Flavopiridol (L86-8275): Selective Antitumor Activity in Vitro and Activity in Vivo for Prostate Carcinoma Cells

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ABSTRACT

We have selected a panel of human tumor xenografts for in vitro and in vivo studies that allows an indication of selectivity of action of novel chemotherapeutic agents. We report here the antitumor activity of the flavone flavopiridol (previously designated L86-8275), which has been selected for further studies based in part on its behavior in the anticancer drug screening system of the United States National Cancer Institute. Eighteen human tumor and five cell line-derived xenografts established by serial passage in nude mice in our laboratory were used as tumor models for in vitro investigations using a modified double-layer soft agar assay. In vivo investigations were completed in nude mice bearing advanced-stage s.c. growing prostate cancer xenografts. Antitumor activity in vitro (test/control ≤ 30%) of flavopiridol was observed at the very low concentration of 0.1 ng/ml in three of four prostatic xenografts and in one melanoma xenograft. Overall, in 14 of 23 (61%) tumor xenografts, drug treatment resulted in a LC50 of <10 ng/ml, demonstrating the high antiproliferative potential of flavopiridol. Toxicity to in vitro bone marrow cultures was evident only at 100 ng/ml, indicating potential high selectivity for susceptible tumor cells. Comparison of tumor cells with bone marrow samples tested showed clear prostate carcinoma and moderate melanoma selectivity. In vivo studies of flavopiridol confirmed antitumor activity in both prostatic cancer xenografts investigated. At the maximal tolerated dose of 10 mg/kg/day administered p.o. on days 1-4 and 7-11, flavopiridol effected tumor regression in PRXF1337 and tumor stasis lasting for 4 weeks in PRXF1369. We conclude that flavopiridol shows strong prostate- and moderate melanoma-specific antitumor activity in vitro. The prostate antitumor activity is also reflected by the two in vivo models studied. Initial clinical efforts with flavopiridol might consider early evaluation in patients with prostate carcinoma.

INTRODUCTION

One of the main objectives in the development of novel antineoplastic agents is the identification of compounds with tumor-specific or tumor-selective antiproliferative activity. With this goal in mind, the National Cancer Institute has developed a primary screening system consisting of 60 human tumor cell lines from eight tumor types (1–3). However, in vitro culture conditions may result in the selection of poorly differentiated cell lines, limiting the predictive value for the in vivo situation. In addition, in vitro screens may identify potent molecules that have little in vivo therapeutic index or that are metabolized readily to inactive species. Thus, whether such a system can detect agents that retain in vivo or clinical activity is uncertain.

The usefulness of human tumor xenografts for studies of tumor biology and cancer therapy, however, has been demonstrated by various groups (4, 5). We have developed a testing procedure that uses human tumors grown by serial passage in athymic mice as the source of cells for in vitro colony assays, and have also selected a panel of human tumor xenografts for primary in vitro studies (5–7). This test system has shown an excellent correlation of drug response in vitro and in vivo (7). We report here the activity of the flavone flavopiridol ([\(\pm\)])cis-5,7-dihydroxy-2-(2-chlorophenyl)-8-[4-(3-hydroxy-1-methyl)-piperidinyl]-4H-1-benzopyran-4-one (Fig. 1; Refs. 8–11) in 23 human tumor xenografts in the colony-forming assays, as well as in human bone marrow in vivo. We then tested the agent in prostate xenografts in vivo.

Flavonoids such as quercetin and genistein have been shown to inhibit tumor cell growth in vitro (12–16). Quercetin inhibits cellular signal transduction (Refs. 15 and 16; e.g., serine/threonine or tyrosine phosphorylation), and this may enhance the potency of “conventional” agents (17). Genistein is a more specific inhibitor of tyrosine kinase activities, such as epidermal growth factor, pp60[Src] and pp110[Src] (13, 18). Other flavonoids are less potent tyrosine kinase inhibitors. The new flavone flavopiridol (formerly designated L86-8275; Fig. 1) is derived by synthesis from a parent structure obtained from Dysosyllum binectariferum (8, 9), a plant native to India. Flavopiridol has recently been recognized as a potent inhibitor (\(K_{ATP} = 45\) nm) of CDK1, the mediator of cell cycle progression from G2 to M phase (19, 20). Subsequent studies (21, 22) have also demonstrated it to be a potent inhibitor of CDK2. A CDK whose activity appears at the G1-S boundary (reviewed in Ref. 23). Therefore, flavopiridol is a structure with potent activity against members of the CDK family of cell cycle

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2 The abbreviations used are: CDK, cyclin-dependent kinase; T/C, test/control; MTD, maximal tolerated dose.

