Flavopiridol, an Inhibitor of Cyclin-dependent Kinases, Induces Growth Inhibition and Apoptosis in Bladder Cancer Cells In Vitro and In Vivo

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Abstract. Flavopiridol is a semi-synthetic flavone analog of the alkaloid, rohitukine, a compound from an Indian tree, Dysoxylum binectariferum. It has been shown to inhibit cyclin-dependent kinases (CDKs), causing cell cycle arrest and growth inhibition. Flavopiridol is reported to have cytotoxic activity against a wide range of cancer cell lines and has demonstrated its efficacy in several clinical trials. Flavopiridol seems a well-suited potential new agent for the treatment of bladder cancer. We, therefore, evaluated whether flavopiridol inhibits growth and induces apoptosis in bladder cancer cells and additionally examined the toxicity and efficacy of this drug in vivo in a rat bladder cancer model. The in vitro experiments showed an IC₅₀ of 50-100 nM in all cell lines tested. However, there was a difference in the response with regard to the grading of the tumor cells at higher doses. The IC₅₀ was found to be 150-350 nM in the well-differentiated RT4 and RT112 cell lines after treatment with flavopiridol, in comparison to an IC₅₀ of 1000 nM for the poorly-differentiated cell lines T24 and SUP. After exposure to flavopiridol, all tumor cell lines underwent significant apoptosis in comparison to untreated cells, beginning at a dose of 50 nM flavopiridol. At high concentrations (500 nM) of flavopiridol, 80-90% of all cells showed severe apoptotic alterations. The treatment of rat urinary bladder cancer with flavopiridol demonstrated the best efficacy with an intermittent treatment of 0.1 mg/kg, 3 times weekly over a total of 3 weeks, resulting in 7/12 animals tumor-free and a trend for the remaining tumors to have lower stage and grade. There seems to be a small advantage in intermittent versus daily application of flavopiridol. In summary, our results indicated that flavopiridol could be a useful therapeutic agent for bladder cancer, inhibiting tumor growth, malignant progression and inducing apoptosis.

Advanced or metastatic bladder cancer still has a poor prognosis and responses to chemotherapy are mostly limited (1). There is an urgent need to identify new active agents, preferably suited for use in a polychemotherapeutic regimen. Flavopiridol, (also known as 5,7-dihydroxy-8-(4-N-methyl-2-hydroxypyridyl)-6'-chloroflavone, HMR-1275, L-868275 or NSC-649890), is a novel semi-synthetic flavone analog of the alkaloid, rohitukine, a leading compound from an Indian tree, Dysoxylum binectariferum (2, 3). It was identified as a novel antineoplastic agent in the primary screen conducted by Aventis Pharma (formerly Hoechst Marion Roussel) and the Developmental Therapeutics Program of the National Cancer Institute. Cyclin-dependent kinases (CDKs) are thought to be common cellular targets of flavopiridol and regulate the passage of cells through the cell cycle. Flavopiridol inhibits CDK1,-2, -3 and -4, causes both G1 and G2 cell cycle arrest and, in some cases, induces apoptosis (4-6). The flavopiridol-induced decline in cyclin D1 expression is an early event that is specific and, at least in part, due to the transcriptional repression of the cyclin D1 promoter (7). In addition to the CDKs, some other potential targets have also been explored. Flavopiridol down-regulates bel-2 mRNA and bel-2 protein expression (8) and interacts with multidrug resistance protein 1 (MDR1) in a potent way (9). Flavopiridol may also have anti-angiogenic as well as direct antiproliferative effects (10). Furthermore, flavopiridol has recently been shown to inhibit other intracellular targets, notably PTEFb (11), glycogen phosphorylase (12) and DNA (13), which suggests

Abbreviations: i.p., intraperitoneal; PI, propidium iodide; MTT, 3-(4,5-dimethyl-thiazoyl-2-yl)-2,5-diphenyl-tetrazolium bromide; nM, nanomolar; TCC, transitional cell carcinoma.

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that flavopiridol may not be a pure CDK inhibitor and that multi-targets may be involved.

