Characterization of a Human Colorectal Carcinoma Cell Line with Acquired Resistance to Flavopiridol

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ABSTRACT
Flavopiridol is a broad-spectrum inhibitor of cyclin-dependent kinases (cdks) and represents the first in this anticancer class to enter clinical trials. In anticipation of the likelihood that, as with other cancer drugs, acquired resistance may limit the drug’s efficacy, an acquired resistance model has been established by in vitro drug exposure of the human colon carcinoma cell line HCT116. This stably resistant line, possessing 8-fold resistance to flavopiridol, showed a lack of cross-resistance to the anticancer agents etoposide, doxorubicin, paclitaxel, topotecan, and cisplatin, and notably to other chemical classes of cdk inhibitors: the aminopurines rosirovine and purvalanol A, 9-nitropaullone, and hymenialdisine. Resistance did not seem to be related to differences in the levels of multidrug resistance drug efflux proteins, P-glycoprotein, and MRP1. Moreover, there were no changes in overall drug accumulation between the resistant and sensitive cell lines. Flavopiridol induced cell cycle arrest, apoptosis, and inhibition of retinoblastoma gene product phosphorylation on serine 780 in both parental and resistant lines, but the latter required 8-fold higher concentrations to achieve these effects. Cyclin E protein levels and cyclin E-associated kinase activity were increased in the resistant line, suggesting that overexpression of cyclin E may be the mechanism of resistance to flavopiridol. However, transfection of cyclin E to increase expression of this protein did not result in an increase in resistance to flavopiridol. Thus, up-regulation of cyclin E alone does not seem to cause resistance to this cdk inhibitor.

The cell cycle is a highly regulated process that controls cell division. Core regulators are the cyclins and cyclin-dependent kinases (cdks) (Musunuru and Hinds, 1997). Because the cell cycle is deregulated in many cancers, it has been targeted for the development of new anticancer agents (Fry and Garrett, 2000), particularly with respect to inhibitors of cdks. Flavopiridol is a semisynthetic derivative of the alkaloid rohitukine found in the bark of the Indian tree Dysoxylum binefacterium. It is a potent cdk inhibitor, and studies have shown it to inhibit cdk1, cdk2, cdk4, cdk7, cdk8, and cdk9 with similar potency (Carlson et al., 1996; Chao et al., 2000). It acts as a competitive inhibitor of the ATP binding site formed by a cleft between the C- and N-terminal domains of the cdk (De Azevedo et al., 1996). Although it was first believed to exert only cytostatic effects (Kaur et al., 1992), it has since been found to cause cytotoxicity and apoptosis in both resting and proliferating cells (Bible and Kaufmann, 1996). Cells treated with flavopiridol undergo apoptosis independently of p53 and p16 status (Brusselbach et al., 1998; Patel et al., 1998). After phase I evaluation (Senderowicz et al., 1998), flavopiridol is currently undergoing phase II clinical trials, including combination studies with paclitaxel and cisplatin, for which evidence of preclinical synergy has been observed (Bible and Kaufmann, 1997).

Drug resistance is a major obstacle in curing cancer. Mechanisms of resistance described for the currently used cytotoxic anticancer drugs include decreased drug accumulation, altered drug inactivation, increased DNA repair, and changes in drug target (Vendrik et al., 1992). Resistance to most currently used anticancer agents does occur, so it seems very likely that it will also appear with novel classes of drugs, such as flavopiridol. To our knowledge, only one cell line has been described that shows acquired resistance to flavopiridol (Robey et al., 2001). This article describes the generation and characterization of a human colon carcinoma cell line that exhibits acquired resistance to flavopiridol after continuous exposure to this agent.

Materials and Methods

Drugs and Chemicals. 9-Nitropaullone, purvalanol A, and hymenialdisine were kindly provided by L. Meijer (Station Biologique, Centre National de la Recherche Scientifique, Roscoff, Bretagne, France). Doxorubicin and etoposide were purchased from Sigma.

ABBREVIATIONS: cdk, cyclin-dependent kinase; PBS, phosphate-buffered saline; PgP, P-glycoprotein; MRP, multidrug resistance-associated protein; Rb, retinoblastoma gene product; SRB, sulforhodamine B; PARP, poly ADP-ribose polymerase.
Chemical (Poole, Dorset, UK) and Bristol-Myers Squibb Company (Hounslow, UK), respectively. Flavopiridol (kindly provided by E. Sausville, National Cancer Institute, Bethesda, MD) and topotecan (Royal Marsden Hospital Pharmacy, Sutton, UK) were dissolved in distilled water; cisplatin (Sigma) in 0.9% saline; raltitrexed (Tomudex; Astra-Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK) in 0.15 mM sodium bicarbonate; roscovitine (Calbiochem, Nottingham, UK), 9-nitopaullone, purvalanol, and hymenialdisine in dimethyl sulfoxide; and paclitaxel (Calbiochem) in ethanol. Other chemicals were obtained from Sigma unless otherwise stated.

**Cell Culture.** All cells [human colon cancer cell lines BE, COLO205, DLD1, HCT116, HT29, KM12, HCT116/5FP, human ovarian carcinoma cell lines SKOV3-puro and SKOV3-3 S2 (Sharpe et al., 1998), and CHI/doxR (Sharpe et al., 1994)] were grown as monolayers in Dulbecco’s modified Eagle’s medium (Invitrogen, Paisley, Scotland) augmented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, minimal essential medium nonessential amino acids (both from Invitrogen), and 0.5 μg/ml hydrocortisone in a 6.5% CO2/93.5% air atmosphere.

