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Research Article

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Flavopiridol, a Novel Cyclin-dependent Kinase Inhibitor, Suppresses the Growth of Head and Neck Squamous Cell Carcinomas by Inducing Apoptosis

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Abstract

Flavopiridol (HMR 1275) has been identified recently as a novel antineoplastic agent in the primary screen conducted by the Developmental Therapeutics Program, National Cancer Institute. Flavopiridol inhibits most cyclin-dependent kinases (cdks) and displays unique anticancer properties. Here, we investigated whether this compound was effective against head and neck squamous cell carcinomas (HNSCC). Exposure of HNSCC cells to flavopiridol diminished cdc2 and cdk2 activity and potently inhibited cell proliferation (IC_{50} 43–83 nM), which was concomitant with the appearance of cells with a sub-G_1 DNA content. Moreover, DNA fragmentation and TUNEL (terminal deoxynucleotidyl transferase-mediated nick end labeling) reaction confirmed that flavopiridol induces apoptosis in all cell lines, even on certain HNSCC cells that are insensitive to apoptosis to DNA-damaging agents (γ-irradiation and bleomycin). A tumorigenic HNSCC cell line was used to assess the effect of flavopiridol in vivo. Treatment (5 mg/kg per day, intraperitoneally) for 5 d led to the appearance of apoptotic cells in the tumor xenografts and caused a 60–70% reduction in tumor size, which was sustained over a period of 10 wk. Flavopiridol treatment also resulted in a remarkable reduction of cyclin D1 expression in HNSCC cells and tumor xenografts. Our data indicate that flavopiridol exerts antitumor activity in HNSCC, and thus it can be considered a suitable candidate drug for testing in the treatment of refractory carcinomas of the head and neck. (*J. Clin. Invest.* 1998. 102:1674–1681.) Key words: antineoplastic agents • cell cycle • cell death • oral xenografts • oral cancer

Introduction

Head and neck squamous cell carcinomas (HNSCC)[1] represent 6% of all cancers diagnosed each year in the United States (1) resulting in ~ 12,000 deaths. In spite of the recent advances in surgical procedures and treatment modalities, the 5-yr survival rate for these patients, ~ 50%, has remained largely unchanged for > 30 yr (2). The poor prognosis can be attributed to the fact that the early stages of the disease manifest minimal signs and symptoms and, when diagnosed, the lesions are usually well advanced and generally respond poorly to cancer therapies (3).

The available treatment options are dependent on the site, size, and the stage of these lesions, and current modalities include surgery, γ-irradiation, and chemotherapy (4). In general, lesions that respond favorably to either surgery or γ-irradiation usually heal without any significant functional impairment. However, advanced tumors have a much poorer prognosis and a combined modality of surgery and γ-irradiation and chemotherapy is often used in these cases, resulting in severe orofacial dysfunction and disfigurement, thus reducing the quality of life of these patients. Furthermore, conventional chemotherapy for the treatment of HNSCC is limited (5, 6). Favorable prognosis in those patients that have responded to treatment is often compounded by recurrences and secondary neoplastic lesions, which are generally less sensitive to further therapy. Clearly, new treatment strategies are needed to treat and to aid in the management of HNSCC.

Since 1990, to help in the search for novel antineoplastic agents, the Developmental Therapeutics Program of the National Cancer Institute (NCI) has screened ~ 72,000 compounds for their biological activities on a panel of 60 human cancer cell lines (7, 8), cataloguing parameters such as cytotoxicity and cytostasis. This information has been used to search for novel drug candidates and to help identify suitable molecular targets for pharmacological intervention in cancer (9). Five compounds have been identified from this primary screen as displaying novel antineoplastic activities. They are advanced for their preclinical and clinical evaluation based on their in vitro and in vivo activity and pharmaceutic and toxicological properties. Of these five compounds, flavopiridol (HMR 1275) exhibited a number of interesting novel features. These include a potent antiproliferative and cytotoxic effect in vitro and antitumor activity in vivo, in a variety of cell types and human tumor xenografts, respectively (10–13). These biological effects of flavopiridol correlate with its ability to inhibit potently the activity of cyclin-dependent kinase (cdk) 1 (also known as cdc2), cdk2, and cdk4 (14–17). Phase I clinical trials have been completed recently in patients with refractory neoplasms (18). Based on favorable results, phase II trials in several tumor types are now being considered.

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1. *Abbreviations used in this paper:* cdk, cyclin-dependent kinase; HNSCC, head and neck squamous cell carcinoma; T/C, mean tumor size/mean size of control; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

The feasibility of using flavopiridol in the treatment of HNSCC has not been explored, partly due to the fact that the NCI 60 cell line panel does not include those derived from HNSCC and only a few models of oral tumor xenografts have been described (19). Recently, we characterized a panel of HNSCC cell lines derived from primary and secondary cancer lesions of contrasting clinical staging (20). These cell lines have been studied extensively for the presence of molecular alterations in the tumor suppressor genes, *p53* and *p16,* and for the expression and functional activity of key cell cycle components (21–23). Aberrant forms of p53 and p16 were present in all the cells lines tested with the exception of HN30, which was found to express wild-type p53, thus displaying normal p53 function (22). Therefore, using these cell lines as a model system, we sought to examine whether flavopiridol exhibited antiproliferative activity in squamous cell carcinomas of the head and neck.

In this study we report, for the first time, that flavopiridol has a potent antiproliferative effect in HNSCC in vitro by promoting apoptosis independent of p53 function. Furthermore, we observed that flavopiridol exhibits a potent antitumor activity in a HNSCC xenograft, likely to result from drug-induced programmed cell death.

