Abstract

Purpose: We examined the efficacy of flavopiridol, a cyclin-dependent kinase inhibitor that is undergoing clinical trials, on primary cancer cells isolated from the ascites or pleural fluids of patients with metastatic cancers.

Experimental Design: Metastasized cancer cells were isolated from the pleural fluids (n = 20) or ascites (n = 15) of patients, most of whom were refractory to chemotherapy. These primary cancer cells were used within 2 weeks of isolation without selecting for proliferative capacities. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide viability assay was used to characterize the response of these cancer cells to commonly used chemotherapeutic agents, and their response to flavopiridol was compared with rapidly dividing cultured cell lines.

Results: The primary cancer cells displayed phenotypes that were different from established cell lines; they had very low replication rates, dividing every 1 to 2 weeks, and underwent replicative senescence within five passages. These primary tumor cells retained their resistance to chemotherapeutic drugs exhibited by the respective patients but did not show cross-resistance to other agents. However, these cancer cells showed sensitivity to flavopiridol with an average LD_{50} of 50 nmol/L (range, 21.5–69 nmol/L), similar to the LD_{50} in established cell lines. Because senescent cells also showed similar sensitivity to flavopiridol, it suggests that the mechanism of action is not dependent on the activity of cyclin-dependent kinases that regulate the progression of the cell cycle.

Conclusion: Using cancer cells isolated from the ascites or pleural fluids, this study shows the potential of flavopiridol against cancer cells that have developed resistance to conventional chemotherapeutic agents.

Flavopiridol is a synthetic flavone that has shown potential as an antineoplastic agent and is currently undergoing clinical trials for the treatment of cancer (1). Biochemical studies have shown that it is a potent inhibitor of cyclin-dependent kinases (cdks), with a LD_{50} of ~50 to 100 nmol/L, and can inhibit other kinases at higher concentrations (reviewed in ref. 2). Crystallography shows that flavopiridol binds to the ATP-binding domain of cdks. Because of its broad inhibition of cdks, flavopiridol can induce cell cycle arrest and affect other cellular functions, such as transcription and apoptosis (2–4).

Flavopiridol reduces transcriptional elongation through inhibition of cdk9 activity on RNA polymerase II, causing the reduction in expression of several genes, including those required for the cell cycle. Other effects of flavopiridol include inhibition of angiogenesis through the down-regulation of vascular endothelial growth factor and the induction of cellular differentiation (2). Because flavopiridol kills cells that are cycling as well as quiescent (5–7), the compound is most likely acting through a mechanism that does not involve the cdks that function to regulate cell cycle.

Experiments with flavopiridol on tumor xenografts in animal models showed activity against several solid tumors, and similar promising results were obtained in phase I clinical trials. However, in the phase II clinical trials that have been reported with flavopiridol thus far, the results have been mixed for its use as a single-agent treatment against solid tumors (8–10). Several in vitro experiments showed that flavopiridol may potentiate the effects of anticaner agents, and clinical trials are under way to determine its effectiveness in combination therapy (2, 10, 11).

To date, studies demonstrating the cytotoxicity of flavopiridol have mainly been done with established cell lines that divide rapidly. In this study, we report that primary cancer cells isolated from the ascites and pleural fluid of patients can be used to study sensitivity and resistance to antineoplastic agents. These primary cancer cells were isolated from patients with tumors of different origins, most of whom had developed resistance to the anticancer drugs used to treat the disease.
primary cancer cells showed sensitivity to flavopiridol with LD50 values of ~50 nmol/L, comparable with that seen with the MCF-7 human breast cancer cell line.

Materials and Methods

**Cell culture and medium.** Cell lines used in the study were the human breast cancer MCF-7, osteosarcoma Saos-2, prostate cancer DU-145, cervical cancer HeLa, and normal human diploid fibroblast HSF-55. These cells were grown in DMEM containing 10% fetal bovine serum and antibiotics (100 units/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B) at 37°C in a 5% CO2 incubator. Primary cancer cells isolated from ascites or pleural fluid were cultured in the same medium, except that the dose of antibiotics was doubled.

