Characterization of an Ovarian Carcinoma Cell Line Resistant to Cisplatin and Flavopiridol

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ABSTRACT

Flavopiridol, the first inhibitor of cyclin-dependent kinases to enter clinical trials, has shown promising antineoplastic activity and is currently undergoing Phase II testing. Little is known about mechanisms of resistance to this agent. In the present study, we have characterized an ovarian carcinoma cell line [OV202 high passage (hp)] that spontaneously developed drug resistance upon prolonged passage in tissue culture. Standard cytogenetic analysis and spectral karyotyping revealed that OV202 hp and the parental low passage line OV202 shared several marker chromosomes, confirming the relatedness of these cell lines. Immunoblotting demonstrated that OV202 and OV202 hp contained similar levels of a variety of polypeptides involved in cell cycle regulation, including cyclin-dependent kinases 2 and 4; cyclins A, D1, and E; and proliferating cell nuclear antigen. Despite these similarities, OV202 hp was resistant to flavopiridol and cisplatin, with increases of 5- and 3-fold, respectively, in the mean drug concentrations required to inhibit colony formation by 90%. In contrast, OV202 hp and cisplatin, paclitaxel, topotecan, 1,3-bis(2-chloroethyl)-1-nitrosourea, etoposide, doxorubicin, vincristine, and 5-fluourouracil, respectively, in the mean drug concentrations required to confirm the relatedness of these cell lines. Immunoblotting demonstrated that OV202 and OV202 hp contained similar levels of a variety of polypeptides involved in cell cycle regulation, including cyclin-dependent kinases 2 and 4; cyclins A, D1, and E; and proliferating cell nuclear antigen. Despite these similarities, OV202 hp was resistant to flavopiridol and cisplatin, with increases of 5- and 3-fold, respectively, in the mean drug concentrations required to inhibit colony formation by 90%. In contrast, OV202 hp and cisplatin, paclitaxel, topotecan, 1,3-bis(2-chloroethyl)-1-nitrosourea, etoposide, doxorubicin, vincristine, and 5-fluourouracil, suggesting that the spontaneously acquired resistance was not attributable to altered P-glycoprotein levels or a general failure to engage the cell death machinery. After incubation with cisplatin, whole cell platinum and platinum-DNA adducts measured using mass spectrometry were lower in OV202 hp cells than OV202 cells. Similarly, after flavopiridol exposure, whole cell flavopiridol concentrations measured by a newly developed high performance liquid chromatography assay were lower in OV202 hp cells. These data are consistent with the hypothesis that acquisition of spontaneous resistance to flavopiridol and cisplatin in OV202 hp cells is due, at least in part, to reduced accumulation of the respective drugs. These observations not only provide the first characterization of a flavopiridol-resistant cell line but also raise the possibility that alterations in drug accumulation might be important in determining sensitivity to this agent.

INTRODUCTION

Flavopiridol is the first CDK3 inhibitor to undergo widespread clinical testing. Preclinical studies have demonstrated the ability of this agent to inhibit CDKs 1, 2, 4, and 7 (1–5), presumably as a result of competing for the ATP binding sites of these kinases (2, 6). Consistent with these effects, flavopiridol has been observed to induce cell cycle arrest in breast and lung cancer cells in vitro (1, 3, 7, 8). Although flavopiridol was initially considered to be strictly cytostatic, additional studies have demonstrated that this agent kills cycling and noncycling cells (9–11). Flavopiridol-induced apoptosis has been demonstrated in several human cell lines (9, 12–15), as well as chronic lymphocytic leukemia cells in vitro (16). Moreover, flavopiridol-induced apoptosis has been observed in nude mice bearing human leukemia cells (11) or head and neck squamous cell carcinoma xenografts (17). Based upon promising antitumor activity in a number of xenograft models (11, 18, 19), as well as its unique mechanism of action, Phase I testing of flavopiridol as a 72-h infusion (20) or a 24 h infusion has been initiated. Phase II trials using the 72-h schedule are currently in progress in a number of different malignancies (21).

Additional studies have demonstrated that flavopiridol can dramatically enhance the cytotoxicity of other antineoplastic agents in vitro, particularly when administered as the second agent in the sequence (8, 14). In the clinical setting, administration of paclitaxel followed by flavopiridol has resulted in responses in paclitaxel-refractory tumors. Collectively, these observations raise the possibility that flavopiridol might have...
important modulating properties in addition to its intrinsic antineoplastic activity. Additional clinical studies of flavopiridol-containing combinations are planned or in progress.