Methods

Materials. [^{3}H]Thymidine was purchased from ICN Pharmaceuticals Inc. (Costa Mesa, CA), [γ-^{32}P]ATP from DuPont/NEN (Boston, MA), DMEM from GIBCO-BRL (Gaithersburg, MD), FBS from HyClone Laboratories, Inc. (Logan, UT), ECL from Amersham International (Arlington Heights, IL), Gammabind G Sepharose from Pharmacia Biotech Inc. (Piscataway, NY), In Situ Cell Death Detection Kit, histone H1, and RNase A from Boehringer Mannheim Biochemicals (Indianapolis, IN), and all other reagents from Sigma Chemical Co. (St. Louis, MO).

Drug. Flavopiridol (HMR 1275) was provided by Hoechst Marion Roussel (Kansas City, MO) to the Developmental Therapeutics Program of the NCI. A 10 mM stock solution of the compound was prepared in DMSO and diluted further, to the working concentration, in the same diluent. The final concentration of DMSO in the culture medium was $\leq 0.1\%$.

Cell lines and culture conditions. Culture conditions of HNSCC cells used in this study have been described in detail elsewhere (20–22). In brief, cells were cultured on a lethally irradiated feeder layer of Swiss 3T3 murine fibroblasts in DMEM supplemented with 10% FBS and 0.4 μg/ml hydrocortisone, at 37°C in 95% air/5% CO_2. Feeder cells were removed before subculturing by standard technique (21). HaCaT cells (a gift from Dr. S.I. Chung, National Institute of Dental Research) were cultured as described above, but in the absence of fibroblast support.

Thymidine incorporation. Cells ($1–2 \times 10^4$/well) were grown overnight in 24-well plates and treated with flavopiridol (1–1,000 nM) or DMSO (0.1%, final concentration) for the control wells in medium containing 0.5% FBS. After 24–48 h the cells were pulsed with [^{3}H]thymidine (1 μCi/well) for 4–6 h, fixed (5% trichloroacetic acid), and solubilized (0.5 M NaOH) before scintillation counting. Experiments were performed in triplicates.

Cell cycle analysis. Cell cycle analysis was performed as described previously (24). In brief, control and treated cells were harvested, washed in cold PBS, and fixed in 70% ethanol. DNA was stained by incubating the cells in PBS containing propidium iodide (50 μg/ml) and RNase A (1 mg/ml) for 30 min at 37°C. The DNA content of the cells was analyzed on a Becton Dickinson FACScan®, using Cell Quest software (Becton Dickinson Immunocytometry Systems, San Jose, CA).

cdc2 and cdk2 kinase activity and immunoblotting. Cells exposed to flavopiridol (0–500 nM) or DMSO for 12–14 h were washed with ice-cold PBS and lysed in NP-40 lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 20 mM EDTA, 0.5% NP-40, 1 mM PMSF, 5 μg/ml aprotinin, and 5 μg/ml leupeptin) on ice for 10 min. Lysates containing 500 μg of total cellular protein and 1 μg of polyclonal antibody to human cdc2 and cdk2 (sc-954 and sc-163, respectively; Santa Cruz Biotechnology Inc., Santa Cruz, CA) were immunoprecipitated, and immunocomplex kinases assays were performed as described (25), using histone H1 as a substrate. Samples were resolved as described (25), and gels were subjected to autoradiography and PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA). 5 μl each of cdc2 and cdk2 immunoprecipitates or 50-μg aliquots of total cell lysates were resolved as described and subjected to Western blot analysis using appropriate antibodies (cdc2, cdk2, as above; cyclin D1 and cyclin E, sc-246 and sc-247, respectively; Santa Cruz Biotechnology Inc.) and detected by horseradish peroxidase–conjugated secondary antibodies and ECL.

DNA fragmentation assay. DNA fragmentation analysis on treated (flavopiridol, 300 nM; bleomycin, 10 μg/ml; γ-irradiation, 2 Gy) and control HNSCC and HaCaT cells was carried out as previously described (26). In brief, the total cell population (adherent and detached cells) was collected and lysed in 500 μl of lysis buffer (Tris-HCl pH 7.4, 10 mM EDTA, and 0.5% Triton X-100) and centrifuged to separate intact from fragmented DNA. The resulting supernatant containing the fragmented DNA was digested with RNase (0.1 mg/ml) and proteinase K (0.2 mg/ml), respectively, each for 2 h at 37°C. After precipitation with 5 M NaCl (to a final concentration of 0.5 M) and 0.5 vol of 100% isopropanol, the DNA was collected and resuspended in 20 μl of TE buffer and electrophoresed in a 2.0% agarose gel. Low molecular weight DNA bands were stained with ethidium bromide and visualized under ultraviolet light.

TUNEL analysis and DAPI staining. In situ labeling of apoptosis-induced DNA strand breaks was carried out using the TUNEL (terminal deoxynucleotidyl transferase-mediated nick end labeling) technique and the In Situ Cell Death Detection Kit, available from Boehringer Mannheim Biochemicals. Labeling was carried out according to the manufacturer's recommendations. In brief, HNSCC and HaCaT cells were grown to 80% confluence on glass coverslips and treated for 12–18 h with 300 nM of flavopiridol or DMSO. Cells were washed in PBS, fixed, and permeabilized in paraformaldehyde (4% in PBS containing 0.05% Tween 20) at 37°C for 15 min. DNA strand breaks were end-labeled with fluorescein-conjugated nucleotides, by terminal deoxynucleotidyl transferase. After rinsing in PBS, the coverslips were further incubated for 5 min with DAPI stain (0.1 μg/ml), washed (three times), mounted on glass slides with Vectashield (Vector Laboratories, Burlingame, CA), and analyzed under fluorescence light. TUNEL labeling of paraffin-embedded tissue sections was carried out according to the manufacturer's recommendation.

