville, MD).

Antiserum Development. Peptides with an additional NH²-terminal cysteine were synthesized corresponding to the COOH terminus of CDK2 (CHPFFQDVTKPVPHRL-COOH) or the COOH terminus of CDK4 (COH-SYLYHKDEGNPE-COOH) using the F-moc strategy, cleaved from the support resin as described previously (45) and purified using a linear gradient of increasing acetonitrile:water containing 0.01% trifluoroacetic acid. An additional CDK2 peptide (HPFFQDVTKPVPHRL-COOH), which lacks the NH₂-terminal cysteine, was synthesized for comparison in competition studies (peptide 2; Fig. 6). The cysteine-containing peptides were conjugated to a...
carrier protein, maleimide-activated keyhole limpet hemocyanin (Pierce Chemical Co., Rockford, IL) before immunizing New Zealand White rabbits. The resulting CDK2 antiserum was affinity purified over protein G columns, and both CDK2 and CDK4 antisera were demonstrated to be specific for both immunoprecipitation and Western blotting by comparison with preimmune serum and competition with the immunizing peptide.

Recombinant CDK4 Kinase Reactions. CDK4-cyclin D1 kinase activity was determined in microtiter plates as follows. Forty μg Gst-Rb (46) were mixed with different amounts of Flavopiridol and unlabeled ATP. Reactions were then started by the addition of an ammonium sulfate cut of the S100 fraction obtained from insect cells expressing recombinant human CDK4 and cyclin D1 (47). The final reaction conditions were 10 mM MgCl₂, 50 mM Tris-HCl (pH 7.5), and 1 mM DTT. The final concentration of ATP was adjusted accordingly. Radiolabeled ATP (NEN002Z) was used as a phosphoryl donor. The reaction was carried out for 2.5 min at 30°C after addition of enzyme and then terminated with the addition of EDTA. The Gst-Rb was then captured with glutathione-Sepharose (Pharmacia, Piscataway, NJ), and the incorporated radioactivity was determined by liquid scintillation counting.

Immune Complex Kinase Determination. Cells were lysed with buffer containing 10 mM phosphate, 100 mM NaCl, 1 mM Na₂VO₄, 0.5% sodium deoxycholate, 1.0% Triton X-100, 20 μg/ml aprotinin and leupeptin, and 2 mM phenylmethylsulfonyl fluoride, and the lysates were clarified by centrifugation (10 min at 10,000 × g) prior to immunoprecipitation of CDK2 from 400 μg soluble protein. The immune complexes were pelleted with protein A-Sepharose and washed three times with kinase assay buffer [20 mM Tris (pH 7.5) containing 0.1 mM EGTA, 1 mM DTT, and 10 μg/ml MgCl₂]. Kinase assays were done at 30°C for a 20-min incubation period immediately after immunoprecipitation using a synthetic peptide (AAAKTPKKAKK-CONH₂) containing the proline-directed phosphorylation site (48) and a final ATP concentration of 400 μM with a specific activity of 150-200 cpm/pmol.

CDK4 immunoprecipitates were derived from 400 μg protein from cells lysed with buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 0.1% Tween 20, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin, 20 units/ml aprotinin, 10 mM β-glycerophosphate, 1 mM NaF, and 0.1 mM Na₂VO₄. Lysates were sonicated on ice and clarified by centrifugation (10 min at 10,000 × g). Following immunoprecipitation, the protein A beads were washed three times with kinase assay buffer [50 mM HEPES (pH 7.5) containing 1 mM DTT, 2.5 mM EGTA, 10 mM β-glycerophosphate, 1 mM NaF, and 10 mM MnCl₂] unless otherwise specified, and the kinase reactions were carried out in a final volume of 40 μl containing 0.4 μg Gst-Rb and 20 μM ATP (specific activity, 35,000 cpm/pmol). Reactions were incubated for 30 min at 30°C and terminated by the addition of 10 μl 5X Laemmli’s loading buffer. Using a plasmid construct (kindly provided by Dr. W. Kaelin, Dana-Farber Institute), the Gst-Rb fusion protein used in these assays was expressed and purified as described previously (49).