Flavopiridol is reported to have cytotoxic activity against a wide range of cancer cell lines, including HCT8 ileocecal adenocarcinoma, T98G glioblastoma, MCF-7 breast adenocarcinoma, HL-60 leukemia cells (14), head and neck squamous cell carcinomas (15), various ovarian cancers (16), esophageal adenocarcinomas (SKGT-2, SKGT-4 and SKGT-5), epidermoid carcinoma (HCE-4) cells (17), human chronic lymphocytic leukemia (18), hematopoietic cell lines (19), seven non-small cell lung cancers (20) and MDA-MB-435 breast cancer cells (21). In vivo, flavopiridol has demonstrated antitumor activity against several tumor xenografts, including prostate cancer (22), head and neck squamous cell carcinomas (15) and hematopoietic neoplasia (23). Combined administration of flavopiridol with other antineoplastic agents seems highly schedule-dependent. Flavopiridol enhances the cytotoxic effect of several chemotherapeutic agents when combined. Synergy was shown for mitomycin C, cisplatin, paclitaxel, cytarabine, topotecan, doxorubicin and etoposide (24-26). Flavopiridol was the first CDK inhibitor in early clinical trials for the potential treatment of renal, prostate, gastric and colon cancers, and non-Hodgkin’s lymphoma. Taken together, flavopiridol seems well-suited for evaluation as a potential new agent for the treatment of bladder cancer, especially in a multidrug regimen. We evaluated whether flavopiridol inhibits growth and induces apoptosis in bladder cancer cells. Additionally, the toxicity and efficacy of this drug was examined in vivo in a rat bladder cancer model. First, bladder cancer cell lines were exposed to flavopiridol at concentrations ranging from 10-1000 nM in order to determine the inhibition concentrations IC\(_{20}\), IC\(_{50}\) and IC\(_{80}\). Then, the apoptosis induction properties were evaluated by flow cytometric analysis of Annexin-V and propidium iodide.

Materials and Methods

**Tumor cell lines and culture conditions.** The human transitional cell carcinoma cell lines RT4 (grade 1), RT112 (grade 1), T24 (grade 3) and SUP (grade 4) were obtained from the ATCC (Rockville, MD, USA) and maintained in RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% nonessential amino acids, 2 mM L-glutamine, 100 units/ml penicillin G, 100 μg/ml streptomycin and a vitamin solution (Gibco BRL, Life Technologies Inc., Frederick, MD, USA). For experiments, 1x10^6 tumor cells were seeded in 25-cm\(^2\) culture flasks (Costar, Cambridge, USA) and incubated for 48 h to allow maximum confluence, thus providing optimal cell-cell contact. The cells were routinely subcultured by trypsinization (0.05% Trypsin, 1 mM EDTA) (Gibco BRL).

**Reagents and chemicals.** Flavopiridol was resuspended in NaCl 0.9%. Aliquots of the stock solution were stored at –20°C. For the animal tumor induction, the animals received 0.15 ml (1.5 mg) of a MNU (Sigma, St. Louis, MO, USA) solution dissolved in 100 ml of normal saline.

**Cell culture treatment scheme.** Bladder cancer cell lines were exposed to flavopiridol at concentrations ranging from 10 - 1000 nM (10, 50, 100, 500, 1000 nM) in order to determine the inhibition concentrations (IC) of IC\(_{20}\), IC\(_{50}\) and IC\(_{80}\) after 24, 48 and 72 h. IC is defined as the concentration where growth of cells is inhibited as compared to untreated cells (IC\(_{50}\): 50% growth inhibition, IC\(_{80}\): 80% growth inhibition). Flavopiridol was added to the culture medium of cells in log-phase growth. Control experiments were performed with normal saline.

**Cytotoxicity assay.** The MTT (Micro culture tetrazolium) assay was used to assess cell viability after exposure to flavopiridol. In brief, trypsinized tumor cells were resuspended in medium at 5x10^5 cells/ml, after verifying cell viability by trypan blue dye exclusion. One hundred μl of the cell suspension was distributed into each well of a 96-well flat-bottomed microtiter plate and each plate was incubated for 24 h to allow adherent cell growth. Following incubation, the medium was removed and 200 μl of the different reagent solutions were distributed and incubated for 24, 48 and 72 h. Thereafter, 20 μl MTT dye working solution (Sigma) was added and incubated for 4 h. The supernatant in the wells was decanted and replaced by 200 μl 2-propanol, supplemented with 0.05 N HCl to dissolve the reactive dye. The absorbance (A) values were read at 550 nm, using an automatic multiwell spectrophotometer (340 ATTCL SLT, Cailasheim, Germany). The percentage of growth inhibition was calculated using the background-corrected absorbance as follows: % Growth Inhibition = ([A of experimental well]/A of positive control well] *100. Each experimental data-point represents the average values obtained from six replicates; the experiments were performed in duplicate.