Animal studies. Animal studies were performed under an approved NIH animal care and use protocol. Female athymic (*nu/nu*) nude mice (Harlan Sprague Dawley, Frederick, MD), 5–6 wk old and weighing 18–20 g, were used. The animals were housed in appropriate sterile filter-capped cages and fed and watered ad libitum. All handling and transplantation procedures were conducted in a laminar-flow biosafety hood.

Establishment of tumor xenografts in athymic nu/nu mice. HNSCC cell line, HN12, was cultured to subconfluence, trypsinized, washed in PBS, and resuspended in DMEM, at a concentration of 10^7 viable cells/0.5 ml. Cell viability was determined by the Trypan blue exclusion test. 0.5 ml of the cell suspension was transplanted subcutaneously in the neck region of the athymic mice and monitored twice weekly for tumor formation. Precise tumor measurements were made with calipers and experiments on tumor-bearing mice were performed when the tumor volume was calculated to be ~ 0.9 cm^3 (27).

In vivo treatment of tumor xenografts with flavopiridol. Tumor-bearing mice were randomly separated into control ($n = 8$) and test

groups ($n = 16$), which were treated subsequently with an equal volume of DMSO (0.1%) or flavopiridol (5 mg/kg per day), respectively. Treatment strategy was a single injection per animal, given intraperitoneally for five consecutive days, as described for the non-Hodgkin's lymphoma model (11). One animal from each group was killed 1 h after injection on days 2 and 5, for photographic records and retrieval of tumor xenografts. The excised tumor mass was weighed, part fixed, and snap-frozen for apoptosis and immunohistochemical analysis. After the treatment period, tumor size and body weight were assessed twice weekly using calipers and balance, respectively. Tumor weight was calculated by using the formula as described (27), whereby tumor volume ($LW^2/2$, where L and W represent length and the width of the tumor) was converted to weight, assuming unit density. Changes in tumor weight (Δ weights) for each treatment (T) and control (C) groups were determined for each time point by subtracting the median tumor weight on the first day of treatment (staging day) from the median tumor weight on the indicated observation day. These values were used to calculate a percent mean tumor size/mean size of control (T/C) as $\%T/C = (\Delta T/\Delta C) \times 100$ (27). Currently, optimum (minimum) values (optimal %T/C) $< 40\%$ (60% reduction) are being considered as predictive of antitumor activity (27).

Statistical analysis. Results of the animal experiments are expressed as mean±SEM. Student's t test was used to determine the difference between the treated group and the control. $P < 0.05$ was considered to be significant.

Immunohistochemical analysis of tumor xenografts. Using appropriate antibodies, serial sections of tumor xenografts from control and treated animals (days 2 and 5) were analyzed for expression of cyclin D1 and cyclin D3 (P2D11F11 and DCS-22, respectively; Novocastra Laboratories, Burlingame, CA), and cyclin E (HE12; PharMingen, San Diego, CA) as previously described (28). In brief, after antigen retrieval using microwave heating, serial tissue sections were stained using the ABC method, in an automated immunostainer (Ventana Medical Systems, Inc., Tucson, AZ).

Results

Antiproliferative effect of flavopiridol on HNSCC cells. Previous work has demonstrated flavopiridol to have a potent antiproliferative effect in vitro on cells from a number of tissues, including breast, prostate, and lung carcinomas (10, 13). Therefore, we sought to explore whether HNSCC cell lines were sensitive to flavopiridol by assaying the incorporation of [^{3}H]thymidine into cellular DNA of cells exposed to increasing concentrations of this compound. HaCaT cells, a spontaneously immortalized and nontumorigenic keratinocyte cell line, were used as control. As shown in Fig. 1, a 24-h exposure of the cells to flavopiridol was sufficient to inhibit [^{3}H]thymidine incorporation in a dose-dependent manner (1–1,000 nM). All HNSCC cell lines tested were sensitive to flavopiridol in the nanomolar range with similar IC_{50} values (HN4, 65.2 nM; HN8, 42.9 nM; HN12, 72.8 nM; HN30, 82.7 nM; and HaCaT, 56.7 nM). The maximal effect of this compound for all the cell lines was observed between 300 and 1,000 nM. These results indicate that flavopiridol may exert an antiproliferative effect in HNSCC cells. Based upon these results, we chose to use 300 nM of flavopiridol, a concentration that gave $\sim 80\%$ reduction in [^{3}H]thymidine uptake, for subsequent experiments.

Effects of flavopiridol on the cell cycle in HNSCC cells. To investigate the antiproliferative effects of flavopiridol, we analyzed cell cycle profiles of asynchronously growing HNSCC cells exposed to flavopiridol. As a control, we used γ-irradiation, which in these cells causes a characteristic G_2/M block before undergoing apoptosis (Fig. 2). Of interest, one of the cell lines, HN30, exhibited a block in G_1 and G_2/M phase of the cell cycle in response to γ-irradiation, but remained insensitive to apoptosis despite normal p53 function (22). By contrast, all flavopiridol (300 nM) treated cells (HaCaT, HN12, and HN30) exhibited a decrease and an increase in the cell population in the S and sub-G_1 phase, respectively, when compared with control cells (Fig. 2). Similar results were obtained for the remaining HNSCC cell lines (HN4 and HN8, data not shown). Interestingly, upon longer exposure to flavopiridol (> 24 h), most HNSCC cells were found in the sub-G_1 phase (data not shown). The results indicate that flavopiridol affects the cell cycle profile of HNSCC cells and causes the appearance of a fraction of cells with a sub-G_1 DNA content, which is suggestive of apoptosis.