The failure of some patients in Phase I and Phase II trials to respond to flavopiridol suggests that resistance to this agent will be observed in the clinical setting. Despite the widespread clinical interest in flavopiridol, little is presently known about potential mechanisms of resistance to this agent. In contrast, resistance to other anticancer drugs has been widely studied. Cellular resistance to cisplatin, for example, can arise as a consequence of diminished cellular uptake, enhanced cellular export, accelerated detoxification by reaction with glutathione or metallothioneins, increased removal of platinum-DNA adducts by the nucleotide excision repair machinery, or diminished mismatch repair (reviewed in Refs. 22–25).

The present report describes a primary ovarian epithelial carcinoma cell line that developed spontaneous resistance to both flavopiridol and cisplatin after prolonged culture in vitro. In an effort to determine the mechanism(s) contributing to this resistance, a recently developed technique for measuring platinum-DNA adducts by mass spectrometry and a newly developed method for measuring flavopiridol by HPLC were applied to the sensitive low passage line (OV202) and its less sensitive counterpart (OV202 hp) after drug treatment. To our knowledge, this is the first characterization of a flavopiridol-resistant cell line.

MATERIALS AND METHODS

Materials. Flavopiridol and BCNU were provided by the Pharmaceuticals Resources Branch of the National Cancer Institute (Bethesda, MD). Topotecan was kindly provided by Dr. Randall K. Johnson (SmithKline Beecham, King of Prussia, PA). Oxaliplatin from Sanofi Research (Malvern, PA) was provided by Dr. Charles Erlichman (Mayo Clinic). Cytarabine, 5-fluorouracil, etoposide, paclitaxel, doxorubicin, and cisplatin were purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies to cyclin A, cyclin E, cdc2/CDK1, CDK2, and CDK4 (26) were kindly provided by Dr. Robert Abraham (Duke University, Durham, NC). Antibodies to the following polypeptides were purchased from the indicated suppliers: cyclins B, D1, and D3 from Transduction Laboratories (Lexington, KY); cyclin D1 from Santa Cruz Biotechnology (Santa Cruz, CA); and Rb from PharMingen (San Diego, CA). All other reagents were obtained as described previously (8, 27).

Stock (1000-fold concentrated) solutions of flavopiridol, cytarabine, paclitaxel, topotecan, doxorubicin, etoposide, and 5-fluorouracil were prepared in DMSO and stored at −20°C prior to use. Cisplatin, oxaliplatin and BCNU were prepared immediately before use as 1000-fold concentrated solutions in DMSO. The absorption spectrum of flavopiridol was determined using a Beckman (Palo Alto, CA) DU7400 diode array spectrophotometer using 100 μl sample cells and 1% aqueous triethylamine (pH 5.0) as a “blank.”

Tissue Culture and Colony-forming Assays. OV202 and OV202 hp cells were cultured in MEM with Earle’s salts and nonessential amino acids containing 20% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 2 mM glutamine (medium A). Cells were passaged once weekly and maintained at 37°C in an atmosphere containing 95% air-5% CO2 (v/v). To determine population doubling times, 1 × 105 cells were seeded in replicate 60-mm tissue culture plates, incubated for 24–96 h, trypsinized, and counted on a hemacytometer.

Colonies-forming assays were performed as described previously (9). In brief, subconfluent cells were released with trypsin, plated at a density of 3000 cells/plate in multiple 35-mm dishes containing 2 ml of medium A and incubated for 14–16 h at 37°C to allow cells to attach. Graded concentrations of each drug or equivalent volumes of DMSO (0.1%) were then added to triplicate plates. After a 24-h treatment, plates were washed twice with serum-free MEM and incubated in drug-free medium A for an additional 14 days. Alternatively, flavopiridol was added on the day after plating and left in the culture. In either case, the resulting colonies were stained with Coomassie Blue and counted. Diluent-treated control plates typically contained 175–225 colonies.

Cytogenetic Studies. Routine cytogenetic analyses were performed using in situ culture and robotic harvesting as described previously (28). Spectral karyotype analysis of previously G-banded metaphase preparations was performed by the method of Veldman et al. (29) using reagents, instrumentation, and software purchased from Applied Spectral Imaging (Carlsbad, CA).

Immunoblotting. OV202 and OV202 hp cells grown to 50% confluence in triplicate 100-mm tissue culture dishes were washed four times in situ with 10 ml volumes of calcium- and magnesium-free PBS and solubilized in alkylating buffer [6 M guanidine hydrochloride, 250 mM Tris-HCl (pH 8.5 at 21°C), and 10 mM EDTA supplemented immediately before use with 150 mM [β-mercaptoethanol and 1 mM α-phenylmethylsulfonyl fluoride]. Samples were then processed for SDS-PAGE and subsequent immunoblotting using techniques described in detail previously (27).