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regulatory kinases. It is considerably less potent against every other kinase family thus far studied, including the epidermal growth factor receptor kinase, cyclic AMP-dependent protein kinase, protein kinase C, and mitogen-activated protein kinase (11).\(^3\) Flavopiridol has potent antiproliferative effects in vitro, with IC\(_{50}\) ranging from 25 to 150 nM in a series of breast and lung carcinoma cell lines (24). The mechanism of this effect is not clear, although the capacity of the compound to inhibit cell cycle progression in G\(_1\), G\(_2\), or S, depending on the cell-synchronization protocol used (24) and to alter selectively the phosphorylation state of CDK1 in living cells (19) is concordant with a mechanism directed at the CDK family of cell cycle regulatory kinases. In our disease-oriented panel of human tumor xenograft cells tested in vitro in a soft agar colony-forming assay, we were able to demonstrate a clear prostate cancer antitumor effect. The prostate antitumor activity was also demonstrated in vivo.

**MATERIALS AND METHODS**

**Drug.** Flavopiridol (Behringwerke L86-8275; National Service Center 649890) was originally supplied by Hoechst India and Behring AG, Marburg, Germany, to the National Cancer Institute. Other chemicals were obtained from commercial sources.

**Nude Mice and Tumors.** Eighteen human tumors established in serial passage in nude mice (NMRI genetic background) in our laboratory and five cell line-derived xenografts were used as tumor material (25, 26). The animals were housed in macronol cages set in laminar flow racks. Tumor designation and tumor types are shown in Fig. 2. Tumor models were selected out of more than 300 regularly growing xenografts of different histology that we established in serial passage (26). Characterization of these models included histology, immunohistochemistry (carcinoblastic antigen, human chorionic gonadotropin, epithelial membrane antigen, B2-microglobulin, cytokeratin, and others; Refs. 27 and 28), and growth behavior, as well as chemosensitivity to standard anticancer drugs in vitro and in vivo (29, 30), isoenzyme phenotype analysis (31), hormone receptor analysis, and DNA histogram as generated by flow cytometry (32).

**In Vitro Studies.** A modification of the double-layer soft agar assay as described by Hamburger and Salmon (33) was used as described earlier (5). The target cell population is the stem cell population, which is responsible for the unlimited growth of a tumor. An excellent correlation of drug response in the patient and in the clonogenic assay has been published by various groups (34, 35).

**Preparation of a Single-Cell Suspension.** Solid human tumor xenografts were mechanically disaggregated and subsequently incubated with a disaggregating solution consisting of 0.05% collagenase, 0.07% Dnase, and 0.1% hyalurondase in RPMI 1640 at 37°C for 30 min. Cells were washed twice with PBS and passed through sieves of 200- and 50-μm mesh size. The percentage of viable cells was determined in a hemocytometer using trypan blue exclusion. The freshly prepared tumor cell suspension was used for clonogenic assay.

**Human Bone Marrow.** Human bone marrow cells were aspirated from the iliac crest of healthy volunteers into preservative-free heparinized syringes. Mononuclear cells with a density of less than 1.007 g/ml were separated by density centrifugation in Ficoll-Paque, washed, and plated in 24-multiwell plates as described below. Granulocyte macrophage colony-stimulating factor (1000 U/ml) was added to stimulate the growth of granulopoietic colonies (colony forming unit, granulocyte macrophage).

**Culture Methods.** The tumor cell suspension was plated into 24-multiwell plates over a bottom layer consisting of 0.2 ml of Iscove's modified Dulbecco's medium with 20% FCS and 0.7% agar. A total of 20,000 to 200,000 cells was added to 0.2 ml of the same culture medium and 0.4% agar and plated onto the base layer. Flavopiridol was applied by continuous exposure (drug overlay) in 0.2 ml of medium. In each assay, six control plates received the vehicle only, and drug-treated groups were plated in triplicate in six concentrations ranging from 0.1 ng/ml to 10 μg/ml.

Cultures were incubated at 37°C and 5% CO\(_2\) in a humidified atmosphere for 6–18 days and monitored closely for colony growth using an inverted microscope. Within this period, in vitro tumor growth led to the formation of colonies with a diameter of ≥50 μm. At the time of maximum colony formation, counts were performed with an automatic image analysis system (Bausch & Lomb OMNICON FAS IV). Twenty-four h before evaluation, vital colonies were stained using a tetrazolium chloride dye.

Drug effects were expressed in terms of the percentage of survival, obtained by comparison of the mean number of colonies in the treated plates with the mean colony count of the untreated controls (colony count T/C × 100). A compound was considered active if it reduced colony formation to less than 30% of the control group value (T/C ≤ 30%). Furthermore, effective IC\(_{50}\), IC\(_{70}\), and IC\(_{90}\) were calculated corresponding to T/C values of 50, 30, and 10%, respectively.