**Growth Inhibition Assay.** The sulforhodamine B (SRB) assay (Skehan et al., 1990) was used to determine growth inhibition by flavopiridol and other agents. Briefly, 4 × 10^4 cells/well were seeded into 96-well microtiter plates in 160 μl of growth medium. After overnight incubation, serial dilutions of drug were added to quadruplicate wells, and cells were exposed for 96 h. Quantitation of cell growth was assessed using 0.4% SRB dissolved in 1% acetic acid. IC_{50} values were determined graphically. Resistance factors were calculated for each individual experiment by the ratio of IC_{50} values of HCT116/5FP cells to IC_{50} values of HCT116 cells; mean and S.D. values were then determined.

**Clonogenic Survival Assay.** Cells were seeded at 500 cells/well into 6-well plates and left to attach overnight. They were then exposed to serial dilutions of flavopiridol for 96 h. Cells were washed twice with PBS before addition of fresh growth medium. Cells were left to grow for 10 days to form colonies. Colonies of at least 50 cells were counted and expressed as a percentage of control cells. IC_{50} values were determined graphically.

**Doubling Times.** Cells were seeded at a concentration of 1 × 10^5 into 25-cm² flasks. The cells were harvested by trypsinization, and the cells were counted using a hemocytometer after 24, 48, 72, and 96 h of incubation. The doubling times were calculated for the exponential growth phase.

**Western Blot.** Western blot analysis of proteins was carried out as described previously (Sharpe et al., 1994) with detection by enhanced chemiluminescence (PerkinElmer Life Sciences, Boston, MA). Antibodies were obtained from BD PharMingen (San Diego, CA) (cyclin A (BF683), cyclin B1 (GNS-1), cyclin D2 (DCS3.1), cyclin E (HE12), cdk4 (ACD1), and caspase 3); Santa Cruz Biotechnologies (Santa Cruz, CA) (caspase 3 (BF683); cyclin A (cyclin A); cyclin B1 (GNS-1); cyclin D2 (DCS3.1); cyclin E (HE12); cdk4 (ACD1), and caspase 3); Santa Cruz Biochemicals (Santa Cruz, CA) (cyclin D2 (18B6–10), cdk2 (D-12); New England Biolabs (Hitchin, UK) (total Rh, phospho Rh serine 780); CLONTECH (Palo Alto, CA) (PARP); Centecor Diagnostics (Malvern, PA) (Pgp (C219)); and Monosan (Uden, the Netherlands) (MRP (MRPm6)). Secondary antibody was obtained from Amersham Pharmacia Biotech. For analysis of apoptosis by caspase 3 and PARP cleavage, detached cells were harvested by centrifugation after 48-h exposure to either 0.45 μM (HCT116) or 3.9 μM (HCT116/5FP) flavopiridol.

**Flavopiridol Uptake.** Asynchronous cells (~10^6) were treated for 2 h with equimolar (0.25 μM; 5 times the IC_{50} value) or equitoxic (0.25 μM for HCT116 and 2.0 μM (5 times the IC_{50} value) for HCT116/5FP) concentrations of flavopiridol and were either lysed immediately or washed three times with PBS and incubated in drug-free medium for an additional hour before lysing. Cells were lysed in H_2O and sonicated for 15 s at half of maximum power (MSE Soniprep 150, Fisons Plc, Loughborough, UK). An aliquot of 50 μl was taken for protein analysis and lysed in 4 volumes of 1 N NaOH at 37°C overnight before determining protein content by the Bradford protein assay (Sigma). For the samples used for flavopiridol analysis, protein was precipitated with two volumes of methanol followed by centrifugation. The supernatant (20 μl) was injected for analysis by liquid chromatography/tandem mass spectrometry chromatography. The separation was achieved on a 50-mm (4.6 i.d.) × 5 μm ABZ+ column (Supelco, Poole, Dorset, UK). A 3-min gradient of 80% formic acid/20% methanol was used. The mass spectrometer used was a triple mass spectrometer (TSQ700, Thermoquest Ltd., Hemel Hempstead, Herts, UK) with an electrospray ionization source. The capillary temperature was 250°C and the spray voltage was 4.5 kV. Detection was achieved in positive-ionization mode by selected-reaction monitoring of the sum of the daughter ions of the pseudomolecular ion [M + H+] 402.2 (340.9 atomic mass units). The collision gas flow was 1.6 mtorr and the collision energy was −20 V. Standard curves were made in the cell extracts spiked with flavopiridol analyzed at levels of 4, 10, 40, 100, and 400 ng/ml, and the limit of quantitation was 4 ng/ml. Peak area was determined using Prism 2.01 software (GraphPad Software, San Diego, CA) and increased linearly with concentrations.

**Flow Cytometry.** Flow-cytometry analysis of cell cycle distribution was carried out as described previously (Ormerod, 1994) using asynchronously growing cells in log-phase with the use of a Coulter Elite flow cytometer (Beckman Coulter, Buckinghamshire, UK) equipped with an argon-ion laser (Spectra Physics, San Jose, CA) with an output of 200 mW at 488 nm. Typically, data from 2 × 10^5 cells were analyzed for forward and orthogonal scattered light together with red fluorescence (peak and integrated area). Pulse-shape analysis was performed to eliminate any cell clumps, and data were gated on light scatter before recording a histogram of red fluorescence. Histograms were generated using the WinMDI2.8 (Windows Multiple Document Interface Flow Cytometry Application (http://www.uwm.ac.uk/uwcm/hg/hoy/index.html)) with the cell cycle data calculated using software with a Watson algorithm (Ormerod et al., 1987).