Flow Cytometry. OV202 and OV202 hp cells were grown to 30–40% confluence in 100-mm tissue culture dishes, treated with the indicated concentrations of flavopiridol, released by trypsinization, and sedimented at 200 × g for 5 min. Samples were then washed in PBS at 4°C, fixed in 50% ethanol, digested with RNase A, and stained with propidium iodide as described (8). Flow cytometry was performed on a Becton Dickinson FACScan (San Jose, CA) using an excitation wavelength of 488 nm and an emission wavelength of 585 nm. Histograms were analyzed using ModFit software (Verity Software House, Topsham, ME).

Assessment of Cellular Platinum Accumulation and Platinum-DNA Adducts. For the assessment of whole cell platinum accumulation, OV202 or OV202 hp cells grown to 50% confluence on quadruplicate 100-mm tissue culture dishes were treated with cisplatin or oxaliplatin (added from freshly prepared 1000-fold concentrated stocks). After incubation for the indicated period of time, cells were washed in situ four times with ice-cold PBS and solubilized by direct addition of 3 ml of 70% nitric acid to the first of each set of four plates followed by serial transfer of this solution to each additional plate in each set to solubilize the remaining cells. After a 24 h incubation at 20–22°C, aliquots were removed for estimation of protein and elemental platinum. To assess protein content, samples were

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diluted 1:2 or 1:4 in nitric acid and assayed for absorbance at 358 nm. Results were compared to absorbance of known amounts of BSA in the same diluent (30). To assess platinum content, aliquots containing 1 ml of nitric acid extract were heated to 100°C for 5 min, combined with 1 ml of 30% H₂O₂, heated to 100°C for 5 min, and subjected to mass spectrometry as described below.

For the assessment of platinum-DNA adducts, OV202 or OV202 hp cells grown to 50–80% confluence on four to six 100-mm tissue culture dishes were treated with cisplatin or oxaliplatin as described above. At the completion of the incubation, cells were scraped from the plates without removal of drug-containing medium, sedimented at 200 × g for 10 min, washed four times with 10-ml volumes of ice-cold PBS, and lysed in 5 ml of TEN buffer [10 mM Tris-HCl (pH 7.4 at 21°C), 10 mM EDTA, 150 mM NaCl] containing 0.4% SDS and 1 mg/ml proteinase K. After proteinase K digestion, highly purified DNA was isolated by extraction with phenol/chloroform and chloroform, ethanol precipitation, digestion with RNase A, and extraction with phenol/chloroform and chloroform as described previously (31).

Elemental platinum in cell lysates and purified DNA was determined by inductively coupled plasma mass spectrometry using a Perkin-Elmer Sciex Elan 6000 mass spectrometer (Norwalk, CT) as recently described in detail (32). Platinum species were determined at 194 and 195 a.m.u. 50 times/reading. Platinum standards at 194 and 195 a.m.u. were summed using a program that generated results were used in all calculations. This approach was 10–100-fold more sensitive than the atomic absorption spectroscopy method used previously (31).

Assessment of Intracellular Flavopiridol Concentrations. To measure cellular flavopiridol concentrations, flavopiridol was released from washed cells by PCA treatment, extracted into chloroform, and subjected to HPLC. Briefly, cells grown to 50% confluence in 50–100 10-cm tissue culture dishes were treated with 9 μM flavopiridol for 24 h. At this time point, >98% of cells remained adherent and excluded trypan blue, consistent with the delayed death reported previously in solid tumor cells treated with flavopiridol (9). After treatment, cells were washed rapidly in situ four times with 3-ml volumes of ice-cold PBS and scraped into 3 ml of 0.5 mM PCA. The PCA extracts were combined on ice, sonicated, and sedimented at 200 × g for 10 min to remove macromolecules. The resulting supernatant (~150 ml) was extracted four times with 25-ml volumes of chloroform. The PCA supernatant was then neutralized to pH 7.4 with 0.5 volumes of 0.33 M phosphate buffer (pH 7.4) that was supplemented with 1 M KOH (31). After sedimentation to remove precipitated potassium perchlorate, the supernatant was extracted again four times with 25-ml volumes of chloroform.