**Assessment of apoptosis by Annexin-V and propidium iodide.** To evaluate the extent and time-course of apoptosis after incubating cells with flavopiridol, Annexin-V and propidium iodide (PI) (both Boehringer, Ingelheim, Germany) were used in a double stain technique. The cells were counterstained with PI as a vital dye to distinguish between early (Annexin-V-positive, PI-negative) and late apoptosis (Annexin-V-positive, PI-positive), and cell disintegration (Annexin-V-negative, PI-positive), respectively. Controls and 3x10^5 treated cells (all attached and detached cells in supernatant) were resuspended in 200 μl of culture medium without phenol red, incubated with 5 μl FITC-conjugated Annexin-V and 10 μl PI for 20 min at room temperature in the dark, and then were immediately analyzed by flow cytometry. A minimum of 10,000 events was acquired for each sample.

**Animal model.** Animal experiments were carried out in accordance with the principles of laboratory animal care and protection of animals after approval by German law (Approval no: 23.203.2-bn 29, 22/97). The model utilized was initially described by Hicks and Wakefield (27), and modified by Steinberg et al. (28) and our group, as described (29, 30). Forty-six female Fischer 344 rats (aged 6-7 weeks and weighing 120-150 g) were purchased from Charles River (Sulzfeld, Germany) and housed 5 rats per cage at a temperature of 23°C in a controlled dark-light-rhythm of 12 h under pathogen-low conditions. All rats used in the experiments were acclimatized for 2 weeks under routine laboratory conditions before starting any experiments.

**Treatment schedules.** The animals were anesthetized with ether inhalation narcosis prior to intraperitoneal (i.p.) injection of
flavopiridol. For the toxicity evaluation, 10 animals received daily doses of flavopiridol, starting at 0.01 mg/kg/day and escalating to 5 mg/kg/day i.p.. For the efficacy studies, a dose of 0.1 mg/kg flavopiridol dissolved in normal saline was administered, beginning on the 16th week after tumor initiation, daily for 5 days (group A), or 0.1 mg/kg, 3 times weekly over a total of 3 weeks (group B). As control, rats with tumor were treated by intravesical instillation of normal saline in the same schedules. The animals were sacrificed 2 days after the end of therapy by carbon dioxide narcosis. The urinary bladder was excised completely. The liver, lungs, kidneys and ureters were inspected for pathological conditions and, if suspicious, removed for further histological examination. All organs were fixed in isopentane and then frozen in liquid nitrogen. Sections were cut transversely through the midportion of the bladder, and 4-µm sections were taken to adequately sample the entire bladder. All the sections were reviewed by two investigators and evaluated for histological grading and stage according to the World Health Organization/International Society of Urological Pathology consensus classification of urothelial neoplasms of the urinary bladder (31). Hyperplasia and atypia were not considered to be tumor. Comparison of the treatment results was done by assessing the stage and grade, as well as by evaluating proliferation and apoptosis by MIB-1 (KI-67 antigen) immunostaining and TUNEL assay, respectively.

**MIB-1 immunostaining.** All tumor specimens were stained with MIB-1 to assess the proliferation fraction. Five-µm sections were incubated with blocking solution (Dako, Denmark), and then incubated for 30 min with anti-Ki-67, clone MIB-1 (Dianova, Hamburg, Germany) at a dilution of 1:20. These sections were thereafter covered with normal swine serum to reduce non-specific staining. The sections were then processed at room temperature as follows: PBS wash, 30-min incubation with biotinylated rabbit-anti-goat IgG 1:50 (Dako), PBS wash, followed by staining with diaminobenzidine tetrahydrochloride (DAB) solution (Fluka, Germany), and then incubated with blocking solution (Dako) to block endogenous peroxidase was added, the sections were then washed in PBS, 30-min incubation with ABCComplex/HRP (avidin and biotinylated horseradish peroxidase) (Dako), PBS wash, followed by staining with diaminobenzidine tetrahydrochloride (DAB) solution (Fluka, Germany). Finally, the sections were counterstained with hematoxylin and examined.

