Induction of Apoptosis by Flavopiridol in Human Neuroblastoma Cells Is Enhanced under Hypoxia and Associated With N-myc Proto-oncogene Down-Regulation

Maura Puppo,1 Sandra Pastorino,4 Giovanni Melillo,3 Annalisa Pezzolo,2 Luigi Varesio,1 and Maria Carla Bosco1

1Laboratory of Molecular Biology, and 3Laboratory of Oncology, Giannina Gaslini Institute, Genoa, Italy; 2Developmental Therapeutics Program-Tumor Hypoxia Laboratory, Science Applications International Corp.-Frederick, Inc., National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Maryland; and 4Neuro-Oncology Branch, National Cancer Institute, NIH, Bethesda, Maryland

ABSTRACT

Purpose: Neuroblastoma is the most common extracranial solid tumor of children that arises from the sympathetic nervous system. Survival rates for neuroblastoma patients is low despite intensive therapeutic intervention, and the identification of new effective drugs remains a primary goal. The cyclin-dependent kinase inhibitor, flavopiridol, has demonstrated growth-inhibitory and cytotoxic activity against various tumor types. Our aim was to investigate flavopiridol effects on advanced-stage, N-myc proto-oncogene (MYCN)-amplified human neuroblastomas and the modulation of its activity by hypoxia, a critical determinant of tumor progression and a major challenge of therapy.

Experimental Design: Cell viability was monitored by 3-(4,5 dimethyl-2 thiazolyl)-2,5 diphenyl-2H tetrazolium bromide (MTT) and trypan blue dye exclusion assays; DNA synthesis was assessed with the bromodeoxyuridine pulse-labeling technique; apoptosis was studied by Giemsa staining, DNA fragmentation, terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling reaction, flow cytometric determination of hypodiploid DNA content, and evaluation of caspase activity and cytochrome c (CytC) release; MYCN expression was determined by Northern and Western blotting.

Results: Flavopiridol caused dose- and time-dependent decreases in neuroblastoma viability by inducing apoptosis, as confirmed by morphologic and biochemical criteria. Cell death was preceded by DNA synthesis inhibition and G1-G2 arrest, reversed by the pancaspase inhibitor, zVAD-fmk, and associated with caspase-3 and -2 activation and CytC increase. Moreover, flavopiridol strongly down-regulated MYCN mRNA and protein expression. Exposure to hypoxia enhanced both the extent of apoptosis and flavopiridol effects on CytC, caspase 3, and MYCN.

Conclusions: These results indicate that flavopiridol has growth-inhibitory and apoptotic activity against advanced-stage neuroblastomas in vitro and is worthy of further investigation for the treatment of this disease.

INTRODUCTION

Neuroblastoma is the most common pediatric solid tumor; it derives from the neural crest and arises in the adrenal medulla or paraspinal ganglia (1). Neuroblastomas exhibit clinical and biological heterogeneity, ranging from rapid progression associated with metastatic spread and poor clinical outcome to occasional, spontaneous, or therapy-induced regression or differentiation into benign ganglioneuroma (2). Several transformation-linked genetic changes have been identified in neuroblastoma (1, 2). Amplification of the N-myc proto-oncogene (MYCN), which occurs in a subset of tumors and results in enhanced MYCN expression, is the most typical genetic feature of advanced-stage neuroblastoma and an adverse prognostic indicator (1–3). MYCN amplification correlates with a more malignant course of the disease, induction of angiogenesis, resistance to therapy, and poor clinical outcome (3–6), suggesting that it may be a progression-related event and a potential therapeutic target (2).