Using these evaluation parameters, previous studies have demonstrated that the majority of clinically established anticancer agents were active at drug concentrations of <1 μg/ml. Some drugs, such as nitrosoarcines or 5-(3,3-dimethyl-1-triazethyl)-1H-imidazole-4-carboxamide, required dose levels of 6–30 μg/ml, respectively. An assay was considered fully evaluable if

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\[^3\] E. Sausville, unpublished results.
**Fig. 2** Selectivity of flavopiridol in the colony-forming assay. Variations of individual IC₇₀s (drug concentration needed to reduce colony formation to 30% of control value) from mean value are expressed as bars in the logarithmically scaled axis. Bars to the left demonstrate IC₇₀s lower than the mean value; bars to the right demonstrate higher values. The mean potency of the compound was 8 ng/ml. *HBM*, human bone marrow; *CXF*, colorectal xenograft; *LXF*, lung xenograft; *L*, large cell; *S*, small cell; *MAXF*, mammary xenograft; *MEXF*, melanoma xenograft; *OVXF*, ovarian xenograft; *PRXF*, prostate xenograft; *RXF*, renal xenograft.

The following quality control criteria were fulfilled (36): (a) Mean number of colonies in the control group wells were ≥20 colonies, with a colony diameter of ≥50 μm. (b) Initial plate counts on day 0 or day 2 were =20% of the final control group count. (c) The positive reference compound 5-fluorouracil (at the toxic dose of 1000 μg/ml) had to reduce colony survival to ≤30% of the controls. (d) Coefficient of variation in the control group was ≤50%.

**Prostate Cancer Xenografts.** Two human prostate cancer xenografts, PRXF1337 and PRXF1369, grown s.c. in nude mice were used. Histological examination showed a moderately differentiated cribriform adenocarcinoma (PRXF1337) and a poorly differentiated adenocarcinoma with solid components (PRXF1369), respectively. Both xenografts are androgen dependent, with growth in nude mice occurring only with androgen supplementation. These models have a moderate tumor growth rate, with tumor volume doubling times between 10 and 17 days.

**Nude Mouse Tests.** Male nude mice 6–8 weeks old of NMRI genetic background were used for all experiments. Tu-
mors were implanted s.c. in both flanks of athymic nude mice. Treatment was started as soon as the tumors reached a median tumor volume of 500 mm³ for PRXF1337 and 300 mm³ for PRXF1369, depending on the individual tumor doubling time between days 10 and 17 (5). Mice were randomly assigned to treatment groups and control group (8–10 tumors per group).

Flavopiridol was dissolved in water and administered p.o. by gavage on days 1–4 and days 7–11. Tumor size was measured weekly by two-dimensional measurement with calipers. Tumor volumes were calculated according to the formula $V = \frac{(a \times b^2)}{2}$, where $a$ is the larger diameter and $b$ is the smaller. Data evaluation was performed using specifically designed software by plotting relative tumor volumes against time. Relative tumor volume values were calculated for each single tumor by dividing the tumor volume day $X$ by the tumor volume day 0 at the time of randomization. Tumor doubling time of test and control groups was defined as the period required to double the initial tumor volume. Growth curves were analyzed in terms of maximal tumor inhibition (treated/control, T/C) and growth delay (the difference in days to double the initial tumor volume of the test minus the control group). Toxicity was assessed by lethality of tumor-bearing nude mice. At the maximal tolerable dose, the mice were allowed approximately a LD$_{10}$ (or a body weight loss with recovery within 2 weeks after the last injection).

**Statistical Analysis.** The statistical data analysis of the in vivo investigations was performed by the Wilcoxon test. Data of relative tumor volumes of each treatment group were compared with control group.

**RESULTS**

**Screening Studies.** Initial screening studies of flavopiridol in the National Cancer Institute in vitro cell line screen (2) revealed evidence of potent growth-inhibiting effect, with an average IC$_{50}$ of 25 ng/ml evaluated in 60 cell lines. Against the PC3 and DU145 cell lines, IC$_{50}$ of 32 and 30 ng/ml, respectively, were observed in a 2-day growth inhibition assay (data not shown). Therefore, it became of interest to investigate in an independent panel of cell lines evidence for a potent and selective antiproliferative effect.

**Detailed in Vitro Studies.** Table 1 and Fig. 2 show the cytotoxic activity of flavopiridol using the clonogenic assay in 23 human tumor cell models. Cytotoxicity (T/C < 30%) begins to be apparent at concentrations as low as 0.1 ng/ml. In three prostate cancer xenograft (PRXF1369, PC3MX, and LNCaP)- and one melanoma xenograft (MEXF1341)-derived clonogenic cell types, we observed potent colony inhibition, even at the lowest concentration tested (0.1 ng/ml). Human bone marrow colony formation was significantly growth inhibited only between 10 and 100 ng/ml. Growth in 14 of the 23 tumor cell types was more potently affected than bone marrow. MCF7XAD, resistant to doxorubicin, was as potently inhibited as its parental cell type, suggesting indirectly that the multidrug resistance phenotype was not likely a major determinant of drug efficacy. Cytotoxicity in most of the other xenografts was observed by 10 ng/ml.