**TUNEL assay.** Apoptotic cells in the urothelium and stroma of the specimen were detected by an *in situ* cell death detection kit (Boehringer Mannheim, Germany), based on the terminal-deoxynucleotidyltransferase-mediated dUTP nick-end labelling method (TUNEL). Frozen tissue was cut into 5-µm sections, then dried and fixed in 3% paraformaldehyde. After washing the sections, blocking solution (Dako) to block endogenous peroxidase was added for 15 min at room temperature and, after repeat washing with PBS, immersed in terminal deoxynucleotidyltransferase reaction mixture containing enzyme and fluorescein-labelled dUTP, at 37 °C for 1 h. Then, the anti-fluorescein antibodies, Fab fragments from swine, conjugated to horseradish peroxidase, were applied to the sections for 15 min to detect the labelled nucleotides. Binding was localized with diaminobenzidine and the sections were slightly counterstained with hematoxylin. Apoptotic cells in the sections were counted by microscopic examination with the “hot-spot” procedure, as described by Weidner (32).

**Analysis and statistics.** For analysis of MIB-1 and TUNEL staining, areas with pronounced apoptotic or proliferative activity were examined at 200-fold magnification by light microscopy, as described by Weidner (32). With a counting frame of 0.0092 mm², all positive as well as all negative nuclei were counted. From these results, percentages were calculated. The counting procedure was repeated with 5 “hot-spots”. The results of the MIB-1 staining and TUNEL assay were statistically evaluated by the Wilcoxon U-test. A *p*-value of 0.05 or less was considered significant.

**Results**

**Cytotoxicity.** Flavopiridol inhibited, effectively and dose-dependently, the proliferation in all bladder cancer cell lines. The IC₂₀ was 50-100 nM in all cell lines tested. However, there was a difference in the response with regard to the grading of the tumor cells at higher doses. The IC₅₀ was found to be 150-350 nM in the well-differentiated RT4 and RT112 cell lines after treatment with flavopiridol, in comparison to a IC₅₀ of 1000 nM for the poorly-differentiated cell lines T24 and SUP (*p*<0.05) (Figure 1a-d). No cytotoxic response was seen in concentrations of 10 nM flavopiridol nor could an IC₈₀ be achieved. Normal saline had no effects on the cell lines.

**Apoptosis induction.** After having shown the growth inhibitory effect of flavopiridol, the induction of apoptosis in the cell lines was evaluated. After exposure to flavopiridol, all tumor cell lines underwent significant apoptosis (*p*<0.05) in comparison to untreated cells, beginning at a dose of 50 nM flavopiridol. At high concentrations (500 nM) of flavopiridol, 80-90% of all cells showed massive apoptotic alterations (Figure 2). There was no significant difference to be found between the cell lines regarding the grade and extent of apoptosis.

**Tumor induction and histopathological progression of MNU-treated rat bladders.** 1.5 mg intravesical administered MNU, at the schedule described, induced transitional cell cancer of the urinary bladder in 10 out of 12 rats by the 16th week (data not shown).

**Toxicity.** The toxicity study, with escalating doses of up to 0.1 mg/kg/day i.p. flavopiridol, revealed neither major nor minor side-effects in the animals. At a dose of 1 mg flavopiridol, dose-related leukopenia and drug-related lesions in the spleen and bone marrow were observed. The gastrointestinal and bone marrow toxicity was dose-limiting over a period of 3 weeks. The maximal tolerated dose was 3 mg/kg/d in rats.

**Efficacy.** The efficacy results showed in the examined treatment groups, 0.1 mg/kg daily for 5 days [group A] and 0.1 mg/kg, 3 times weekly over a total of 3 weeks [group B], a significant decrease of tumor incidence. The best antitumor efficacy was found in group B, with 7 out of 12
animals completely tumor-free, another 3 animals showing dysplasia only. In group A, 4 out of 12 animals were tumor-free, while 3 animals were found to have dysplasia. Additionally, a trend towards a lower tumor stage and a lesser grade was found in the remaining tumors of the flavopiridol-treated animals. A compilation of the data, with all histological stages of the treatment and control group animals, is depicted in Figure 3 a and b.