Rapidly expanding neuroblastoma tumors present areas of low O2 tension (hypoxia) and metastasize to hypoxic sites, such as bone and bone marrow (7). Hypoxia is a common denominator of many pathologic processes and a critical determinant of tumor cell growth, susceptibility to apoptosis, and resistance to radio- and chemotherapy (8–10). Hypoxia activates the hypoxia-inducible transcription factor-1 (HIF-1), a heterodimeric complex composed by the basic-helix-loop-helix PAS proteins, HIF-1α (or the homologues HIF-2α/3α), which is the hypoxia-responsive subunit, and HIF-1β, also known as the aryl hydrocarbon receptor nuclear translocator (ARNT), and constitutively expressed in unstimulated cells (9, 11). HIF-1 transactivates the hypoxia-responsive element (HRE) present in the promoter or enhancer elements of many genes encoding angiogenic, metabolic and metastatic factors (10, 12, 13), inducing their expression and contributing to the acquisition of tumor cell aggressive phenotypic changes (8–10). Accumulation of HIF-1α/2α proteins was observed in vitro in advanced-stage,
metastatic human neuroblastoma cell lines exposed to hypoxia and in the hypoxic areas of experimental neuroblastoma xenografts grown in mice (14, 15). Recent findings have demonstrated that hypoxia has a profound impact on neuroblastoma aggressive behavior. Increased production of the angiogenic mediator, vascular endothelial growth factor (VEGF), and of the Id2 protein, an inhibitor of the antiproliferative function of the retinoblastoma (Rb) tumor suppressor gene (16), was observed in hypoxic neuroblastoma cells both in vitro and in vivo (14, 17). Furthermore, hypoxia down-regulates several neuronal/endoctrine marker genes in neuroblastoma cells and, conversely, up-regulates genes expressed in neural crest sympathetic progenitors, leading to neuroblastoma cell differentiation and acquisition of an immature and more aggressive neural-crest-like phenotype (14).

Despite the array of chemotherapeutic agents presently available (2, 18) and multimodality therapeutic protocols that use high-dose chemotherapy associated with autologous bone marrow or stem-cell transplantation (2, 7, 19), the prognosis of children who develop metastatic, stage IV neuroblastoma over the age of 1 year has only marginally improved, and the overall long-term disease-free survival rate of this tumor remains low (2, 7, 19–21). Thus, the search for effective treatments for neuroblastoma, either at advanced stage or at minimal residual disease, remains a primary goal. The regulation of neuroblastoma growth, differentiation, and apoptosis has become an area of active research in the last decade with the aim of identifying novel and more specific therapeutic agents. Advanced-stage, MYCN-amplified neuroblastomas usually present defects in the expression and function of elements of the apoptotic machinery, such as deletion/inactivation of genes involved in cell death induction and/or constitutive activation/overexpression of genes counteracting the apoptotic process (for a review, see ref. 22) that have been implicated in neuroblastoma development, malignant progression, and resistance to traditional cytotoxic agents (22–25). Much work has consequently focused on the possibility of restoring the normal rate of apoptosis in neuroblastoma, and the identification of novel agents capable of triggering programmed cell death in this type of tumor has become an important therapeutic objective (2, 22).

One potential candidate in this line of research is flavopiridol (Flp), a semisynthetic flavone derivative of the alkaloid rohitukine (26) that has raised considerable interest in the past few years because of its potent antineoplastic activity against a variety of tumors both in vitro (27–31) and in vivo in human xenografts models (27–29), including melanomas and small-cell lung carcinomas, that share with neuroblastoma the neuroectodermal origin (28, 32, 33). Preclinical evidence also indicates that this agent can potentiate the therapeutic effects of conventional chemotherapeutics (34, 35) and is endowed with antiangiogenic and antimetastatic properties that may contribute to its cytotoxic activity in vivo (13, 17, 36). Flavopiridol is currently undergoing Phase II/III clinical trials for the treatment of various refractory neoplasms, both as a single agent and in combination with other antineoplastic drugs (for a review, see ref. 36), and promising therapeutic activity associated with mild toxicity has been observed (36–38). Numerous in vitro studies have shown that flavopiridol can inhibit tumor cell growth either by causing cell cycle arrest at the G1 and G2 phases, through the suppression of cyclin-dependent kinase (CDK) activity (36, 39, 40), or by triggering apoptosis (27, 29–31, 33, 35, 41). Remarkably, flavopiridol apoptotic activity can be exerted on both cycling and noncycling tumor cells (30, 40) and is refractory to various genetic alterations commonly found in human neoplasias (42–44), a property that makes this compound a promising therapeutic agent for the treatment of malignancies resistant to other chemotherapeutic drugs.