Inhibition of cdc2 and cdk2 activity in HNSCC by flavopiridol. Since the cell cycle effects may be, in part, responsible for the antiproliferative response to flavopiridol, we sought to examine whether cdc2 and cdk2 activity was altered in response to flavopiridol. cdc2 and cdk2 immunoprecipitates were prepared from cells (HaCaT, HN12, and HN30) treated with increasing concentrations of flavopiridol for 12–14 h, and in vitro immunocomplex kinase reactions were performed, using histone H1 as a substrate. All three cell lines demonstrated a similar decrease in activity in both cdc2 and cdk2, whereas the corresponding protein levels were unaltered, as represented for HN12 (Fig. 3 *A*). Furthermore, a significant reduction of both cdc2 and cdk2 activity was achieved with 300 nM of flavopiridol, and PhosphorImager analysis demonstrated that the mean reduction of this activity in the three cell lines (HaCaT, HN12, and HN30) was $\sim 75\%$ (Fig. 3 *B*). Of note, cdc2 activity in all cell lines was more sensitive to flavopiridol

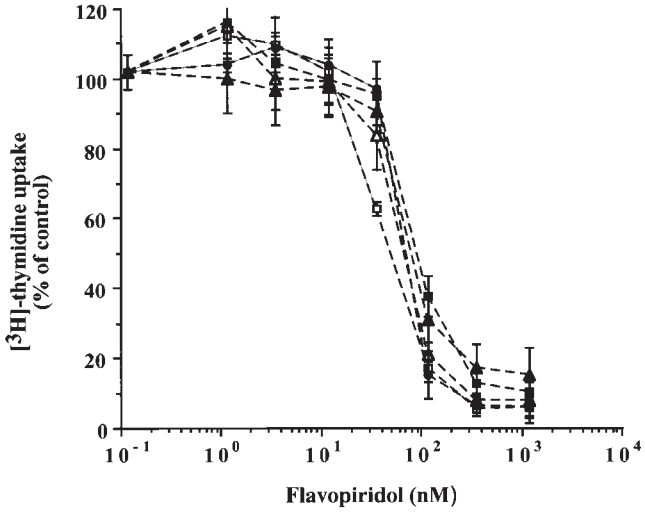

Figure 1. Antiproliferative effect of flavopiridol on HNSCC cells. Cells (HaCaT, *open boxes*; HN4, *open triangles*; HN8, *filled circles*; HN12, *filled triangles*; and HN30, *filled boxes*) were seeded ($1–2 \times 10^4$/well) into 24-well plates and grown for 12–18 h. Cells were further grown for 24 h in the presence of flavopiridol (1–1,000 nM) or DMSO (0.1%) and DNA synthesis was subsequently assessed by the cell's ability to incorporate [^{3}H]thymidine. The growth inhibitory effect of flavopiridol is shown as percent inhibition of [^{3}H]thymidine incorporation relative to control. The results are the mean (±SEM) of three independent experiments and were used to calculate the IC_{50} of each cell line.

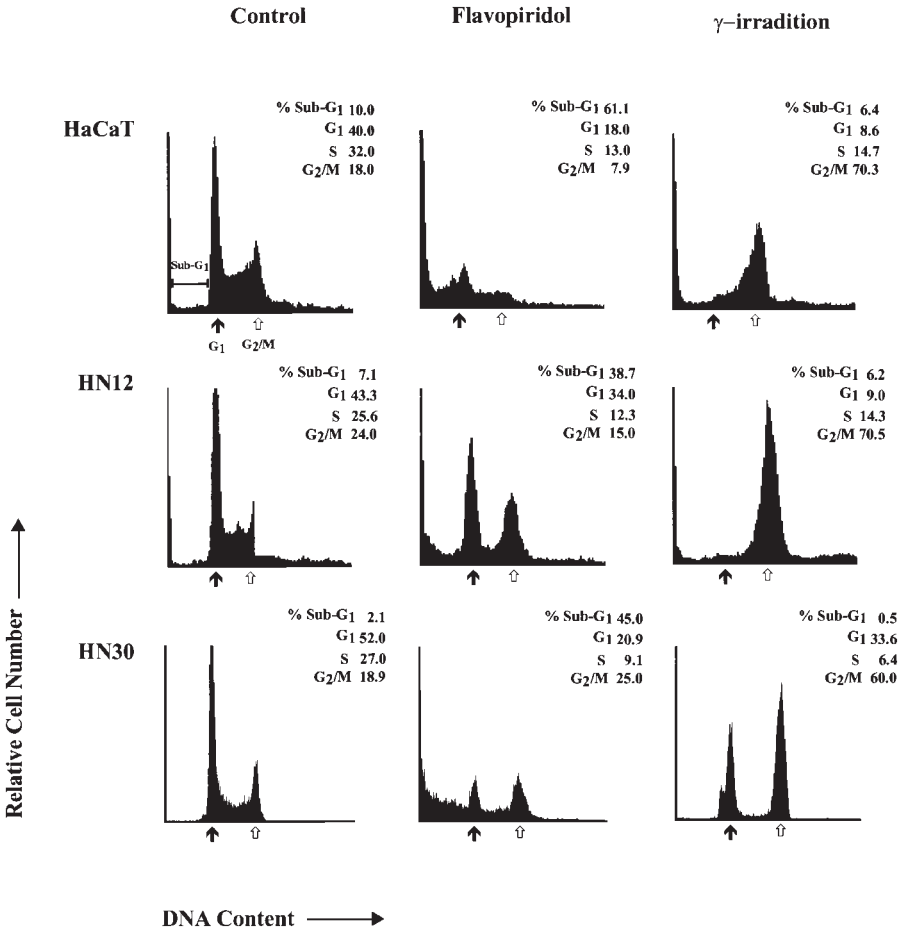

Figure 2. Flow cytometric analysis of HNSCC cells exposed to flavopiridol or γ-irradiation. Asynchronous HN12, HN30, and HaCaT cells were grown to 40–50% confluence and then harvested after 12–18 h of treatment with either flavopiridol (300 nM), γ-irradiation (2 Gy), or DMSO. Cell fixation, RNA hydrolysis, and DNA staining with propidium iodide were carried out as outlined in Methods. Histograms of cellular DNA content were obtained by flow cytometry and the positions of G_1 (*black arrows*), G_2/M (*white arrows*), and DNA content (%) for each phase of the cell cycle are indicated. The data shown are representative of three independent experiments.