**Isolation of cancer cells.** The process of obtaining consent from the patient was approved by the Thunder Bay Regional Health Sciences Centre Research Ethics Team (Thunder Bay, Ontario, Canada), and all required signed documents were obtained from patients before processing of patient samples. Tumor cells were isolated from the ascites of patients with ovarian cancer (n = 5), colon cancer (n = 2), breast cancer (n = 1), pancreatic cancer (n = 2), and one each of gastric cancer, mesothelioma, non-Hodgkin’s lymphoma, cholangiocarcinoma, and adenocarcinoma. Tumor cells were also isolated from the pleural fluid of patients with lung cancer (n = 8), ovarian cancer (n = 3), breast cancer (n = 3), colon cancer (n = 2), and one each of mesothelioma, renal cancer, hepatocarcinoma, and non-Hodgkin’s lymphoma. Table 1 lists all the cells that were isolated for use in the study. Ascites or pleural fluids drained from patients with metastatic cancers were transferred to 50 mL Falcon tubes and centrifuged at 2,500 rpm for 10 minutes to pellet the cells. The cells were resuspended in PBS, pooled, and recentrifuged to pellet the cells. Erythrocytes in the pellet were lysed by mixing each 5 mL of the ascites suspension with 40 mL of erythrocyte lysis buffer (155 mmol/L NH4Cl, 10 mmol/L NH4HCO3, 100 µmol/L EDTA) and incubating at room temperature for 30 minutes. After centrifuging at 2,500 rpm for 10 minutes, the final cell pellets were washed once with PBS, pooled, resuspended in regular growth medium, transferred to 150 mm tissue culture dishes, and cultured at 37°C with 5% CO2. The growth medium was changed every 2 or 3 days to remove cellular debris and any residual blood cells. After 2 weeks, the cells that remained adhered to the dish were trypsinized, counted with a Coulter cell counter (Mississauga, ON, Canada), and plated out for drug sensitivity studies. The yield of cancer cells from the ascites or pleural fluids varied between patients but typically ranged between 2 x 10^6 and 1 x 10^7 cells from 2 to 4 liters of fluid.

**Bromodeoxyuridine incorporation.** Incorporation of bromodeoxyuridine (BrdUrd) into DNA was used to correlate the proliferative capacity of the cells with drug sensitivity. The cells were seeded onto 18 mm cover glasses (Fisher, Ottawa, ON, Canada) and incubated with 30 µmol/L BrdUrd for 6 days before fixation with ice-cold methanol for 10 minutes. Immunostaining for BrdUrd was done according to the method described by MacDougall et al. (12). Briefly, following methanol fixation and one wash in PBS, the cells were incubated in 2 N HCl at 37°C for 20 minutes and neutralized in sodium borate/boric acid buffer. After a wash in PBS, mouse monoclonal anti-BrdUrd antibody (Sigma, Oakville, ON, Canada) was added at a dilution of 1:500 and incubated for 45 minutes at room temperature. This was followed by four 10-minute washes in PBS, and Cy3-conjugated anti-mouse antibody (Molecular Probes, Eugene, OR) was added at a dilution of 1:200 and incubated for a further 45 minutes. After four washes with PBS, the cells were counterstained with 1 µg/mL 4,6-diamidino-2-phenylindole, mounted onto a microscope slide in mounting medium (90% glycerol, 1 x PBS, containing 1 mg/mL p-phenylenediamine), and viewed with a Zeiss microscope (North York, ON, Canada) with epifluorescence. To score the percentage of replicating cells, two images of the cancer cells from different regions of each cover glass were taken using a CCD video camera (Sony, Toronto, ON, Canada), and the percentage of BrdUrd-labeled cells relative to the total number of 4’,6-diamidino-2-phenylindole–stained cells was determined for each image. Depending on whether the cells divided, each field of view typically contained between 80 and 100 cells, and the overall percentage of BrdUrd-labeled cells was averaged from the two fields.

**Summary of the cancer cells that were tested for sensitivity to flavopiridol**

<table>
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<tr>
<th>Aspites</th>
<th>Tissue</th>
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<th>Sex</th>
<th>LD50 (nM)</th>
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<td>Non-Hodgkin's Lymphoma</td>
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<td>M</td>
<td>52.9</td>
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</table>

**Table 1. Summary of the cancer cells that were tested for sensitivity to flavopiridol**

NOTE: LD50 values were taken from the graphs of MTT assays showing sensitivity of cancer cells to flavopiridol.
Canada), taxol (Bristol-Myers Squibb, Montréal, PQ, Canada), and Adriamycin (Pharmacia, Mississauga, ON, Canada) were then added at increasing concentrations and incubation continued for 3 days for cultured cells lines or 6 days for the primary cancer cells. Four wells were used for each treatment, and the average was used for LD50 determination. Measurement of viable cells remaining in each well was done using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method described by Carmichael et al. (13). MTT reagent was dissolved in PBS, added to each well to a concentration of 0.5 mg/mL, and incubated for 4 hours. The medium containing the MTT reagent was then aspirated, DMSO (100 μL) was added to each well, and the absorbance was measured in a microplate reader at a wavelength of 490 nm.