The pooled chloroform extracts were evaporated to dryness under anhydrous nitrogen. The resulting residue was solubilized in 100 μl of 1% (v/v) aqueous triethylamine adjusted to pH 5.0 with acetic acid (mobile phase A), a solvent chosen because of low flavopiridol solubility at higher pH. After centrifugation at 200 × g for 10 min to remove insoluble material, 60 μl of the resulting supernatant were subjected to HPLC analysis using a Beckman 125 dual pump gradient system equipped with 507e autosampler, 168 diode array detector, and IBM 350 personal computer with Beckman Gold Nouveau software. A Brownlee MPLC Newguard C18 precolumn (3.2 mm × 15 mm × 7 μm) and a Beckman Ultrasphere ODS column (4.6 mm × 250 mm × 5 μm) preequilibrated for at least 30 min with 80% mobile phase A and 20% methanol were used for all analyses. Separation was accomplished using a flow rate of 2 ml/min and the following elution gradient: 0–10 min of 80% mobile phase A, 20% methanol; 10–20 min of linear ramp to 20% mobile phase A, 80% methanol; 20–25 min of linear ramp to 100% methanol; 25–35 min of 100% methanol.

As indicated below, the retention time of flavopiridol used in this system was 18.6 min. Peak areas were measured at 311 nm, a λmax of flavopiridol in mobile phase A. Detection at 311 nm allowed baseline chromatographic resolution in comparison to using detection at 254 nm or other wavelengths. Standard curves of peak area at 311 nm versus flavopiridol concentration generated with each experiment by assay of multiple concentrations of authentic flavopiridol dissolved in mobile phase A uniformly gave linear correlation coefficients >0.99. To provide a basis for comparison of the flavopiridol contents, protein concentrations were determined by solubilizing the PCA pellets in 6 ml of 70% nitric acid, incubating for 24 h at 20–22°C, and comparing the absorbance of the resulting solutions at 358 nm with that of known amounts of BSA solubilized under the same conditions (30).

Statistics. Differences between cell lines were assessed using two-sided t tests and pooled estimates of variance.

RESULTS

Characterization of OV202 and OV202 hp Cell Lines. During the course of studies on cisplatin sensitivity in a series of recently described primary ovarian carcinoma lines (33), we observed that one of the lines spontaneously developed resistance to flavopiridol and cisplatin upon prolonged passage. The present studies were designed to elucidate the mechanisms of this resistance.

In view of the recent determination that MCF-7/AdR6 cells were probably not derived from MCF-7 cells (34), we first examined the relationship between the parental and hp cells. Cytogenetic analysis of the parental OV202 cells (studied in passage 24) and the highly passaged derivative OV202 hp (studied at passages 100–120) showed that the two lines had multiple chromosomal anomalies, including several marker chromosomes that were shared between the two lines (G-banded karyotypes not shown). Because the markers were difficult to accurately characterize by routine cytogenetic analysis, we also performed spectral karyotyping on metaphase spreads. Table 1

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This drug concentration was chosen to facilitate more precise quantitation of intracellular flavopiridol content despite the relatively low extinction coefficient of the flavopiridol chromophore.
summarizes the results of these analyses. The two lines shared several abnormalities, confirming their relationship to each other. For example, both cell lines contained a chromosome 20 derivative with a HSR derived from chromosome 19. Fluorescence in situ hybridization analysis demonstrated that this HSR contains multiple copies of the *AKT2* gene (data not shown), an oncogene that is sometimes amplified in ovarian cancer (35). In addition, both lines shared additional marker chromosomes derived by translocations involving chromosomes X and 9, 1 and X, 1 and 13, 2 and 6, 3 and 5, 3 and 6, 5 and 21, 4 and 11, 9 and 12, 3 and 13, and 5 and 18. Despite these shared marker chromosomes, several differences were also observed. OV202 hp, for example, contained an unbalanced 3;11 translocation and two different types of chromosome 5 deletions that were not observed in OV202. Additional differences are summarized in Table 1.

Analysis of the growth characteristics of these cell lines revealed that OV202 hp cells proliferated more rapidly, with a mean population doubling time about half that of OV202 cells (45.6 ± 14.14 versus 86 ± 10 h; *P* < 0.05). At confluence, OV202 cells formed a uniform monolayer, whereas OV202 hp cells piled up, leading to a 50% higher cell density for OV202 hp at confluence. The plating efficiencies of the two lines were essentially the same (OV202, 5.4 ± 0.2%; OV202 hp, 6.3 ± 1.1%; *P* > 0.2).