Western Blotting. Phosphotyrosine Western blotting was done as described previously (2). The retinoblastoma protein was immunoprecipitated and visualized by Western blotting using specific monoclonal antibodies (Oncogene Science, Uniondale, NY) and the enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL). Polyclonal antibodies against cyclin E (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and cyclin A (PharMingen, San Diego, CA) were used for Western blotting.
RESULTS

Previous studies (1) have indicated that Flavopiridol can block cell cycle progression in either G1 or G2. Because CDK4-cyclin D is known to promote progression through G1, we first decided to determine the effect of Flavopiridol on the kinase activity of CDK4-cyclin D1. Using partially purified, recombinant human CDK4-cyclin D1, Flavopiridol produced a potent inhibition that appeared competitive with respect to ATP from Lineweaver-Burk plots (Fig. 1A). Nonlinear regression analysis of the kinetic data obtained over a concentration range of 10–600 μM ATP and an inhibitor concentration of 0–5 μM estimated the apparent KiATP to be 64.8 nM when a model of competitive inhibition with respect to ATP was used. Comparison with other models of inhibition (noncompetitive, uncompetitive, or mixed competitive) indicated that competitive inhibition was the most appropriate. The fitted curves to the derived kinetic parameters from nonlinear regression analysis of the rate data are shown in Fig. 1B. The CDK4-cyclin D1 kinase activity could be blocked by the addition of recombinant inhibitory protein p16 to the reaction (data not shown).

We next measured CDK4 kinase activity from breast carcinoma cells using an immune complex kinase assay. This assay used a specific polyclonal antiserum raised against a peptide from the COOH terminal of CDK4 conjugated to keyhole limpet hemocyanin (see "Materials and Methods"). The pRb-negative MDA-MB-468 cells possess no or very little CDK4 activity relative to the pRb-positive MCF-7 cells (see Fig. 2A). This is consistent with the expression of CDK4, cyclin D and p16 that has been determined by others in these cell types (35, 50). Specificity of the CDK4 immunoprecipitation and kinase activity is shown by loss of immunoprecipitated kinase activity following preincubation of the antisera with the immunizing peptide and by inhibition of CDK4 activity through addition of the specific CDK4 inhibitor p16 (Fig. 2A). The requirement for the divalent cations Mg2+ or Mn2+ is shown, and an improved signal:noise ratio was obtained when using Mn2+, which was used in all subsequent CDK4 kinase assays.

CDK4 kinase activity immunoprecipitated from exponentially growing MCF-7 cells following exposure to 300 nM Flavopiridol for various times is shown in Fig. 2B with quantitation of pRb phosphorylation shown in Fig. 2C. Within the initial period of Flavopiridol exposure, there is increased kinase activity found in the immunoprecipitates (approximately 3-fold at 3 h), but virtually complete loss of activity at later times (12–24 h), which is also when cells are arrested in G1 (data not shown).

The phosphorylation mediated by both CDK4 and CDK2 of the Rb protein is required for the cells to progress from G1 into S-phase in those cells possessing a functional pRb. As an indication that Flavopiridol is inhibiting the CDKs within the cell, we have determined the phosphorylation status of the Rb gene product following exposure to Flavopiridol in exponentially growing MCF-7 cells. Over a 24-h exposure to Flavopiridol, we found that the Rb protein changed from a mostly hyperphosphorylated form at 0 h, to equivalent hyperphosphorylated and hypophosphorylated pRb at 6 h, to a completely dephosphorylated form at 24 h (Fig. 3). Attempts to synchronize the MCF-7 cells revealed that a significant fraction of cells would stop in G0 after completion of mitosis, complicating analysis. However, in asynchronous cells, we were able to show reduction of the S-phase population from 24% in the untreated control cells to 14% in those cells exposed to Flavopiridol (300 nm) for 12 h. The G1 population in the same period was found to increase from 57% in the untreated controls to 67% in those treated with Flavopiridol. Tumor cells that are mutant for Rb generally lack expression of the required D type cyclins for CDK4 kinase activity (36). The pRb-negative MDA-MB-468 breast carcinoma cells, which lack detectable CDK4 kinase.
activity (Fig. 2A), provide a model to determine the ability of Flavopiridol to inhibit cell cycle progression through $G_1$ in the absence of CDK4 kinase activity. When asynchronous, exponentially growing MDA-MB-468 cells are exposed to Flavopiridol; cells within $G_1$ appear to be blocked from entry to S-phase; and those cells within S-phase progress into and remain within $G_2$ (Fig. 4, compare A and B; see Ref. 1). Following release from nocodazole synchronization, cells exposed to Flavopiridol pass into $G_1$ and are inhibited from progression into S-phase (Fig. 4, compare C and D).