To measure the effect of exposure periods, the cells were seeded in the 96-well plates and incubated with varying concentrations of flavopiridol for up to 5 days for the MCF-7 cell line and up to 6 days for the A-Br27 breast cancer cells. For cells that were treated for less than the maximum number of days, the flavopiridol-containing medium was removed after incubation of the desired number of days and replaced with drug-free medium, and incubation was continued. MTT assays were done on the fifth day for the MCF-7 cells or the sixth day for the A-Br27 cells.

**Western blot analyses.** Cultured cell lines and primary cancer cells were incubated for 24 hours with 70 nmol/L flavopiridol and collected by trypsinization. Cells were lysed and extracts prepared for Western blot analysis as described in Derjuga et al. (14). Depending on the cells, total cellular protein (20-50 μg) was loaded in a 12% SDS-polyacrylamide gel and transferred onto a polyvinylidine difluoride membrane (Millipore, Billerica, MA) and immuno-blotted for bcl-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

### Results

**Growth characteristics of cells cultured from ascites and pleural fluids.** The cancer cells isolated from the ascites or pleural fluids of patients with metastatic cancers grew very slowly, taking 1 to 2 weeks to double, and would typically undergo no more than three to five passages before entering a quiescent state. With such low replication rates, we used incorporation of BrdUrd into DNA as indication of cell division rates. With freshly isolated cancer cells that were cycling, incubation with BrdUrd for 3 days labeled <20% of the cells and a 6-day incubation period was required to get labeling in ~80% of the cells (data not shown). With increased subcultures, the percentage of BrdUrd-positive cells decreased to <5% as they approached senescence.

**Sensitivity of cancer cells to flavopiridol.** Consistent with studies that have been done on established cell lines, we found that a 3-day treatment with 50 to 70 nmol/L flavopiridol killed ~50% of the cells in several cell lines (Fig. 1, top). When we extended the study to >40 isolates of primary cancer cells prepared from the ascites or the pleural fluid, the concentration of flavopiridol required to kill 50% of the cells in 6 days was found to be within an order of magnitude of that seen with cultured cell lines, ranging from ~20 nmol/L for a pancreatic cancer cell to ~60 nmol/L for ovarian cancer cells isolated from the pleural fluid (Fig. 1, middle). With another isolate of ovarian cancer cells from the ascites of a different patient, the LD50 was found to be ~20 nmol/L. These results show that the much reduced growth rates of the primary cancer cells did not affect their sensitivities to flavopiridol as was expected because of its specificity toward cdks. The results also showed that cancer cells isolated from the same disease site but from different patients could have a different LD50 for flavopiridol but remain within one-log value. As an example, we found the LD50 for eight ovarian cancer cells and eight lung cancer cells ranging between 20 and 60 nmol/L.

In our study of >40 isolates of cancer cells, three were found resistant to flavopiridol, displaying LD50 values that were >100 nmol/L (Fig. 1, bottom). One such cancer cell (A-Pan22) was from a patient with pancreatic cancer, with ~60% of the cells remaining viable after 6-day continuous incubation with 100 nmol/L flavopiridol. A similar level of

![Fig. 1. Sensitivity of cultured cell lines and cancer cells to flavopiridol. Cytoxic effects of flavopiridol on several cultured cell lines as measured by the MTT cell viability test (top). At concentrations >10 nmol/L flavopiridol (20 nmol/L for DU-145), the survival in all cells was significantly decreased compared with untreated controls using a one-tailed Student's t test (*P* < 0.05). Effects of flavopiridol on several of the cancer cells isolated from patients with metastatic cancers (middle). These cancer cells were isolated from the ascites of patients with pancreatic cancer (A-Pan45) or ovarian cancer (A-Ov40) or from the pleural fluid of a patient with ovarian cancer (P-Ov20). The decrease in survival in all treated cells was statistically significant (*P* < 0.05). Among the cancer cells studied, three isolates were found to be resistant to flavopiridol (bottom). These cancer cells were isolated from the ascites of patients with ovarian cancer (A-Ov15) or pancreatic cancer (A-Pan22) or from the pleural fluid of a patient with lung cancer (P-Lu20). Although there were significant declines in survival of A-Pan22 cells at all flavopiridol concentrations and of P-Lu20 cells when treated with higher concentrations of flavopiridol (*P* < 0.05), 75% of the cells were not killed and were resistant to the treatment.
resistance was seen with P-Lu20 cells isolated from a patient with lung cancer. A great level of resistance was seen with A-Ov15 ovarian cancer cells, where >90% of the cells remained viable after 6-day exposure to 100 nmol/L flavopiridol. When tested for cross-resistance, these cells also had increased tolerance to cisplatin and taxol, showing 3- to 5-fold increase in LD50 (data not shown).