than cdk2 (Fig. 3 *B*). In these cell lines, we did not observe any transient increase in cdk activity (1–3 h) as previously reported (14). Taken together, our results indicate that flavopiridol may interfere with the normal phosphorylating activity of cdc2 and cdk2 of HNSCC cells, possibly resulting in altered cell cycle control and subsequent apoptosis.

Effects of flavopiridol on expression of cyclin D1. Since our previous results suggested that flavopiridol may function by targeting cdk activity, we were interested in exploring whether other cell cycle components were also affected. For this, we analyzed lysates from control and flavopiridol-treated cells for expression of cyclin D1 and cyclin E. The results show that while the expression levels of cyclin E were unaffected in HaCaT and all HNSCC cells, those of cyclin D1 were significantly reduced in each cell line when exposed to flavopiridol (Fig. 4). To investigate whether this effect was specific to flavopiridol, lysates from cells treated with bleomycin or γ-irradiation were analyzed in parallel for the expression of cyclin D1 and cyclin E. As shown in Fig. 4, levels of these proteins were largely unchanged by these treatments. Similar results were obtained for the remaining HNSCC cell lines (HN4 and HN8, data not shown). Thus, these data suggest that flavopiridol may be preferentially targeting cyclin D1 for reduced protein expression and that it might contribute to the cell cycle effects elicited by this novel compound.

Flavopiridol induces apoptosis in HNSCC cells. Since we had observed an increase in a sub-G_1 population of HNSCC cells treated with flavopiridol, we investigated whether these cells had undergone apoptosis. As an approach, we determined the ability of flavopiridol to cause DNA fragmentation in HNSCC cells, using bleomycin and γ-irradiation, known to induce apoptosis in these cell types, as controls. As previously reported, HN30 cells were insensitive to γ-irradiation and subsequently bleomycin, whereas both HaCaT and HN12 were sensitive to both apoptotic agents (Fig. 5). By contrast, 18 h of exposure to flavopiridol was sufficient to induce apoptosis in all cell lines tested (Fig. 5).

As a complementary approach, flavopiridol-treated cells (HaCaT, HN12, and HN30) were labeled for DNA strand breaks using the TUNEL technique and analyzed microscopically. When compared with the controls (Fig. 6, *A*, *C*, and *E*), treated cells (Fig. 6, *B*, *D*, and *F*) were positively labeled by TUNEL, and these apoptotic cells were also detected when counterstained with DAPI (data not shown). In addition, HaCaT and HN12 cells treated with the DNA-damaging agents bleomycin and γ-irradiation were also labeled positively by TUNEL, but no labeling was observed in similarly treated HN30 cells (data not shown). Collectively, these results indicate that flavopiridol potently induces apoptosis in this cell type, even in cell lines that are insensitive to other apoptotic stimuli, such as to certain DNA-damaging agents and γ-irradiation.

Effects of flavopiridol on HNSCC xenografts. Since flavopiridol displays antiproliferative and apoptotic effects on HNSCC lines in vitro, we set out to investigate whether this compound would have antitumor activity in HNSCC tumor xenografts.