**Kinase Assay.** Cell lysates (50 mM HEPES, 250 mM NaCl, 0.1% NP40, 10 mM β-glycerophosphate, 1 mM NaF, 1 mM EDTA, 1 mM Pefabloc (Roche Molecular Biochemicals, Mannheim, Germany), 1 mM dithiothreitol (Roche), 0.1 mM NaVO_3, 10 μg/ml aprotinin, and 20 μg/ml leupeptin) were prepared from exponentially growing cells exposed to equimolar or equitoxic doses of flavopiridol for 24 h before harvesting. Protein (300–400 μg) was immunoprecipitated using 4 μg of antibody to cdk2 (Neomarker Ab-3; Labvision, Fremont, CA), cyclin E (Neomarker Ab-1), cyclin A (Santa Cruz H432), or cyclin B1 (Santa Cruz GNS1) and bound to protein A Sepharose beads. After washing, a kinase assay was carried out at 30°C for 15 min in kinase buffer containing 50 mM Tris, pH 7.5, 10 mM MgCl_2, 1 mM dithiothreitol (Roche), 50 μM ATP (Roche), 5 μg histone H11, (Roche), 32P-ATP (specific activity, 5 Ci/μmol, 10 μCi/ml; Amersham Pharmacia Biotech). The reaction was stopped with 2× Laemmli buffer (20% glycerol, 10% mercaptoethanol, 4.6% SDS, 62.5 mM Tris, pH 6.8, and 0.05% bromphenol blue). Samples were run on a Tris-glycine gel and visualized using a Storm PhosphorImager (Molecular Dynamics, Sevenoaks, UK).

**Confocal Microscopy.** Cells were treated for 48 h at 10 times their respective 96-h IC_{50} value (0.45 μM and 4.0 μM for HCT116 and HCT116/5FP, respectively), and detached cells were harvested by centrifugation. Cells were fixed in 4% paraformaldehyde in PBS. Cells were stained with propidium iodide after treatment with Triton X-100 and RNase. Samples were analyzed at 488 nm on a confocal microscope (TCS-SP; Leica, Milton Keynes, UK).

**Vector Construction and Transfection.** The DNA for cyclin E was amplified from a cytomegalovirus expression vector (kindly provided by Dr. Michelle Garrett, Institute of Cancer Research, London, UK) by polymerase chain reaction using proofreading polymerase Pwo (Roche) according to the manufacturer’s instructions with cycles of 94°C for 2 min, 10 cycles of 94°C for 15 s, 55°C for 45 s, and 72°C for 90 s followed by 15 cycles of 94°C for 15 s, 55°C for 45 s, and 72°C for 90 s, with cycle elongation of 20 s per cycle and prolonged...
elongation at 72°C for 10 min. After blunt cloning into PCR-Script CAM vector (Stratagene, Amsterdam, the Netherlands) according to the manufacturer's instructions, the gene of interest was cut out as an EcoRI/NheI (New England Biolabs) fragment and cloned into the vector F373 (Hobs et al., 1998).

Statistics. As appropriate, statistical significance was determined using an unpaired, two-tailed Student's t test. All values given are means of at least three experiments with the corresponding standard deviation given, unless otherwise stated.

Results

Growth Inhibition by Flavopiridol. A small panel of human colorectal cell lines was initially used to investigate the effects of flavopiridol. Figure 1A shows the 96-h IC50 values (i.e., the concentration of drug that inhibits 50% growth). BE and HT29 cell lines were the most resistant to flavopiridol (IC50 = 0.15 and 0.17 μM, respectively). HCT116 and KM12 were the most sensitive (IC50 = 0.042 and 0.052 μM, respectively), and DLD1 and COLO205 showed intermediate sensitivity (IC50 = 0.069 and 0.059 μM, respectively).

Generation of a Subline with Acquired Resistance to Flavopiridol. We selected HCT116 cells to generate a subline with acquired resistance to flavopiridol because it was the most sensitive line in the small panel tested. Over a period of 3 months, HCT116 cells were exposed continuously to increasing concentrations of flavopiridol. Concentrations started at 100 nM (2 times the IC50 value) and were doubled each time the dose was increased until 400 nM was reached. By this means, we obtained a subline that showed stable 8-fold increase in resistance to flavopiridol (Table 1). This subline was designated HCT116/8FP. Resistance was observed using the SRB growth inhibition assay and confirmed in a clonogenic assay (Fig. 1B). The mean resistance factor was calculated by the IC50 of HCT116/8FP cells/IC50 of parental cells for each repeated analysis and was 8.42 ± 2.42 (mean ± S.D.) as determined by the SRB growth inhibition assay and 7.20 ± 1.47 (mean ± S.D.) as measured by the clonogenic assay. The resistant cells were stable in drug-free medium for at least 3 months (data not shown). Doubling times were assessed and found not to be significantly different (12.6 ± 2.2 h and 15.8 ± 3.8 h for HCT116 and HCT116/8FP, respectively; p = 0.11).

Using the 96-h SRB assay, the sensitivity of the parent and resistant subline and cross-resistance were determined for a series of standard anticancer drugs, together with four additional cdk inhibitors of different chemical types: the trisubstituted aminopurines roscovitine (Meijer et al., 1997) and purvalanol A (Gray et al., 1998), the paullone 9-nitropaulnone (Zaharevitz et al., 1999), and the marine sponge constituent hymenialdisine (Meijer et al., 2000). Notably, no cross-resistance to any of the alternative cdk inhibitors investigated was observed, and neither was any statistically significant cross-resistance seen with cisplatin, doxorubicin, etoposide, paclitaxel, or raltitrexed. Cross-resistance was only observed with the topoisomerase inhibitor topotecan, which showed a modest but statistically significant 1.7-fold increase in resistance (p = 0.027) (Table 1).