The purpose of this study was to assess the effects of flavopiridol on neuroblastoma cells, with particular attention to the induction of apoptosis and the biochemical mechanisms that may regulate this process. Moreover, we were interested in investigating the modulation of flavopiridol activity by hypoxia. We demonstrate that pharmacological concentrations of flavopiridol (in the 200–300 nmol/L range) arrest cell growth and trigger programmed cell death in advanced-stage, MYCN-amplified neuroblastoma cells lines. Flavopiridol-induced apoptosis involves both caspase activation and cytchrome c (CytC) release and is enhanced on cell exposure to hypoxia. Moreover, we show a correlation between flavopiridol cytotoxic activity and MYCN down-regulation.

MATERIALS AND METHODS

Cells and Culture Conditions. The human neuroblastoma cell lines GI-LI-N, LAN-5, ACN, GI-ME-N, IMR-32 (purchased from the Bank of Biological Material Interlab Cell Line Collection, Advanced Biotechnology Center, Genoa, Italy), and HTLA-230 (45) were isolated from patients with metastatic, stage IV neuroblastoma tumors and were characterized for the presence of molecular alterations (1). With regard to MYCN status, amplification was observed in GI-LI-N, LAN-5, IMR-32, HTLA-230, but not in ACN and GI-ME-N cell lines (1, 45). Cells were maintained in the logarithmic phase of growth in RPMI 16140 (Euroclone Ltd., Celsbio, Milan, Italy), supplemented with 10% heat-inactivated fetal bovine serum (Sigma, Milan Italy), 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin (Euroclone Ltd), at 37°C in a humidified atmosphere. Oxygen tension in the medium was measured with a portable, trace oxygen analyzer (Oxi 315i/set, WTW; VWR International, Milano, Italy). Cells were maintained with 10% heat-inactivated fetal bovine serum (Sigma, Milan Italy), 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin (Euroclone Ltd), at 37°C in a humidified atmosphere. Oxygen tension in the medium was measured with a portable, trace oxygen analyzer (Oxi 315i/set, WTW; VWR International, Milano, Italy). For cell growth and apoptosis determination, adherent cells were detached from culture plates with a solution of 0.05% trypsin and 0.02% EDTA in PBS (Euroclone Ltd) and were plated at subconfluent densities 2 days before the addition of the reagents.

Reagents. Flavopiridol (obtained from the Developmental Therapeutics Program, Drug Synthesis and Chemistry Branch, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, MD), was dissolved in DMSO as a 10-mmol/L stock solution and was stored in aliquots at −80°C. Flavopiridol was diluted in culture medium and was used at final concentrations ranging from 50 to 300 mmol/L. zVAD-fmk, a pancaspase inhibitor that irreversibly binds to the cata-
lytic site of caspases 1 to -9 inhibiting apoptosis, was purchased from Becton Dickinson (Milan, Italy), dissolved in DMSO at a concentration of 10 mmol/L, and stored in aliquots at -20°C. zVAD-fmk was used at a final concentration of 50 μmol/L.

**MTT Assay.** Exponentially growing LAN-5 and GI-LI-N cells were plated in triplicate wells of 96 flat-bottomed-well Corning plates (Celtibio) at a density of 7 × 10⁴ and 2 × 10⁴ per well, respectively, and were exposed to increasing flavopiridol concentrations or to DMSO for different time points. After the incubation period, 3-(4,5 dimethyl-2 thiadiazolyl)-2,5 diphenyl-2H tetrazolium bromide solution (MTT; Sigma) was added to each well to a final concentration of 0.5 mg/mL and was incubated for 4 hours to allow MTT reduction. The formazan crystals were dissolved by adding a solubilization solution containing 0.01 M HCl/10% SDS, and absorbance was measured at the dual-wavelengths of 570 and 630 nm with a SPECTRAFluor Plus spectrophotometer (TECAN Italia, S.r.l., Cologno Monzese, Milan, Italy). The data, subtracted of background MTT absorbance, are expressed as percentages of viable cells relative to control cells at each time point. In some experiments, cell viability was assessed by trypan blue dye exclusion test. Cells were harvested at the indicated time points after drug exposure and were stained with 0.2% trypan blue. Live (trypan blue-excluded) and dead cells (trypan blue-stained) were then counted on a hemocytometer.

**Morphologic Evaluation of Apoptosis.** One × 10⁵ cells were applied to Polysine glass slides by cytocentrifuging at 900 rpm for 10 minutes, were fixed in methanol, and were stained with May-Gruenwald-Giemsa dye (Sigma). Stained cytospin preparations were examined with a phase contrast microscope (Olympus Italia second s.r.l., Segrate, Milan, Italy). Photomicrographs were taken with a Zeiss camera (Zeiss, Jena, Germany).