When cell cycle regulatory proteins were examined by immunoblotting (Fig. 1A), levels of cyclin A, cyclin E, CDK2, CDK4, and proliferating cell nuclear antigen were indistinguishable in the OV202 and OV202 hp cells. Cyclin D1, which was at the limit of detection with two separate antibodies, likewise appeared to be equally expressed in the two cell lines. In contrast, levels of cyclin D1, cdc2/CDK1 and Rb were approx-

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**Table 1** Comparison of cytogenetic abnormalities in OV202 and OV202 hp

<table>
<thead>
<tr>
<th>Abnormalities present only in parental line</th>
<th>Abnormalities present in both parental and high pass lines</th>
<th>Abnormalities present only in high pass line</th>
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<td>64–79 chromosomes</td>
<td>–X</td>
<td>63–68 chromosomes</td>
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<td><em>a</em></td>
<td>der(X)(X;9)(q13;q11)</td>
<td><em>b</em></td>
</tr>
<tr>
<td><em>a</em></td>
<td>der(1)(X;1)t(p13q15) dup(1)(q11q25) dup(1) (q11q21)(q11q22)</td>
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</tr>
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<td><em>a</em></td>
<td>der(1)(1;13)(q11q21.2)</td>
<td><em>b</em></td>
</tr>
<tr>
<td><em>a</em></td>
<td>der(2)(2;q)(q13;?)</td>
<td><em>b</em></td>
</tr>
<tr>
<td>del(2)(q31)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>der(3)(3;13)(p13q22)</td>
<td>der(3)(3;13)(p13q22)</td>
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</tr>
<tr>
<td>-4, -4</td>
<td>del(5)(5q21)</td>
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<tr>
<td></td>
<td>del(6)(q15q21)</td>
<td><em>b</em></td>
</tr>
<tr>
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<td>der(11)(4;11)(q25p15 del(11)(q11)</td>
<td><em>a</em></td>
</tr>
<tr>
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<td>der(11)(4;11)(q12q13)</td>
<td><em>a</em></td>
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<tr>
<td></td>
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<tr>
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<tr>
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<tr>
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<td>der(22)(12;22)(p11.2;14;22)</td>
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<td></td>
<td>(q11.2q13)</td>
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* Two copies of the abnormality in the center column were present.

a One copy of the abnormality in the center column was present.
response to flavopiridol, OV202 hp cells did not (Fig. 1 and quotations of whole cell lysates containing 50 (lanes 1 and 4), 25 (lanes 2 and 5), or 12.5 (lanes 3 and 6) $\mu$g of protein were subjected to SDS-PAGE followed by immunoblotting using antibodies that recognize the indicated polypeptides. Immunoblots are representative of three independent experiments. B, cell cycle distributions of untreated and flavopiridol-treated OV202 and OV202 hp cells. Cells grown to 30% confluence were treated for 24 h with the indicated concentration of flavopiridol and harvested for flow cytometry. Data shown are representative of three independent experiments.

Fig. 1 Comparison of OV202 and OV202 hp cells. A, immunoblot analyses of various polypeptides in OV202 and OV202 hp cells. Aliquots of whole cell lysates containing 50 (lanes 1 and 4), 25 (lanes 2 and 5), or 12.5 (lanes 3 and 6) $\mu$g of protein were subjected to SDS-PAGE followed by immunoblotting using antibodies that recognize the indicated polypeptides. Immunoblots are representative of three independent experiments. B, cell cycle distributions of untreated and flavopiridol-treated OV202 and OV202 hp cells. Cells grown to 30% confluence were treated for 24 h with the indicated concentration of flavopiridol and harvested for flow cytometry. Data shown are representative of three independent experiments.

Sensitivity of OV202 and OV202 hp to Chemotherapeutic Agents. The sensitivity of OV202 and OV202 hp cells to various chemotherapeutic agents was further assessed using colony-forming assays. After a 24 h flavopiridol treatment, OV202 and OV202 hp cells displayed IC$_{50}$ of 220 ± 23 and 1100 ± 120 nM, respectively, indicating a 5-fold decrease in flavopiridol sensitivity in OV202 hp cells (Fig. 2A; $P < 0.001$). When cells were instead exposed to flavopiridol continuously, OV202 and OV202 hp displayed IC$_{50}$ of 71 ± 10 and 145 ± 23 nM, respectively ($P < 0.02$; Fig. 2B). Additional experiments demonstrated that the diminished sensitivity was also observed when cell death was directly measured by determining uptake of the nonpermeable dye trypan blue or by assessing the area of the sub-diploid peak by FACS analysis at various times after a 24-h flavopiridol exposure. Likewise, after a 24-h cisplatin treatment, OV202 and OV202 hp cells displayed IC$_{50}$ of 850 ± 50 and 2900 ± 100 nM, respectively, in colony-forming assays, indicating a 3-fold decrease in cisplatin sensitivity in OV202 hp cells (Fig. 2C; $P < 0.001$). In contrast, OV202 and OV202 hp cells exhibited indistinguishable sensitivities to oxaliplatin, paclitaxel, topotecan, BCNU, 5-fluorouracil, doxorubicin, etoposide, and cytarabine (Fig. 2, D–F, and data not shown). As discussed below, this pattern of selective resistance cannot be explained by up-regulation of P-glycoprotein, enhanced nucleotide excision repair, or a general failure of OV202 hp cells to engage the cell death machinery. These considerations prompted us to search for drug-specific factors that might account for the resistance.