Drug Accumulation and the Potential Role of Efflux Pumps. Reduced drug accumulation is often associated with resistance to anticancer drugs and frequently involves drug efflux pumps such as PgP and MRP (Germann, 1996; Borst et al., 2000). Expression of PgP and MRP1 was studied in HCT116 and HCT116/8FP cells using Western blot analysis. Results showed no detectable expression of either of these protein efflux pumps compared with the positive control cells CHDoxR for PgP and SKOV3-S2 for MRP1 (data not shown). However, other efflux pumps may potentially be involved in decreasing the amount of drug in cells (Borst et al., 2000). To investigate whether reduced drug accumulation plays a role in this model, the levels of flavopiridol were measured in cells after 2-h drug exposure at concentrations that were either equimolar (0.45 μM) or equitoxic (0.45 μM and 4.0 μM for HCT116 and HCT116/8FP, respectively, representing 10 times the 96-h IC50 value). Concentrations were determined by the highly sensitive method of liquid chromatography/tandem mass spectrometry. The results (Fig. 2) showed that the parent and the resistant cells accumulated similar amounts of flavopiridol at 120 min (14.00 ± 3.12 and 15.10 ± 5.10 ng flavopiridol/mg protein for HCT116 and HCT116/8FP, respectively; p = 0.67) when exposed to equimolar amounts of flavopiridol. When treated with an equitoxic concentration, the amount of flavopiridol taken up by the resistant cells increased 4-fold to 59.4 ± 6.6 ng flavopiridol/mg protein. One hour after drug removal, neither cell line

<table>
<thead>
<tr>
<th>Drug</th>
<th>HCT116 IC50 μM</th>
<th>HCT116/8FP IC50 μM</th>
<th>Resistance Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roscovitine</td>
<td>5.67 ± 0.64</td>
<td>7.85 ± 2.95</td>
<td>1.38 ± 0.16</td>
</tr>
<tr>
<td>Purvalanol A</td>
<td>3.18 ± 0.38</td>
<td>2.48 ± 0.19</td>
<td>0.78 ± 0.07</td>
</tr>
<tr>
<td>9-Nitropaulnone</td>
<td>0.22 ± 0.03</td>
<td>0.21 ± 0.01</td>
<td>0.99 ± 0.14</td>
</tr>
<tr>
<td>Hymenialdisine</td>
<td>3.40 ± 0.85</td>
<td>2.63 ± 0.38</td>
<td>0.82 ± 0.30</td>
</tr>
<tr>
<td>Etoposide</td>
<td>0.41 ± 0.15</td>
<td>0.44 ± 0.17</td>
<td>1.19 ± 0.54</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.34 ± 0.37</td>
<td>0.19 ± 0.08</td>
<td>1.28 ± 0.92</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>2.78 ± 1.40</td>
<td>4.35 ± 1.70</td>
<td>1.42 ± 0.22</td>
</tr>
<tr>
<td>Raltitrexed</td>
<td>0.01 ± 0.00</td>
<td>0.01 ± 0.00</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>3.90 ± 3.50</td>
<td>5.90 ± 1.90</td>
<td>2.20 ± 1.5</td>
</tr>
<tr>
<td>Topotecan</td>
<td>7.40 ± 1.64</td>
<td>12.33 ± 3.19</td>
<td>1.7 ± 0.61</td>
</tr>
</tbody>
</table>
showed any detectable amounts of flavopiridol at equimolar doses. Exposure to higher concentrations of drug resulted in 9.28 ± 2.04 ng flavopiridol/mg protein remaining in the resistant cells 1 h after drug removal.

Because previous studies have implied a role for the drug efflux pump MRP1 in the cellular response of flavopiridol (Hooijberg et al., 1997, 1999), we determined the sensitivity of an isogenic pair of cell lines that were transfected with either empty vector (SKOV3-puro) or the same vector containing the MRP gene (SKOV3-S2) (Sharp et al., 1998). SKOV3-S2 cells are 3-fold less sensitive to etoposide (a known MRP substrate) than SKOV3-puro cells with a 96-h IC_{50} value of 3.22 ± 0.62 μM for SKOV3-puro and 10.75 ± 3.30 μM for SKOV3-S2. A comparison of the IC_{50} values after 96-h exposure to flavopiridol showed no significant difference between the two cell lines (IC_{50} = 0.265 ± 0.046 μM for SKOV3-puro and 0.265 ± 0.029 μM for SKOV3-S2), suggesting that flavopiridol is not a substrate for this efflux pump. Using flavopiridol as a potential modulator of MRP1 at a nontoxic dose (100 nM; <10% cell kill), the sensitivity to etoposide (Cole and Deeley, 1998) was investigated. The sensitivity ratios (IC_{50} of etoposide + flavopiridol/IC_{50} of etoposide alone) for both cell lines were 1.05 ± 0.4 and 1.11 ± 0.2 for HCT116 and HCT116/8FP, respectively (p = 0.83). This result is also consistent with a lack of significant interaction of flavopiridol with MRP1.

**Cell Cycle Distribution.** The cell cycle distribution of the parent and resistant cell line was studied in the presence and absence of flavopiridol. Cells were exposed for 24 h to an equitoxic concentration corresponding to 10 times their 96-h IC_{50} value (0.45 and 3.9 μM for HCT116 and HCT116/8FP, respectively). This revealed an increase in the proportion of cells in G_{2}/M and a concomitant reduction in S-phase cell numbers for parent HCT116 cells (Fig. 3A; Table 2). In contrast, HCT116/8FP cells exposed to 0.45 μM flavopiridol showed no change in cell cycle distribution compared with control cells (Fig. 3B, Table 2). Treated at an equitoxic concentration of flavopiridol (0.45 μM for HCT116 and 3.9 μM for HCT116/8FP), HCT116/8FP cells demonstrated a decrease in S-phase fraction together with an increase in G_{2}/M, as seen in the parental cells (Fig. 3C).