Apoptosis and proliferation in the tumors after flavopiridol treatment. After treatment with flavopiridol, the remaining rat bladder tumors in group A showed an elevated apoptotic rate of 49.5%; at the same time the proliferation rate decreased to 1.8% in comparison to the control group, where apoptosis and proliferation were 2.9% and 14.6%, respectively. In group B, the remaining tumors showed, after flavopiridol treatment, an apoptotic rate of 49.8% and a decreased proliferation rate of 3.8%. In the control group, saline-treated bladder tumors showed an apoptotic rate of 3.0% and a proliferation rate of 19.9%. In summary, flavopiridol treatment reversed the balance between proliferation and apoptosis in favor of apoptosis, resulting in a decrease of tumor incidence (Figure 4 a and b).

Discussion

Flavopiridol is an inhibitor of cyclin-dependent kinases. In vitro, the agent is active against a variety of tumor types. The data presented here demonstrated that flavopiridol dose-dependently induced growth inhibition and apoptosis in 4 bladder cancer cell lines. In the cytotoxicity experiments, a difference was found between the responsiveness to flavopiridol in relation to histological grade of the cells, however, not in the apoptosis induction experiments. In a study by Chien et al., the effects of flavopiridol were evaluated in RT4, UMUC-3 and 5637 bladder cancer cells for cytotoxicity and apoptosis induction. The growth inhibitory evaluations in this study showed similar results to our findings, however, the authors found only moderate levels of apoptosis, which additionally required up to 72 h of continuous drug exposure using 1 μM of flavopiridol, which is contrary to our findings. This might be explained by the use of different apoptosis assays and other methodological reasons. Additionally, Chien et al. failed to demonstrate a synergistic effect of flavopiridol with cisplatin and/or irradiation in in vitro bladder carcinoma models (33).

To further confirm our findings, an in vivo model of bladder cancer was used to evaluate the toxicity and efficacy of the drug. The animal model in this study has previously been characterized and reflects clinically observed tumors in humans. The tumors arise only from the urothelium; they are spontaneous and not implanted, and are histologically equivalent to human transitional cell carcinoma (TCC). The treatment of rat urinary bladder cancer with flavopiridol demonstrated the best efficacy for intermittent treatment with 0.1 mg/kg, 3 times weekly over a total of 3 weeks, resulting in 7/12 animals tumor-free and a trend for the remaining tumors.
to lower stage and grade. There seems to be a small advantage in the intermittent versus daily application of flavopiridol. Human clinical trials will have to further clarify the optimal dosage and scheduling. After flavopiridol treatment, the rate of apoptosis in the tumor was increased, whereas the tumor proliferation rate decreased, suggesting that the antitumor effect of flavopiridol is, in vivo, mediated at the same time by induction of apoptosis and inhibition of tumor growth. Furthermore, the animal model showed that flavopiridol at a dose of 0.1 mg/kg is both safe and effective.

In humans, clinical phase I trials have shown that gastrointestinal (secretory diarrhea) and bone marrow toxicities are dose-limiting. A phase I trial was conducted by the National Institutes of Health, USA. Flavopiridol was given as a 72-h infusion every 2 weeks. The MTD of infused flavopiridol was 50 mg/m²/day x 3, with dose-limiting secretory diarrhea at 62.5 mg/m²/day x 3. The MTD was 78 mg/m²/day x 3 in combination with antidiarrheal prophylaxis (ADP), with dose-limiting hypotension at 98 mg/m²/day x 3. Based on chronic tolerability, the recommended phase II dose without ADP was 40-50 mg/m²/day x 3 (34-37).

Although flavopiridol has demonstrated a wide antitumor spectrum against various cancer cells both in vitro and in vivo, the phase II clinical trial results are disappointing (38-41). The reason for this may be found in inadequate study designs, patient selection and poorly-chosen tumor entities, as well as other factors that can affect the clinical outcome. It is still too early to reach conclusions about the clinical potential of flavopiridol. The development of new analogs of flavopiridol and continuing studies with the aim of improving its pharmacokinetic characteristics might be future directions that are worth pursuing. As many chemotherapeutic agents, such as paclitaxel, cytarabine, topotecan, doxorubicin and etoposide, have shown sequence-dependent synergy with flavopiridol (25), another rewarding direction may be the development of combination regimens in a clinical setting. In summary, our results indicate that flavopiridol could be a useful therapeutic agent for bladder cancer, by inhibiting tumor growth, malignant progression and inducing apoptosis. Flavopiridol treatment may offer a new therapeutic option for bladder cancer patients and should be further evaluated in a clinical trial.

References


Flavopiridol and Apoptosis


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