Whole-cell Platinum Accumulation and Platinum-DNA Adducts after Treatment with Cisplatin or Oxaliplatin. To evaluate the hypothesis that cisplatin resistance in OV202 hp cells results from decreased cisplatin-induced DNA damage, covalently bound platinum was measured by mass spectroscopy after purification of DNA from OV202 and OV202 hp cells exposed to cisplatin for 24 h, the same incubation time used in the colony-forming assays. Cisplatin treatment of OV202 hp cells consistently produced fewer platinum-DNA adducts in comparison to OV202 cells (120 ± 20 versus 200 ± 20 pg of platinum/$\mu$g of DNA; $P < 0.01$; Fig. 3A). Additional experiments involving cisplatin exposure followed by incubation in drug-free medium indicated that the rates of removal of platinum-DNA adducts in the two lines were similar, with persistence of ~75% of the covalently bound platinum in both cell lines 48 h after cisplatin removal.

To determine whether the lower levels of platinum-DNA adducts in cisplatin-treated OV202 hp cells might result from decreased drug accumulation, the platinum content of whole cell lysates was measured after a 24-h cisplatin treatment. Total cellular platinum content was consistently lower in OV202 hp cells than in parental cells (Fig. 3B; 140 ± 20 versus 230 ± 50 pg of platinum/$\mu$g of protein; $P < 0.02$), suggesting that reduced cisplatin accumulation in OV202 hp cells might contribute to their resistance. This decreased accumulation of cisplatin by OV202 hp cells was evident at drug concentrations as low as approximately 2-fold higher in the OV202 hp cells, whereas cyclin B was approximately 2-fold lower.

Despite the differences in population doubling time and certain cell cycle regulatory proteins, log phase OV202 and OV202 hp cells had similar cell cycle distributions, as assessed by flow cytometry (Fig. 1B). However, after exposure to flavopiridol for 24 h, a treatment designed to mimic one of the flavopiridol administration schedules currently being evaluated clinically, the two lines differed (Fig. 1B). Whereas OV202 cells demonstrated a dose-dependent increase in G2-M population in response to flavopiridol, OV202 hp cells did not (Fig. 1B). These observations raised the possibility that OV202 hp cells might be resistant to the effects of flavopiridol.

K. C. Bible, unpublished observations.
4 μM and at exposure times much shorter than 24 h (see below). In contrast, the two cell lines did not exhibit any difference in platinum-DNA adducts after treatment with oxaliplatin (Fig. 3C; 82 ± 3 versus 86 ± 8 pg of platinum/μg of DNA; P > 0.5). This latter result is consistent with the equal sensitivities of the cell lines to oxaliplatin in clonogenic assays (Fig. 2D) and suggests that the mechanism of resistance to cisplatin does not convey resistance to all agents that form platinum-DNA adducts.

To begin to investigate potential explanations for diminished cisplatin accumulation in OV202 hp cells, the kinetics of cisplatin uptake (Fig. 3D) and efflux (Fig. 3E) were examined. After cells were loaded with cisplatin, the rate of efflux was lower in the OV202 hp cell line (Fig. 3E), arguing against the possibility that overexpression of an efflux pump was responsible for the observed differences in drug accumulation. On the other hand, the amount of drug accumulated at each time point after drug addition was lower in the OV202 hp line than in the OV202 line (Fig. 3D). Collectively, these results suggest that the rate of cisplatin transport into the OV202 hp cells is lower than into OV202 cells.

**Cellular Flavopiridol Concentrations in OV202 and OV202 hp.** To evaluate the possibility that flavopiridol resistance in OV202 hp cells might result from decreased cellular drug accumulation, flavopiridol content was assessed in OV202 and OV202 hp cells exposed to flavopiridol. For these experiments, we developed a new HPLC assay for flavopiridol. Based upon the observation that flavopiridol is much less soluble in aqueous buffers at neutral or alkaline pH, cells were lysed in PCA. Flavopiridol in these lysates was extracted into chloroform, concentrated, and subjected to reverse phase HPLC under slightly acidic conditions (Fig. 4A). Absorbance of the flavopiridol chromophore at 311 nm (Fig. 4B) was used for detection. These conditions yielded good baseline separation of flavopiridol from the few other peaks that were present in the extracts (Fig. 4A) and permitted generation of linear standard curves with correlation coefficients uniformly >0.99.