To investigate whether the cell cycle effects of flavopiridol were reversible, drug was removed after 24-h exposure, and cells were maintained for an additional 24 h in drug-free medium. The parental cell line showed no difference 24 h after drug removal compared with the cell cycle distribution after 24 h of drug treatment. The resistant cells showed signs of a partial recovery by a decrease in the G_{1} population of cells maintained in drug-free medium after drug treatment at 3.9 μM flavopiridol, compared with the cells exposed to flavopiridol. However, the percentage of G_{1} cells in the resistant line after the period in drug-free medium was higher than in the control cells (Fig. 3).

**Apoptosis.** Alterations in programmed cell death have also been shown to be involved in drug resistance (Hickman, 1998). Apoptosis induction by flavopiridol was investigated by confocal microscopy as well as caspase 3 and PARP cleavage. Morphologically, both parent and resistant cells treated at an equitoxic concentration (10 times the 96-h IC_{50} value) showed condensed nuclei indicative of apoptosis (Fig. 4A). This observation was supported by immunoblotting for PARP and caspase 3 (Fig. 4, B and C). Probing for PARP revealed the emergence of the 85-kDa cleaved fragment from the 116-kDa native PARP, and probing for caspase 3 showed a decrease of uncleaved (30 kDa) protein after treatment with flavopiridol for 48 h. Similar measurements of apoptosis using HCT116/8FP cells exposed to 0.45 μM flavopiridol (i.e., the equimolar concentration) could not be carried out because no floating cells could be collected. Taken together, these experiments indicate that flavopiridol induces

![Fig. 2. Flavopiridol uptake in HCT116 and HCT116/8FP cells.](image)

**Fig. 3.** Cell cycle distribution of HCT116 and HCT116/8FP cells after 24-h exposure to flavopiridol with or without a 24-h drug-free period after drug exposure. A, HCT116 treated at 0.45 μM flavopiridol. B, HCT116/8FP treated at an equimolar dose (0.45 μM) of flavopiridol. C, HCT116/8FP treated at an equitoxic dose (3.9 μM).
apoptosis to a similar extent in both the parent and the resistant cell line. However, as with the cell cycle effects, higher concentrations of drug are required to cause apoptosis in the resistant cell line.

**Rb Phosphorylation.** Because the Rb protein is a major target downstream of the cdks in the regulation of the cell cycle (Musunuru and Hinds, 1997), phosphorylation of this protein on serine 780 was also investigated using a phosphospecific antibody. Although the levels of total Rb remained similar (Fig. 5), both parent and resistant cells treated for 24 h with flavopiridol showed a decrease in phosphorylation at equitoxic levels (5 times the 96-h IC50 value). In contrast to parental cells, flavopiridol-resistant cells treated with 0.25 μM flavopiridol (5 times the parental IC50 value) showed no difference in phosphorylation of Rb compared with untreated HCT116/8FP cells (Fig. 5).

**Expression of Cell Cycle Proteins.** Alterations of the levels of the target protein can lead to drug resistance (Vendrik et al., 1992). Because flavopiridol inhibits cdks, expression of cyclin A (60 kDa), cyclin B1 (62 kDa), cyclin D2 (35 kDa), cyclin D3 (34 kDa), cyclin E (50 kDa), cdk2 (33 kDa), and cdk4 (32 kDa) proteins was determined. These proteins were selected because of the commercial availability of good antibody reagents. Western blots revealed no difference in constitutive levels of these proteins between parent and resistant lines for most of the proteins studied. However, cyclin E levels were clearly and reproducibly increased in the resistant cell line (Fig. 6).

**Kinase Activity.** To determine whether the observed change in cyclin E protein levels resulted in an increase in associated kinase activity, kinase assays were performed on immunoprecipitated proteins using histone H1 as a substrate (Fig. 7). Cyclin E and cdk2-associated kinase activity were increased when constitutive levels were compared between HCT116 and HCT116/8FP cells. Exposure of cells for 24 h with equitoxic amounts of flavopiridol (0.25 μM and 2.0 μM for HCT116 and HCT116/8FP, respectively; 5 times the 96-h IC50 value) resulted in a decrease in the kinase activity associated with cyclin E and cdk2, as well as that with cyclin A and cyclin B1. However, when cells were exposed to levels of flavopiridol that were lower than the IC50 value of the respective cell line (0.03 μM and 0.25 μM for HCT116 and HCT116/8FP, respectively), all kinase activity of all proteins investigated (cyclin E, cyclin A, cyclin B1, and cdk2) was elevated compared with the constitutive levels.

**Transfection of Cyclin E.** From these results, it seemed possible that cyclin E was the cause of resistance to flavopiridol. Thus, a vector containing the gene encoding cyclin E protein was transfected into HCT116 cells. Two clones overexpressing cyclin E (HCT116/513 clone 4 and clone 5) were chosen and compared with cells transfected with empty vector alone (HCT116/373). Figure 8A shows the constitutive expression for cyclin E of cells in the exponential growth phase. Both HCT116/513 clones expressed increased levels of cyclin E protein compared with HCT116/373 cells. The increase in cyclin E expression was similar to that observed in the HCT116/8FP cell line. This increased cyclin E protein level correlated with an increased kinase activity associated with cyclin E and cdk2 (Fig. 8B, lanes 1, 3, and 5). Having shown that the transfected cells overexpress functional cyclin E, the sensitivity of these cells to flavopiridol, the structurally different cdk inhibitor roscovitine, paclitaxel, and cisplatin was investigated (Table 3). No difference in sensitivity to flavopiridol was observed when the cyclin E–overexpressing cells were compared with the empty vector control cells. Neither was a difference observed with the other cdk inhibitor investigated or with the anticancer agents paclitaxel and cisplatin.