Cell cycle–independent chemosensitivity of flavopiridol. Because flavopiridol has a high specificity toward cdk5, we compared its cytotoxicity with agents that are dependent on cell division, such as taxol and cisplatin, and correlated their respective effectiveness against growth rates as measured by BrdUrd uptake. As shown in Fig. 2, when ovarian cancer cells were cycling, as indicated by 76% of cells having incorporated BrdUrd, taxol and cisplatin were highly effective, killing the cells with LD50 of ~2 μmol/L. With subsequent passages, when the percentage of BrdUrd-labeled cells declined to ~50%, the LD50 increased to ~7 μmol/L for cisplatin and 9 μmol/L for taxol. When the cells reached senescence and did not take up BrdUrd, the ovarian cancer cells were resistant to both agents.

Unlike taxol and cisplatin, the MTT assays showed that noncycling senescent cancer cells were just as sensitive to flavopiridol as those that were cycling. As shown in Fig. 2 (bottom), flavopiridol was as effective in killing the dividing ovarian cancer cells that took up 76% BrdUrd as the noncycling ones that did not incorporate BrdUrd. This result suggests that flavopiridol is capable of inducing cell death through an alternate pathway that does not depend on the cell division and cdk activities.

Flavopiridol sensitivity and exposure period. To determine the extent of exposure that is required to kill cells, we incubated MCF-7 cells for periods of up to 5 days in varying concentrations of flavopiridol. As shown in Fig. 3 (top), a 1-day treatment of the rapidly cycling MCF-7 cells with the higher concentrations of 75 or 100 nmol/L flavopiridol was sufficient to kill >80% of the cells. At 50 nmol/L, a 5-day incubation killed ~30% of the cells, and the lowest concentration of 25 nmol/L did not result in significant killing. When we compared this with the A-Br27 breast cancer cells isolated from ascites fluid, these slower cycling primary cancer cells showed a wider variation in sensitivity to the exposure period of up to 6 days (bottom). At the lower concentrations of flavopiridol, these cells showed considerable sensitivity, with ~40% to 50% cell death at 25 nmol/L. At the higher concentration of 100 nmol/L, a 1-day treatment was not as effective as that seen with MCF-7 cells, killing ~70% of the cells. At least a 2-day exposure was required to kill ~90%. At the 50 and 75 nmol/L concentrations, increasing the number of days of exposure resulted in correspondingly greater percentages of cell death. Although the results showed that continuous exposure was not required to induce cell death, the extent of incubation period required is dependent on the cells and the concentrations used.

Effect of flavopiridol on chemoresistant cancer cells. The cancer cells used in this study were isolated from the ascites or the pleural fluids that were obtained from patients with tumors that were resistant to standard chemotherapy. The chemotherapy agents employed in this study were used only for some types of cancers, and we examined the records of the patients to determine the agents to which they had developed resistance. When compared with the results of the MT assays, it was found that the cancer cells did not have broad-ranged resistance to all the antineoplastic agents but were resistant only to the drugs that the patients had become refractory to. An example of this selective resistance is shown in the experiment described in Fig. 4. The cells (P-Hep35) were isolated from the pleural fluid of a patient with hepatocellular carcinoma who was treated with, and eventually became refractory to, Adriamycin (Fig. 4, top). The pleural fluid was drained a total of eight times and the cancer cells from earlier isolations showed sensitivity to Adriamycin. However, with subsequent removal of fluids from the patient, the hepatocarcinoma cells showed a gradual increase in resistance to Adriamycin. When tested against other agents, these cells were sensitive to the cytotoxic effects of carboplatin (middle) regardless of when the isolates were prepared. Similar sensitivity was also seen with flavopiridol (bottom).

Expression of bcl-2. The expression of antiapoptotic bcl-2 has been reported to be down-regulated by flavopiridol in
several cell lines (15–18), but this was not consistently seen in other cells (7). In examining the effects of flavopiridol on proteins that affect apoptosis, we saw a down-regulation of bcl-2 in several cell lines (Fig. 5, top). However, with the primary cancer cells, we did not see any correlation between sensitivity to flavopiridol and cellular levels of bcl-2. As shown in Fig. 5 (bottom), the pancreatic cancer cells A-Pan22 and A-Pan45 expressed different levels of bcl-2 that were not affected by the presence of flavopiridol. The MTT viability test showed that A-Pan22 was resistant to flavopiridol and A-Pan45 was sensitive (see Fig. 1). With the ovarian cancer P-Ov30, the expression level of bcl-2 was unaffected by flavopiridol, although these cells were sensitive (see Fig. 1), whereas the resistant ovarian cancer cell A-Ov15 down-regulated their expression of bcl-2 when incubated with flavopiridol.