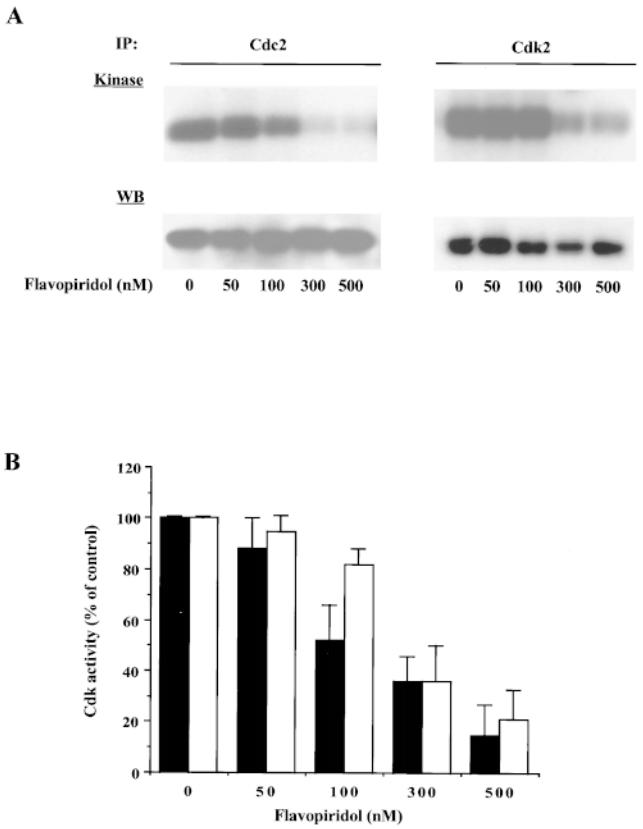

Figure 3. cdk activity in HNSCC cells exposed to flavopiridol. 500-μg aliquots of total cell lysate were prepared from cells (HN12, HN30, and HaCaT) treated with flavopiridol (0–500 nM) or DMSO and used to immunoprecipitate cdk complexes. Immunoprecipitates of HNSCC and HaCaT cells treated with flavopiridol (0–500 nM) and DMSO for 12–14 h were washed extensively, and histone H1 kinase reaction assay was carried out as described. Reactions were resolved by SDS-PAGE, dried, and autoradiographed. cdc2 and cdk2 activities for HN12 are shown (*A*, *top*). Parallel Western blots were performed on the same immunoprecipitates to indicate internal control for cdk activity (*A*, *bottom*). Quantification of cdc2 (*filled bars*) and cdk2 (*open bars*) activity of treated and control cells (HN12, HN30, HaCaT) was performed with a PhosphorImager and the mean values of the three cell lines are shown (*B*). Data shown are representative of those obtained from three independent experiments.

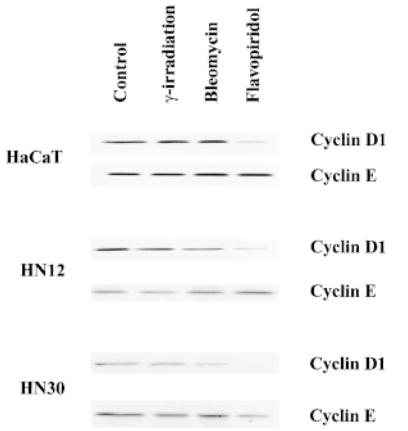

Figure 4. Expression of cyclin D1 in flavopiridol-treated HNSCC cells. 50-μg aliquots of total cellular lysate from cells (HN12, HN30, and HaCaT) treated with flavopiridol (300 nM; 12–18 h), bleomycin (10 μg/ml; 24 h), γ-irradiation (2 Gy; 24 h after treatment), or DMSO were subjected to SDS-PAGE and Western blotted as described in Methods. Blots were probed with antibodies as indicated and detected by ECL. Data shown are similar to those obtained from three independent experiments.

Subcutaneous tumors of HN12 cells were established in athymic *nu/nu* mice within 12 d (7–9 mm in diameter) and were subsequently treated as described. Upon completion, animals from the treated and control groups were killed and tumor tissue and serum were retrieved for analysis, or the animals were monitored for body weight and tumor growth for a further 10 wk. Immediate effect of flavopiridol was noticeable by the end of the treatment period, at which time the treated group demonstrated a reduction in tumor growth by $\sim$23% (Fig. 7). This reduction was sustained, and by 10 wk these lesions were $\sim$60% smaller than those from control animals. Collective data from both groups and representative of three independent experiments are summarized in Fig. 7, where the overall reduction in the tumor growth in the treated group is shown to be significant ($P < 0.05$) where indicated. Analysis of the T/C (ratio of the changes in the median tumor size of treated and control animals) for each time point revealed that the optimal T/C was 26% (74% reduction; $P < 0.001$; day 31). However, assessment of tumor shrinkage in some animals in the treated group was difficult, possibly due to an acute inflammatory response, similar to those observed in patients from a recent phase I clinical trial (18). Currently, the mechanism(s) for this phenomenon is under investigation. Drug toxicity was limited, since when assessed by weight loss, animals in the treated group demonstrated only a marginal reduction (< 10%) during the treatment period and made full recovery within 3–4 d of completion.

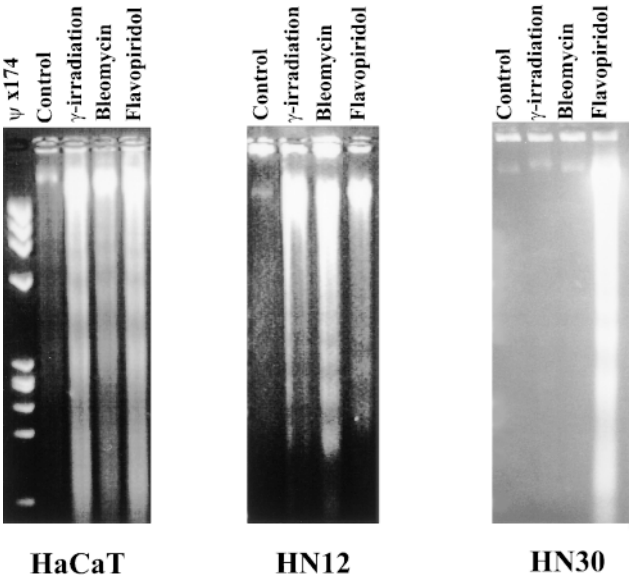

Figure 5. Flavopiridol-induced DNA fragmentation in HNSCC cells. Cells (HN12, HN30, and HaCaT) were treated with flavopiridol (300 nM; 12–18 h), bleomycin (10 μg/ml; 24 h), γ-irradiation (2 Gy; 24 h after treatment), or DMSO. Attached and floating cells were harvested and the fragmented DNA was isolated and analyzed as described in Methods. Similar results were observed in three additional experiments.

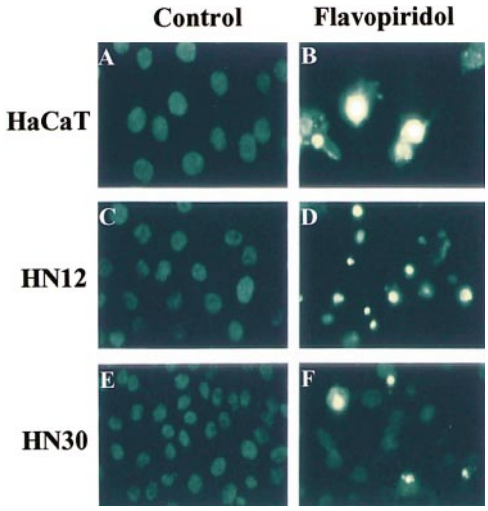

Figure 6. TUNEL staining of HNSCC cells treated with flavopiridol. HN12, HN30, and HaCaT cells were grown overnight on coverslips and exposed to flavopiridol (300 nM) or DMSO for 12–18 h, labeled using the TUNEL assay as described, and analyzed under fluorescence light. Results shown are representative of three independent experiments.