The kinase activity of CDK2 may have a greater role in mediating $G_1$ progression for those cells that lack CDK4 kinase activity (11, 51). Using a specific polyclonal antiserum raised against the COOH-terminal region of CDK2, we could demonstrate that Flavopiridol inhibited potently the kinase activity of CDK2 in an immune complex assay. Under the conditions of the assay with an ATP concentration of 400 $\mu$M, the IC$_{50}$ for inhibition was 100 nM (Fig. 5A). Comparison of CDK2 activity from MDA-MB-468 cells released from the nocodazole block into the medium with either control vehicle or Flavopiridol (300 nM) is shown in Fig. 5B. The kinase activity from the control cells shows the expected increase in activity that is coincident with progression through S-phase. The CDK2 kinase activity from asynchronous, exponentially growing MCF-7 breast carcinoma cells following Flavopiridol treatment is shown in Fig. 5C. The kinase activity of CDK2 (Fig. 5) from both cell types (either synchronized MDA-MB-468 or asynchronous MCF-7), following Flavopiridol exposure was elevated within the initial period of treatment (maximal at 3 h). Subsequent to this point, reduced kinase activity was found from the Flavopiridol treated cells coincident with the development of a $G_1$ block in the MDA-MB-468 cells (compare Figs. 4D and 5B). Immunoprecipitation and Western blotting found that CDK2 and CDK4 levels were not changed in response to treatment of the cells with Flavopiridol (data not shown). In contrast, cyclin levels did change with increasing time of Flavopiridol exposure. Cyclins D, E, and A from MCF-7 cells measured by Western blotting are shown in Fig. 6. Cyclin D decreased to relatively negligible levels by 6 h of Flavopiridol exposure, whereas cyclin E was gradually decreased over the 6–24-h period, as was Cyclin A. The phenomenon of increased kinase activity found in immunoprecipitated CDK2 (Fig. 5) and CDK4 (Fig. 2) derived from Flavopiridol-exposed cells was similar to an earlier observation for CDK1 (2). A concentration-dependent relationship for this effect of Flavopiridol after either a 3- or 8-h exposure on CDK2 kinase activity derived from Flavopiridol-treated MDA-MB-468 cells is shown in Fig. 7A. This effect could not be achieved by addition of the same Flavopiridol concentration to the immunoprecipitation buffers used for immunoprecipitates derived from untreated cells (Fig. 7B). Addition of Flavopiridol only to the immune complex kinase reaction produced the expected inhibition. The phosphotyrosine content of CDK2 was not reduced by adding Flavopiridol to immunoprecipitates derived from untreated cells, but in contrast, phosphotyrosine content was reduced in those immunoprecipitates derived from Flavopiridol-treated cells (Fig. 7C). We were unable to demonstrate phosphorylation on the tyrosine of CDK4 in untreated cells and, therefore, were unable to determine whether Flavopiridol affected this potential site of regulation.
DISCUSSION