Exposure of the control cells and the cyclin E–overexpressing cells to 0.45 μM flavopiridol (10 times the 96-h IC50 value) for 24 h resulted in a decrease in kinase activity associated with both cyclin E and cdk2 (Fig. 8B).

**Discussion**

A human colorectal carcinoma cell line exhibiting acquired resistance to flavopiridol has been established. This cell line, HCT116/8FP, was generated by continuous exposure to increasing concentrations of flavopiridol until a cell line exhibiting stable 8-fold resistance compared with the parent cell line was established. A lack of statistically significant cross-resistance was observed with the clinically used anticancer agents etoposide, doxorubicin, cisplatin, paclitaxel, and raltitrexed. However, there was a modest 1.7-fold cross-resistance to the topoisomerase inhibitor topotecan in the resistant subline HCT116/8FP. Comparing the parent cell line HCT116 and the resistant subline HCT116/8FP revealed no cross-resistance to four other cdk inhibitors: roscovitine, purvalanol A, 9-nitropauloline, and hymenialdisine. These alternative cdk inhibitors are from three structurally different classes: the trisubstituted aminopurines (roscovitine and purvalanol), the paullones (9-nitropauloline), and the marine sponge–derived natural bromopyrroles (hymenialdisine). Thus, the resistance mechanism seemed unique to flavopiridol itself among the drugs studied. This suggests the possi-

**TABLE 2**

Effects of flavopiridol on cell cycle distribution

<table>
<thead>
<tr>
<th>Treatment</th>
<th>G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT116</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 h control</td>
<td>41.43 ± 10.04</td>
<td>43.38 ± 7.04</td>
<td>12.88 ± 6.42</td>
</tr>
<tr>
<td>24 h at 0.45 μM</td>
<td>57.27 ± 6.16</td>
<td>25.97 ± 5.07</td>
<td>15.17 ± 6.42</td>
</tr>
<tr>
<td>24 h at 0.45 μM + 24 h DF</td>
<td>56.61 ± 15.67</td>
<td>27.09 ± 9.95</td>
<td>15.17 ± 4.12</td>
</tr>
<tr>
<td>HCT116/8FP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 h control</td>
<td>49.57 ± 10.43</td>
<td>34.68 ± 13.60</td>
<td>14.46 ± 15.19</td>
</tr>
<tr>
<td>24 h at 0.45 μM</td>
<td>48.95 ± 10.57</td>
<td>31.83 ± 12.49</td>
<td>16.98 ± 3.83</td>
</tr>
<tr>
<td>24 h at 0.45 μM + 24 h DF</td>
<td>43.86 ± 7.24</td>
<td>39.64 ± 8.14</td>
<td>15.39 ± 2.30</td>
</tr>
<tr>
<td>24 h at 3.9 μM</td>
<td>57.92 ± 6.64</td>
<td>29.27 ± 5.08</td>
<td>12.50 ± 3.92</td>
</tr>
<tr>
<td>24 h at 3.9 μM + 24 h DF</td>
<td>52.36 ± 15.94</td>
<td>30.53 ± 13.85</td>
<td>17.83 ± 2.86</td>
</tr>
</tbody>
</table>
bility that alternative cdk inhibitors may retain activity in flavopiridol-resistant cancers. The difference could be because flavopiridol is a broad-spectrum cyclin-dependent kinase inhibitor, whereas the other cdk inhibitors are somewhat more specific. For example, roscovitine, purvalanol, and 9-nitropaullone inhibit cdc2, cdk2, and cdk5 (Meijer et al., 1997; Gray et al., 1998; Zaharevitz et al., 1999), but none of these agents inhibits cdk4 or cdk6. In addition to these cdk inhibitors, some cdk inhibitors (e.g., flavopiridol, paullones, hymenialdisine) may also inhibit GSK-3β (Leclerc et al., 2001). Because flavopiridol also inhibits this enzyme, it does not seem likely that this is the cause of the lack of cross-resistance of the other cdk inhibitors to the flavopiridol-resistant cells. On the other hand, the lack of cross-resistance seen with alternative cdk inhibitor chemotypes may reflect other differences in cellular pharmacology compared with flavopiridol.

Decreased drug accumulation caused by overexpression of drug efflux pumps such as PgP and MRP1 is often associated with multidrug resistance (Germann, 1996; Borst et al., 2000). Therefore, PgP and MRP1 levels were investigated compared with positive control cells. However, no protein expression of PgP was detected in either the parent or the resistant line. Thus, PgP does not seem to play a role in this model of flavopiridol resistance. However, previous studies have shown MRP to be involved in the action of flavopiridol (Hooijberg et al., 1997). In vesicles containing MRP, the ATPase activity associated with these vesicles was stimulated by flavopiridol (Hooijberg et al., 1997). We investigated the role of MRP1 in resistance to flavopiridol by using an isogenic pair of cell lines that differ only in their expression of MRP1. There was no difference in growth inhibition induced by flavopiridol, either as a single agent or as a modulator to etoposide. A previous study also using an isogenic pair of cells differing only in MRP1 status observed a small increase (1.4-fold) in resistance to flavopiridol (Hooijberg et al., 1999). The difference between that study and ours could be explained by different methods of transfection, the vector used, or the cell type. Also, the level of MRP1 protein expressed by the cells could differ between the studies, which could result in differences in responses to cytotoxic agents. In addition, the previous study also showed that inhibition of MRP1 with probenecid did not alter the cytotoxic effect of flavopiridol, thereby questioning the pharmacological significance of the small effect observed (Hooijberg et al., 1999).