**In Vivo Studies.** Dose-finding studies (data not shown) with flavopiridol identified a dose of 10 mg/kg/day administered p.o. on days 1–4 and days 7–11 as the MTD. In tumor-bearing nude mice, we observed 12.5% drug-related deaths at the MTD. Table 2 shows the in vivo antitumor activity of flavopiridol against two prostate cancer xenografts. In PRXF1369, we observed at the MTD of 10 mg/kg/day an optimal T/C of 33% and a growth delay of 30 days. After an initial tumor regression to 85% relative tumor volume, tumor volume increased slightly to 115% at day 28 (Fig. 3A). Flavopiridol administered at the dose level of 15 mg/kg/day was toxic, whereas the dose of 5 mg/kg/day was not active. In PRXF1337, we observed antitumor
N-hydroxy methyl piperidinyl substituent at the 8 position of the a potent inhibitor of the CDK family of cell cycle regulatory correlates closely with the number of hydroxy groups on the molecules CDK1 and CDK2 (20-23). Of great interest will be flavone ring system. Flavopiridol has recently been described as methoxy groups has resulted in a substantial loss of activity flavone ring (16), and the replacement of hydroxy groups with inhibitor. The tyrosine kinase inhibition capacity of flavonoids activities, whereas genistein is a more specific tyrosine kinase in vitro antiproliferative activity of flavopiridol. Three of four show clear prostate-selective and moderate melanoma-selective antiproliferative activity of flavopiridol. Three of four prostate cancer xenograft-derived cell types showed an individual IC50 almost two log-steps lower than the mean. With melanoma cells, we observed notable potency as well, whereas breast, lung, renal, and ovarian cancer showed intermediate sensitivity. Flavopiridol was considerably less potent when directed against human bone marrow, an important result favoring its further clinical development.

However, the ultimate mechanism of in vitro activity is still unclear. Several flavones are known to inhibit tyrosine kinases. Quercetin also inhibits various serine-threonine protein kinase activities, whereas genistein is a more specific tyrosine kinase inhibitor. The tyrosine kinase inhibition capacity of flavonoids correlates closely with the number of hydroxy groups on the flavone ring (16), and the replacement of hydroxy groups with methoxy groups has resulted in a substantial loss of activity (37). Two new types of substituents are present in flavopiridol: a chloro substituent in the 2' position of the aryl ring and a N-hydroxy methyl piperidinyl substituent at the 8 position of the flavone ring system. Flavopiridol has recently been described as a potent inhibitor of the CDK family of cell cycle regulatory molecules CDK1 and CDK2 (20-23). Of great interest will be an examination of the CDK complex of cell cycle regulatory molecules in the prostate carcinoma cells examined here in an effort to understand the basis of the potent activity of flavopiridol. Endogenous activating phosphorylations, as well as novel endogenous intracellular CDK inhibitors, have recently been described (38, 39). Variations in different tumor cell types of the mass of CDK catalytic subunits, the amount or type of regulatory cyclins (40), or the mass of potential endogenous CDK inhibitors could all be a basis for differential sensitivity to the agent. In addition, differential metabolism of flavopiridol in the different cell types obviously could also be a factor in determining susceptibility.

Because flavopiridol demonstrated prostate cancer specificity in vitro, it was studied in vivo in the prostate cancer xenografts PRXF1337 and PRXF1369. PRXF1369 was highly sensitive in vitro, whereas PRXF1337 could not be investigated due to insufficient growth in the clonogenic assay. Flavopiridol was administered p.o. As shown by Czech et al. (41), this application form is as effective as the i.v. application. Also, a sufficient bioavailability of flavopiridol is indicated by similar MTDs for both routes (41). Flavopiridol proved to be active in both prostate cancer xenografts. However, the therapeutic window is small. In both tumors, only the MTD was active, and after the end of therapy both tumors showed regrowth. This means that dosing for more...
than 2 weeks is needed to control tumor growth for a longer time. Nevertheless, the antitumor activity is remarkable because clinical standard cytotoxic agents effect no tumor regressions in our prostate cancer models.

Furthermore, the current cytotoxic chemotherapy has not been effective in the treatment of metastatic prostate cancer (42–44). Thus, the development of new therapeutic agents preferrably with novel mechanisms of action is desirable. These studies, therefore, delineate a basis for the early assessment of flavopiridol in patients with prostate carcinoma. Further studies are necessary to understand the cellular basis for prostate-selective antiproliferative activity seen in vitro and to outline the action of flavopiridol in concert with conventional chemotherapy-agents.

REFERENCES

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