In this study, we show that Flavopiridol inhibits potently the in vitro kinase activity of two CDKs, CDK2 and CDK4, at concentrations that correspond to the growth inhibitory effects of Flavopiridol (IC50: 50–120 nM; see Ref. 1). In the MCF-7 cell type, which possesses functional p53 and Rb protein, the G1 arrest produced by Flavopiridol occurred with an accompanying pRb hypophosphorylation. However, the action of Flavopiridol is not dependent on functional p53 or the presence of pRb, because G1 arrest was demonstrated in the MDA-MB-468 breast carcinoma cells, which possess mutant p53 (52, 53) and have lost the gene encoding the Rb protein (54, 55). Furthermore, no appreciable CDK4 kinase activity was found in the immune complexes from MDA-MB-468 cells, consistent with a previous report on the relative levels of cyclin D1 and D3 and CDK4 protein (36). Under these circumstances, the regulatory interplay between CDK4, D-type cyclins, p16, and pRb would be lost (35, 46, 51, 56–60), indicating that CDK2-cyclin E or CDK2-cyclin A may become an important antiproliferative target for this cell type.

A consistent finding when determining the activity of immunoprecipitated CDK2 and CDK4 from cells exposed to Flavopiridol was an initial increased kinase activity that peaked at 3 h. In the present work, we were able to show an increase in CDK2 activity immunoprecipitated from Flavopiridol-treated cells that correlated with a loss of phosphoserine from the catalytic subunit. Because we were unable to obtain a hypophosphorylated tyrosine form of CDK2 by adding Flavopiridol to the immunoprecipitates derived from untreated cells, it suggests that the increase in kinase activity and reduction of phosphotyrosine content requires the simultaneous presence of Flavopiridol and an intracellular factor or factors. From both analysis of the crystal structure of CDK2 complexed either with or without cyclin A (61, 62) and from the kinetic analysis of inhibition by Flavopiridol, it would appear unlikely that simple binding of Flavopiridol to the catalytic subunit could directly block the inhibitory phosphorylation on threonine-14 and tyrosine-15 phosphorylation sites. It may be suggested that inhibition of the intracellular CDK activity may lead to a compensatory reduction of the inhibitory kinase activity that maintains tyrosine phosphorylation, and the observed activation of CDKs is unmasked after immunoprecipitation and removal of Flavopiridol from the ATP-binding site. The facts that the readdition of Flavopiridol to the CDK2 immunoprecipitate decreased the kinase activity (Fig. 7) and that pRb becomes hypophosphorylated (Fig. 3) indicate that the net result of the action of Flavopiridol is the inhibition of CDK activity in living cells.

Analysis of cyclin levels does not support the contention that the Flavopiridol-induced increase in CDK kinase activity comes from an increased availability of cyclin. However, the dramatic loss of cyclin D in response to Flavopiridol raises further questions regarding the regulation of the CDK4-cyclin D complex and its kinase activity. Currently, the Rb gene product is proposed to increase expression of the D1 cyclin (57), and hyperphosphorylation of pRb produces a feedback inhibition of the D-type CDK activity by the release of a transcription factor that promotes expression of the CDK4 inhibitory protein p16 (35). There is also evidence that in the absence of pRb, increased expression of the p16 protein occurs in human tumor cells in culture (51, 56), which acts to prevent complex formation between CDK4 and cyclin D (56, 58, 59). However, our immunoblot of whole-cell lysates shows that cyclin D is lost (Fig. 6) before pRb becomes significantly hypophosphorylated (Fig. 3), and the CDK4 kinase activity immunoprecipitated after 3 or 6 h of Flavopiridol exposure (Fig. 2) would suggest that p16 or other endogenous inhibitors are not relevant at this stage. The data suggest that the presence of Flavopiridol is promoting the loss of cyclin D from the cell either in response to inhibition of the CDK4 complex or some as yet undefined process. The effect of Flavopiridol on either cyclin E or A in the same cell type is less profound.

We have demonstrated that Flavopiridol is a potent inhibitor of CDK4 and CDK2 kinase activity both in vitro and in living cells. The G1 arrest produced by the drug in either MCF-7 or MDA-MB-468 breast carcinoma cells is consistent with the CDKs being direct targets for the growth inhibitory effect.

REFERENCES

FLAVOPRIDOL INHIBITION OF CDK4, CDK2, AND CELL GROWTH

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