In addition to the above-mentioned drug efflux pumps, many more are known, and at least one other (ABCG2; also known as MXR, BCRP, or ABCP1) has recently been associated with resistance to flavopiridol (Robey et al., 2001). In that study, in

![Fig. 4. Induction of apoptosis by flavopiridol. A, morphology after 48-h treatment at 0.45 μM flavopiridol [HCT116 (1)] or 3.9 μM flavopiridol [HCT116/8FP (2)]. B, PARP cleavage. C, caspase 3 cleavage. 1, HCT116 control; 2, HCT116 + 48 h of 0.45 μM flavopiridol; 3, HCT116/8FP control; 4, HCT116/8FP + 48 h of 3.9 μM flavopiridol. Nontreated attached cells were used as control cells; actin was used as a loading control.]

![Fig. 5. Effect of flavopiridol on Rb phosphorylation at serine 780 (A), Phospho-Rb (B), and total Rb. 1, HCT116 control; 2, HCT116 + 24 h of 0.25 μM flavopiridol; 3, HCT116/8FP control; 4, HCT116/8FP + 24 h of 0.25 μM flavopiridol; 5, HCT116/8FP + 24 h of 2.0 μM flavopiridol.]

![Fig. 6. Western blot analysis of proteins involved in cell cycle regulation in HCT116 and HCT116/8FP cells; actin was used as a loading control.]

addition to flavopiridol, resistance to other substrates of the ABCG2 pump such as topotecan and mitoxantrone was found (Robey et al., 2001). Another recent study described a cell line resistant to flavopiridol that arose spontaneously from the ovarian cell line OV202 (Bible et al., 2000b). In that study, in addition to resistance to flavopiridol, cross-resistance to cisplatin was also observed. The resistance mechanism was attributed to a decrease in drug accumulation.

Therefore, drug concentrations were measured in our parent and in the resistant cell line. Exposure to an equimolar dose of flavopiridol resulted in similar amounts of flavopiridol detected in the parental HCT116 and the resistant HCT116/8FP cells. Exposure to equitoxic doses (5 times the 96-h IC₅₀ value) led to an increased level of flavopiridol detected in the resistant cells compared with the parental cells. One hour after removal of flavopiridol, there were no detectable levels of flavopiridol when HCT116 or HCT116/8FP cells were exposed to an equimolar dose (0.45 μM) of the compound. Taken together with the observations of PgP and MRP1 expression, these data indicate that reduced drug accumulation does not play a role in this model of resistance to flavopiridol. This is in contrast to the two previous reports mentioned above (Bible et al., 2000b; Robey et al., 2001) and may be due to the difference in the mode of generation of the resistant lines.

Flavopiridol has been shown to cause cell cycle arrest at G₁/S and G₂/M with a decrease in S-phase cells (Kaur et al., 1992). These findings were confirmed in the parent HCT116 cell line, which is wild-type for p53 status (O'Connor et al., 1997). Treatment of the resistant cells with equimolar concentrations of flavopiridol that were the equivalent of 10 times the IC₅₀ value of the parental line did not show any difference in cell cycle distribution compared with untreated control cells. However, treatment with an equitoxic concentration resulted in a decrease in S phase and an increase in cells in G₁. The resistant cells exposed to an equitoxic dose of...
flavopiridol and then incubated in drug-free medium started to show some return to normal cell cycle distribution, but this was still incomplete at 24 h. However, the parental cells did not show any recovery.

In addition to cell cycle arrest, we showed that flavopiridol was able to induce apoptosis. Similar observations were found previously in other cells (Li et al., 2000). However, using morphology, caspase, and PARP cleavage to measure apoptosis, we found no difference in the induction of apoptosis when parental or resistant cells were exposed to an equitoxic dose of flavopiridol (10 times the 96-h IC_{50} value). This indicates that differences in apoptosis mechanisms are not involved in this resistance model to flavopiridol. It must be noted that higher concentrations of drug are required to induce apoptosis in the resistant cell line compared with the parental cell line, as with the cell cycle effects.

Having excluded drug accumulation as a cause of drug resistance and demonstrated that the resistant line is able to undergo cell cycle arrest and apoptosis but requires a proportionally higher dose of drug, we next looked at the effects on Rb phosphorylation. Inhibition of cdks would be expected to result in a decrease in Rb phosphorylation, leading to cell cycle arrest. The results showed that flavopiridol reduced Rb phosphorylation in both parent and resistant cell line; these data are consistent with inhibition of cdks in both lines; again, however, a higher drug dose was required to affect the resistant line.

Thus, the cellular and molecular evidence discussed so far is consistent with the hypothesis that flavopiridol induces cell cycle arrest, apoptosis, and cytotoxicity as a consequence of cdk inhibition and the resulting decrease in Rb phosphorylation. However, the resistant cell line requires higher levels of drug to achieve these effects. We hypothesized that the resistant line might express altered levels of cdks or cyclins. We therefore measured the expression of several cdks and cyclins using commercially available antibodies. Constitutive levels of cdk2 and cdk4 and cyclins A, B1, D1, and D2 showed no difference in protein expression. The exception was cyclin E, which was overexpressed in the resistant cell line. Overexpression of cyclin E has been observed in some cancers and has been associated with poor prognosis (Keyomarsi et al., 1994). To determine whether the overexpression of this protein was associated with an increase in activity, kinase assays were carried out on immunoprecipitated proteins. The resistant cell line showed an increased cyclin E-associated kinase activity compared with the parent cell line. A similar pattern emerged when the kinase activity associated with immunoprecipitated cdk2 was investigated. Another interesting result was obtained in which cells exposed to low levels of flavopiridol showed a stimulation of kinase activity for all kinase activities determined. This indicates that flavopiridol may act to stimulate kinase activities at low levels but inhibit them at higher levels. Consistent with these findings is a report demonstrating that kinase activity of cdk4 in MCF7 breast cancer cells increased after 3 h of exposure and decreased only after 12 to 24 h of treatment with flavopiridol (Carlson et al., 1996).