Application of this assay (Fig. 4C) revealed that OV202 hp cells exposed to flavopiridol for 24 h consistently contained lower cellular flavopiridol concentrations than OV202 cells subjected to identical treatment. The mean flavopiridol concentration in OV202 hp cells was 49 ± 21% that in OV202 cells. These data suggest that flavopiridol resistance in OV202 hp cells, like cisplatin resistance, reflects decreased cellular drug accumulation.

**Effect of Flavopiridol Pretreatment on Cisplatin Accumulation.** To assess the possibility that flavopiridol and cisplatin might compete for the same transporter in OV202 cells, cells were exposed to cisplatin in the absence or presence of flavopiridol (Fig. 5). At molar excesses of up to 4-fold, flavopiridol consistently increased rather than decreased whole cell platinum accumulation. These data do not provide any evidence for transport of flavopiridol and cisplatin by a single transporter, but they do provide a potential explanation for the previously described synergy between flavopiridol and cisplatin (8) as discussed below.

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7 K. C. Bible and S. A. Boerner, unpublished observations.
DISCUSSION

In the present study, we have characterized a low passage human ovarian carcinoma line that spontaneously developed resistance to flavopiridol and cisplatin upon prolonged culture in vitro. Comparison of the OV202 and OV202 hp lines revealed that resistance was relatively selective for these two agents. Additional experiments demonstrated diminished levels of cisplatin and flavopiridol in the resistant line. Finally, simultaneous incubation of cells with flavopiridol and cisplatin was observed to enhance rather than diminish cisplatin uptake. Each of these observations has potentially important implications.

Before embarking on the biochemical analysis of drug resistance in this model system, we confirmed that the OV202 hp cells were derived from the OV202 cell line. The recent determination that MCF-7/AdrR cells are not derived from MCF-7 cells (34) highlighted the need to evaluate this relationship in the current cell lines. Routine cytogenetic analysis indicated that OV202 and OV202 hp cells are related but could not accurately identify several of the karyotypic anomalies. Spectral karyotyping analysis, which seemed ideally suited for this application (reviewed in Ref. 36), allowed us to partially characterize the chromosomal composition of the marker chromosomes. In addition, spectral karyotyping clearly demonstrated that several unique anomalies were shared by the two cell lines, including a HSR composed of chromosome 19 material that was shown to contain multiple copies of the AKT2 gene by subsequent fluorescence in situ hybridization analysis. Despite these similarities, several anomalies were unique to OV202 or OV202 hp, suggesting that chromosomal instability is a prominent feature of this particular tumor. It is possible that this instability underlies the acquisition of drug resistance during prolonged passage.

The pattern of drug resistance observed in the OV202 hp cells strongly argues against several common mechanisms of drug resistance. Despite diminished sensitivity to cisplatin and flavopiridol, OV202 hp cells are just as sensitive as parental cells to paclitaxel, etoposide, doxorubicin, and topotecan, four agents that are exported by P-glycoprotein or the multidrug resistance-associated protein MRP. These results are difficult to reconcile with a model in which resistance of OV202 hp cells to flavopiridol is mediated by P-glycoprotein or MRP. These results do not, of course, rule out the possibility that MRP might contribute to flavopiridol resistance in other cells, as suggested by Hooijberg et al. (37). Likewise, although DNA repair capability was not directly measured in nuclear lysates from the two cell lines, the observation that OV202 and OV202 hp lines display similar sensitivities to a variety of DNA damaging...
Resistance to Cisplatin and Flavopiridol

Resistance to cisplatin and flavopiridol is a significant concern in cancer treatment. Recent studies have suggested that the diminution of drug accumulation might remain a possibility that tumors with diminished sensitivity to cisplatin as a consequence of diminished drug accumulation might remain sensitive to oxaliplatin. Additional experiments were performed to examine the possibility that drug-specific factors might contribute to the observed resistance. Mass spectrometry of platinum recovered in highly purified DNA preparations revealed that OV202 hp cells contained fewer platinum-DNA adducts than OV202 cells. Pulse-chase experiments described above indicated that the rates of removal of platinum-DNA adducts from OV202 and OV202 hp cells were low but indistinguishable. In contrast, decreased cisplatin accumulation was observed in the OV202 hp cells. Examination of the kinetics of cisplatin uptake and efflux suggested that the OV202 hp cells had diminished uptake. Although diminished cisplatin accumulation has been observed in many cell lines selected by drug exposure (reviewed in Refs. 22 and 23), the OV202 hp cells are interesting because their cisplatin resistance arose spontaneously upon prolonged passage in vitro. It is not currently known whether a similar selection for spontaneous cisplatin resistance occurs upon prolonged passage in vivo.