Discussion

We describe the use of cancer cells isolated from ascites or pleural fluids for studying responses to anticancer agents that are currently in clinical use and to novel anticancer agents that are undergoing clinical trials. Cancer cells isolated from ascites and pleural fluid have been used to establish cell lines that retain the genotype and phenotype of the cancers of interest. These cells were of clonal origins and were selected for their increased proliferative capacity. In our study, we used the cancer cells directly, without selection for any growth advantages or clonal expansion of any particular subpopulations, and these relatively mixed population of cells should reflect more accurately the clinical situation. There are obvious differences between cultured cell lines and the tumors in a human body, although the biochemical pathways and responses to chemotherapy may be similar. One major difference between the tumor cell lines and the primary cancer cells isolated from ascites or pleural fluid that may influence response to cancer treatment is the growth rate. Cultured cell lines have much shorter doubling times, typically dividing every 18 to 24 hours. The higher metabolic activities would drive the cells to respond faster to anticancer agents. Furthermore, because every cell would traverse every phase of the cell cycle within a day, they would be susceptible to agents that affect S and G2-M phases. With the reduced growth rates and lower metabolism of the primary cancer cells, incubation periods have to be extended for cytotoxic
Flavopiridol have been done on established cell lines. Flavopiridol was the first specific inhibitor of CDKs to undergo clinical trials, and in vitro testing showed antineoplastic activity. However, clinical trials have yielded mixed results. Using cancer cells isolated from the fluids that were drained from patients, we show here that flavopiridol was cytotoxic to the cells that had become resistant to standard anticancer drugs. Flavopiridol was equally effective against actively cycling and nonproliferating cells, suggesting that the compound’s cytotoxic effects were not solely dependent on the inhibition of CDKs that regulate the cell cycle but might have affected CDKs that function in other cellular processes, including transcription (2–4). This observation is consistent with published results with other cell lines and indicates that flavopiridol can induce cell death by other pathways (reviewed in ref. 2). However, in clinical trials, flavopiridol treatments have not consistently resulted in antitumor effects measured by complete or partial response in cancer patients (1, 10). It is possible that rapid clearance from the body can limit flavopiridol from inducing any apoptotic pathways. In a recent study, it has been found that the clearance of flavopiridol can vary by up to 10-fold between patients (24). Our studies with the MCF-7 breast cancer cell line show that a 1-day exposure to 100 nmol/L flavopiridol was sufficient to kill more than 90% of the cells, and the absence of variance between the treatments could reflect the genetic homogeneity of the cultured cell line (Fig. 3). The result was more varied with the breast cancer cells taken from a patient, likely because these cancer cells were genetically heterogeneous as they had not undergone clonal selection and would show some variations in sensitivity to drug treatments. The results shown in Fig. 3 (bottom) indicate that a subpopulation of these cancer cells had greater sensitivity and were eliminated by 25 nmol/L flavopiridol, with the remaining cells displaying varied sensitivities that corresponded to exposure periods. This may have implications in clinical applications of chemotherapy, indicating that an inadequate treatment period with flavopiridol may kill the more sensitive cancer cells in the patient, selecting for the more resistant subpopulation of cancer cells. The results show that, even at the higher concentration of 100 nmol/L, a 1-day treatment would leave ~30% of cells and at least a 2-day treatment was required to kill >90% of the cells. Hence, the variable rate at which patients clear the drug from the body would affect the exposure of the cancer cells to flavopiridol and would affect the efficacy of treatment.

In our study, we were able to analyze cancer cells that were repeatedly drained from a patient with hepatic cancer who was treated with Adriamycin. In the early isolates, the cells were sensitive to Adriamycin. However, with subsequent isolates, the cancer cells showed a steady increase in resistance to Adriamycin. This result suggests that at the early stages of the disease most of the cancer cells had normal sensitivity to Adriamycin, and there may be a very small percentage that was resistant. This resistance may have arisen as a result of random mutations, and this random development of resistance may be a common occurrence during the growth of tumors in the body. With repeated exposures, the sensitive cells were gradually eliminated, allowing for the selection of cells that were resistant and finally leaving the drug-resistant cancers.

In summary, we have shown that cancer cells cultured from the ascites or pleural fluids of patients with metastatic cancers are useful tools for the study of cellular responses to anticancer agents and would complement the work done with established cell lines.
References


Flavopiridol Sensitivity of Cancer Cells Isolated from Ascites and Pleural Fluids

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