The increase in cyclin E and the associated kinase activity in the resistant cell line suggests a plausible hypothesis to explain the mechanism of resistance to flavopiridol in the HCT116/8FP cell line. Increased activity of one of the target kinases could quite conceivably lead to the need for a higher concentration of drug being required to inhibit Rb phosphorylation and to produce equivalent levels of cell cycle arrest, apoptosis, and cytotoxicity to those seen in the parent line. However, it might also be argued that increased cyclin E-associated kinase activity would lead to resistance to other cdk inhibitors, an effect that was not seen with our resistant line. This hypothesis was investigated by transfecting exogenous cyclin E into HCT116 cells. The selected clones showed an increase in cyclin E expression similar to that seen in the HCT116/8FP cells. However, no changes in sensitivity to flavopiridol were observed. Also, no changes in the sensitivity to the other cdk inhibitor studied (roscovitine) or two other anticancer agents, paclitaxel and cisplatin, were seen. These results indicate that cyclin E alone is not the cause of the resistance to flavopiridol. However, cyclin E has recently been shown to increase chromosomal instability when overexpressed (Spruck et al., 1999). Therefore, it is possible that overexpression of cyclin E may be required for further changes in the cell to occur. These additional changes could subsequently lead to resistance. According to this hypothesis resistance would develop only when these additional changes occur after cyclin E up-regulation.

It is quite possible, however, that flavopiridol induces resistance by a variety of mechanisms. We have begun to address this using gene expression microarray analysis of mRNA levels for approximately 5600 genes in the parent HCT116 and resistant HCT116/8FP cells, but this has not revealed any significant differences in constitutive expression between the two cell lines (data not shown). This observation suggests that the resistance mechanism might be the result of post-translational modifications of proteins that alter their activity.

It has recently become apparent that flavopiridol may affect cellular targets in addition to those described previously, including cdk9. Together with cyclin T1, this is a component of the protein kinase P-TEFβ, which controls the elongation phase of transcription by RNA polymerase II (Chao et al., 2000). In addition, cdk9 are not the only target of flavopiridol (Brusselbach et al., 1998). It has been found that flavopiridol can also decrease vascular endothelial growth factor (Melillo et al., 1999), protein kinase A, and epidermal growth factor receptor-associated kinase activity (Czech et al., 1995) as well as bind to DNA (Bible et al., 2000a). In addition, flavopiridol has been implicated in the inhibition of angiogene-

<table>
<thead>
<tr>
<th></th>
<th>Flavopiridol</th>
<th>Roscovitine</th>
<th>Paclitaxel</th>
<th>Cisplatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT116/373</td>
<td>0.042 ± 0.006</td>
<td>9.53 ± 2.54</td>
<td>7.80 ± 1.44</td>
<td>2.37 ± 1.34</td>
</tr>
<tr>
<td>HCT116/513 clone 4</td>
<td>0.043 ± 0.010</td>
<td>8.70 ± 2.44</td>
<td>7.60 ± 1.59</td>
<td>2.70 ± 0.46</td>
</tr>
<tr>
<td>HCT116/513 clone 5</td>
<td>0.039 ± 0.008</td>
<td>8.27 ± 3.29</td>
<td>7.87 ± 2.25</td>
<td>2.88 ± 1.14</td>
</tr>
</tbody>
</table>

TABLE 3
Mean 96-h IC_{50} values for flavopiridol in the cyclin E-overexpressing cells (HCT116/513 clone 4 and clone 5) and the empty vector control (HCT116/373)

Data are shown in micromolar (except paclitaxel, which are in nomomolar) and are expressed as mean ± S.D. (n ≥ 3).
sis (Brusselbach et al., 1998; Kerr et al., 1999). Thus, the antitumor effects of flavopiridol may not be caused simply by an inhibition of cdks. One aspect that has not been fully investigated in this study is the metabolism of flavopiridol, which could be altered in the resistant HCT116/8FP cells compared with the parent HCT116 cells.

In summary, we have generated a stable cell line with 8-fold acquired resistance to flavopiridol. No significant cross-resistance to any of the other drugs studied was observed, including four other cdk inhibitors. The resistant line does not show any increase in PgP or MRP expression. Taken together with direct measurements of flavopiridol accumulation, the results show that altered cellular uptake mechanisms do not play a role in the resistance mechanism in this model. Studies on the cell cycle, apoptosis, and Rb phosphorylation showed a lack of effect when the resistant cells were exposed to equimolar levels of flavopiridol, but similar effects were seen at equitoxic levels compared with the parental cells. Thus the cellular consequences of flavopiridol accumulation remain the same as in the parental line, but a higher concentration of drug is required to bring about these effects. Constitutive levels of cyclin E were increased in the resistant cell line compared with the parental line in contrast to other cyclins and cdks that were studied. This correlated with an increase in cyclin E- and cdk2-associated kinase activity. However, overexpression of cyclin E into HCT116 cells did not result in resistance to flavopiridol. Thus, increased cyclin E alone is insufficient to cause resistance, but it may be involved in the generation of additional changes that do lead to resistance, most likely at the post-translational level. With respect to clinical significance, these results support the view that resistance to flavopiridol may well occur in patients treated with the drug.

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References


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