Analysis of HNSCC tumor xenografts for apoptosis and expression of cell cycle genes. Excised tumor tissues from both control and treated groups were analyzed for apoptosis, using the TUNEL assay. Although increased apoptotic staining was observed as early as day 2 of flavopiridol treatment (Fig. 8, *A* and *B*), the level of TUNEL staining was remarkably elevated upon completion of treatment (Fig. 8, *C* and *D*). To further confirm that the TUNEL staining was indeed specific and was not labeling necrotic tissue, incorporated fluorescein nucleotides were detected by using an antifluorescein antibody conjugated with horseradish peroxidase and analyzed under light microscope after substrate reaction and counterstaining. Control tumor tissue, when stained with hematoxylin and eosin, was observed to be largely undifferentiated with multinucleated cells that demonstrated little or no apoptosis. In contrast, in the treated tumor tissue, a significant increase in apoptotic cells was detected while remaining morphologically similar as control tissue (data not shown). Together, these data suggest that flavopiridol has potent antitumor activity in vivo, possibly by inducing tumor cells to undergo apoptosis.

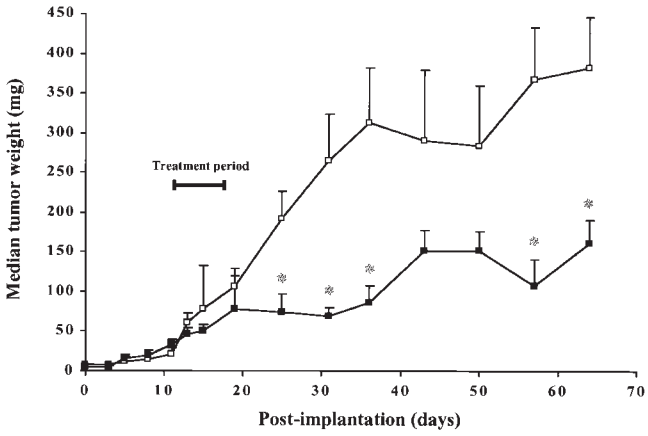

Figure 7. Antitumor activity of flavopiridol. HN12 cells were used to induce xenografts in athymic *nu/nu* mice. Animals were treated on day 12 (intraperitoneally each day for 5 d) with either flavopiridol (*filled squares*; 5 mg/kg; $n = 16$) or equivalent volume of DMSO (*open squares*; control; $n = 8$). The treatment period is indicated. Tumor size in both groups was assessed twice weekly and tumor weight was calculated as described in Methods. The results are expressed as mean±SEM. Student's *t* test was used to determine the difference between the treated control group (*$P < 0.01$). Data are from a representative experiment that was repeated three times with similar results.

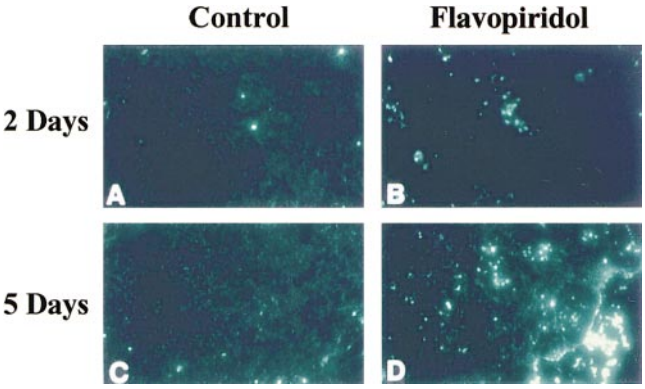

Figure 8. Apoptosis in tumor xenografts treated with flavopiridol. HN12 tumor xenografts from control (*A* and *C*) and treated animals (*B* and *D*) were obtained after 2 and 5 d of treatment, respectively, and paraffin tissue sections were labeled for DNA strand breaks using the TUNEL assay as described, and analyzed using fluorescence light. ×200.

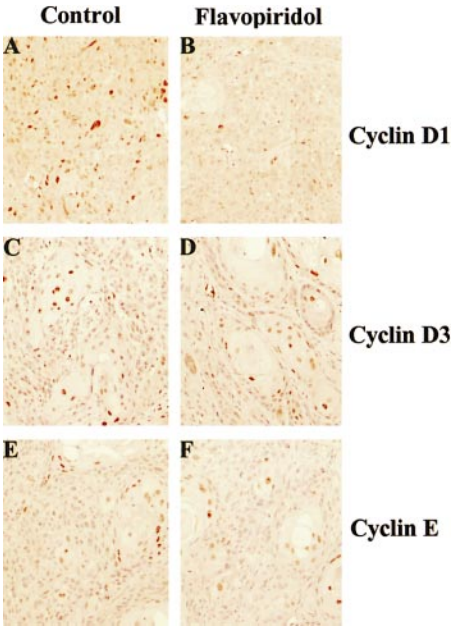

Figure 9. Expression of G_1 cyclins in HN12 xenografts treated with flavopiridol. HN12 tumor xenografts from control (*A*, *C*, and *E*) and 5-d-treated animals (*B*, *D*, and *F*) were analyzed for levels of cyclin D1, D3, and cyclin E. Paraffin sections were probed and stained with appropriate antibodies as indicated. Sections were analyzed under light microscopy and are shown at ×400.