Interestingly, the OV202 hp cells did not demonstrate any alteration in oxaliplatin accumulation or sensitivity. This observation is consistent with previous preclinical and clinical studies, suggesting the relative lack of cross-resistance of oxaliplatin and cisplatin (38–40). In particular, our observations raise the possibility that tumors with diminished sensitivity to cisplatin as a consequence of diminished drug accumulation might remain sensitive to oxaliplatin.

An additional experiment was performed to evaluate the possibility that diminished flavopiridol accumulation might contribute to this cross-resistance. As was the case with cisplatin, flavopiridol levels were diminished in the OV202 hp cells. This decrease might reflect diminished uptake, enhanced efflux, or both. Because of the relatively low level of resistance in the hp line, as well as lack of availability of radiolabeled flavopiridol, experiments to distinguish between these alternatives were not feasible. Likewise, the low level of resistance appears to preclude molecular genetic approaches to identification of the transporter involved in this resistance.

Additional experiments indicated that excess flavopiridol does not inhibit cisplatin accumulation, arguing against the possibility that flavopiridol and cisplatin share a common transporter. Subsequent studies also demonstrated that dipyridamol and 6-aminonicotinamide, two agents that enhance cisplatin accumulation and sensitivity in a variety of tissue culture cell lines (31, 41), have no effect on the flavopiridol sensitivity of OV202 hp cells. Collectively, these observations suggest that the diminished accumulation of cisplatin and flavopiridol might represent alterations in two distinct transport mechanisms.

Despite the lack of cross-resistance to a variety of other agents, OV202 hp cells were resistant to flavopiridol. Development of a HPLC-based assay allowed us to evaluate the possibility that diminished flavopiridol accumulation might contribute to this cross-resistance. As was the case with cisplatin, flavopiridol levels were diminished in the OV202 hp cells. This decrease might reflect diminished uptake, enhanced efflux, or both. Because of the relatively low level of resistance in the hp line, as well as lack of availability of radiolabeled flavopiridol, experiments to distinguish between these alternatives were not feasible. Likewise, the low level of resistance appears to preclude molecular genetic approaches to identification of the transporter involved in this resistance.

Besides helping to rule out the possibility that flavopiridol and cisplatin share a single transporter, the data in Fig. 5 are noteworthy in a second context. Previous studies have demonstrated that most flavopiridol-containing two-drug combinations are more cytotoxic when administered sequentially, with flavopiridol as the second agent (8, 14). In contrast, the combination of cisplatin and flavopiridol also exhibits some degree of cytotoxic synergy when flavopiridol precedes or accompanies cisplatin (8). The present demonstration that flavopiridol enhances cisplatin accumulation (Fig. 4D) provides a potential explanation for the previously unexplained synergy observed when flavopiridol precedes or accompanies cisplatin.

The present demonstration that diminished flavopiridol accumulation contributes to flavopiridol resistance in the OV202 hp cells does not, of course, rule out the possibility of other mechanisms of resistance to this agent. Based on currently
available information on the mechanism of action of flavopiridol, alterations in levels of CDKs or other as-yet-unidentified target kinases, as well as mutations that affect binding of flavopiridol to these targets, might also be expected to alter flavopiridol sensitivity. In the present study, many cell cycle regulatory proteins, including cyclin A, cyclin D, cyclin E, Cdk2, Cdk4, and proliferating cell nuclear antigen were indistinguishable in the OV202 and OV202hp cell lines. In contrast, Cdc2 and Rb were approximately 2-fold higher in the flavopiridol-resistant line. Although these latter changes probably reflect the higher proliferation rate of the OV202 hp cells, we cannot rule out the possibility that the same alterations contribute in some way to resistance of the OV202 hp line as well. Study of additional flavopiridol-resistant cell lines, as well as clinical samples, is required to determine the role of altered cell cycle components and other mechanisms of resistance in model systems and in the clinical setting. The present study, however, suggests that alterations in flavopiridol accumulation should also be examined in these future studies.

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REFERENCES

Characterization of an Ovarian Carcinoma Cell Line Resistant to Cisplatin and Flavopiridol

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