A critical limitation of any antineoplastic agent is the nonselective targeting of normal tissue. To address this we assessed normal oral mucosa from animals from both treated and control groups, for incidence of apoptosis using the TUNEL assay. We observed only a very limited background level of apoptosis in tissues from untreated animals, which was similar to that observed in the flavopiridol-treated group (data not shown). Thus, at least in our model, flavopiridol may be preferentially targeting only tumor cells of squamous origin.

In parallel experiments, paraffin tumor tissues sections (as above) were analyzed for cyclin D1, D3, and cyclin E, and expression of all three gene products was readily detected in the control tissue (Fig. 9, *A*, *C*, and *E*). By contrast, however, a significant reduction in levels of cyclin D1 was observed in the treated tissue, whereas those of cyclin D3 and cyclin E were unaffected (Fig. 9, *B*, *D*, and *F*). Thus, expression of cyclin D1 was specifically diminished both in vitro (see above) and in vivo in response to flavopiridol treatment, thereby providing an indirect biochemical indicator to assess clinically the in vivo effectiveness of this novel chemotherapeutic agent.

Discussion

In this study we have used cell lines derived from a broad range of HNSCC and a HNSCC xenograft model to assess the antineoplastic effects of flavopiridol in HNSCC both in vitro and in vivo. We report that all HNSCC cell lines tested were sensitive to the antiproliferative and cytotoxic effects of flavopiridol with IC_{50} values (43–83 nM) similar to those reported for other cell types (10, 13, 29) and the average (66 nM) obtained from the NCI 60 cell line primary screen (10, 13, and our unpublished observations).

One such cell line (HN12), in addition to its sensitivity to flavopiridol, induces highly undifferentiated tumor xenografts in athymic mice. Thus, we viewed this as a suitable model to assess the antiproliferative and cytotoxic properties of this compound in vivo. The dose and schedule of flavopiridol used in this study were based on those that were safely tolerated in other model systems (1.5–10 mg/kg per day) (11–13). In this system, flavopiridol (5 mg/kg per day, i.p., for 5 d) demonstrated a significant reduction in tumor growth, with an optimal %T/C of 26% ($P < 0.001$; day 31), which is lower than that considered to be of predictive value for antitumor activity (optimal %T/C < 40%) (27). This delay in tumor growth persisted up to 10 wk after treatment, with minimal weight loss (< 10% of total body weight). Thus, the effect of flavopiridol in our model of a poorly differentiated and metastatic HNSCC lesion is encouraging, and could facilitate the development of phase II trials of this compound in patients with this devastating disease.

Since cdks play a pivotal role in cell cycle regulation (30, 31) they are obvious targets for directing inhibitory molecules that can alter the rate of cell proliferation (17). In this regard, flavopiridol, recently identified as a potential drug candidate for cancer treatment, has been demonstrated to behave as a potent and remarkably specific cdk inhibitor (13–16, 32). Similarly, we report that flavopiridol treatment decreased cdc2 and cdk2 activity in HNSCC cells in a dose-dependent manner, and that kinase inhibition correlates with its antiproliferative effects. Of interest, the IC_{50} for inhibition of proliferation (40–80 nM) was marginally lower then that demonstrated for cdk inhibition (100–300 nM), suggesting that additional mechanisms may be responsible for the antiproliferative effects of flavopiridol. One such mechanism may involve a remarkable reduction of expression of cyclin D1 in cultured cells and in xenograft tissue upon flavopiridol treatment. Indeed, this reduction of cyclin D1 expression that was observed was found not to be a general phenomenon associated with apoptotic stimulation, but to be specifically induced in response to flavopiridol treatment. In this regard, recently available data suggest that flavopiridol does not affect the rate of degradation of the cyclin D1 protein, but instead diminishes mRNA levels by a yet unknown mechanism (Senderowicz, A.M., unpublished observations), which is under current investigation. Although how flavopiridol diminishes cyclin D1 levels is still unclear, this finding may be of unexpected clinical relevance as many HNSCC are reported to overexpress cyclin D1, which might contribute to the tumorigenic process (33–35). Furthermore, this drastic reduction of cyclin D1 expression in response to flavopiridol may provide a valuable marker to assess the efficacy of flavopiridol in vivo.

Antineoplastic agents for clinical use are required to selectively target tumor cells, with cell death as a favorable response (36, 37). Although it still remains unclear how flavopiridol may be functioning as an antineoplastic agent, the accumulating evidence suggests that the flavopiridol-induced cytotoxic and antitumor effects may indeed be due to apoptosis (38, 39). In this study, we observed that all HNSCC cells as well as tumor xenografts exhibit a remarkable increase of apoptotic cells upon treatment with flavopiridol, thus indicating that this compound might act as an antineoplastic agent for HNSCC by activating cell death pathways. Of interest, our data suggest these phenomena are independent of p53 and p16 function. These findings may have significant implications for the future use of flavopiridol as a chemotherapeutic agent, since in many cases recurrences of HNSCC after γ-irradiation may result from an in vivo clonal expansion of radiation-insensitive cells, possibly with altered p53 function (40). Together, these data suggest that flavopiridol may be a good candidate for treating HNSCC lesions irrespective of prior radiation therapy.

In summary, the results presented in this report suggest that flavopiridol displays potent antitumor properties, in both in vitro and in vivo models of HNSCC and may be associated with reduced levels of cyclin D1 and, thus, represents an attractive candidate for testing in the treatment of HNSCC, particularly for those lesions insensitive to conventional therapy. These data might provide the basis for the early assessment of flavopiridol in patients with refractory squamous carcinomas of the head and neck.

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