**DNA Fragmentation Analysis.** Cells (2 × 10⁶) were washed with ice-cold PBS, were lysed with TE buffer [10 mmol/L Tris-HCl (pH 8), 1 mmol/L EDTA] containing 0.2% Triton X-100, and were centrifuged for 30 minutes at 13,000 rpm to isolate the cytoplasmic DNA fragments (soluble) from Triton X-100, and were centrifuged for 30 minutes at 13,000 rpm to isolate the cytoplasmic DNA fragments (soluble) from Triton X-100 and 0.1% sodium citrate for 2 minutes at 4°C. After rinsing, slides were incubated with 50 μL of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) reaction mixture, containing TdT- and FITC-labeled dUTP, in a humidified atmosphere for 1 hour at 37°C in the dark. Rinsed slides were then coverslipped with Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) for nuclear counterstaining. TUNEL+ apoptotic cells, which fluoresce bright green, were viewed with a Nikon Eclipse E1000 fluorescent microscope (Nikon Corp., Tokyo, Japan) equipped with a FITC filter. The percentage of apoptotic cells on total cell number was determined for each sample in a blind fashion by counting the number of green fluorescent nuclei among a total of 300 or more DAPI-stained blue nuclei in randomly chosen fields, at a magnification of ×200/0.5 (objective). Photomicrographs were taken with a Zeiss camera. In some experiments, TUNEL labeling was carried out on cells in suspension (2 × 10⁶ per sample) fixed in ice-cold 70% EtOH. Total DNA was then counterstained by resuspending the cells in 0.5 mL PBS containing 5 μg/mL PI and 100 μg/mL DNase-free RNase for 30 minutes at room temperature in the dark. Cells were analyzed for fluoresceinlabeled dUTP incorporation (FITC) versus DNA content (PI) by two-color flow cytometry with a FACScan. FITC and PI fluorescences were carried out on cells fixed in 70% ice-cold EtOH. DNA was extracted sequentially with phenol/chloroform (1:1) and chloroform/isamyl alcohol (24:1). DNA was then precipitated with 3 mol/L sodium acetate (pH 5.2), 1 mol/L MgCl₂, and 100% isopropanol, and was resuspended in TE buffer. DNase-free RNase A (50 μg/mL; Roche Molecular Chemicals, Milan, Italy) was then added for 2 hours at 37°C for RNA digestion. DNA samples were electrophoresed on 1.2% agarose slab gel containing 0.1 μg/mL ethidium bromide. A 1-kb DNA ladder (New England Biolabs, Celebio, Milan) was run in parallel to provide molecular-sized markers. DNA fragments in a ladder pattern were visualized by UV light trans-illumination.

**Flow Cytometric Analysis of Hypodiploid DNA Content.** Cells (5 × 10⁶) were washed with PBS, fixed in ice-cold 70% EtOH, and stained for 1 hour in the dark with a solution containing 0.1% sodium citrate, 0.1% Triton X-100, 50 μg/mL propidium iodide (PI; Sigma), and 100 μg/mL DNase-free RNase in PBS. Monovariate distributions of cell number versus DNA content (PI) were analyzed with a FACScan flow cytometer (Becton Dickinson, Milano, Italy) equipped with a xenon lamp and a filter set for excitation at 488 nm. PI fluorescence intensity was recorded through a 575-nm high pass filter. At least 20,000 events were collected in each histogram, and data were analyzed with CellQuest software (Becton Dickinson). Apoptotic cells could be observed as a “sub-G₁” peak on the DNA histogram.

**TUNEL Assay.** DNA cleavage was assessed by enzymatic end-labeling of DNA strand breaks with a commercial kit (In Situ Cell Death Detection kit, Fluorescent, Roche Molecular Chemicals). Labeling was carried out according to the manufacturer’s instructions. Briefly, 5 × 10⁴ neuroblastoma cells were cytocentrifuged onto Polysine glass slides, fixed in 4% paraformaldehyde in PBS for 1 hour at room temperature, and washed with PBS. Cells were then, permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate for 2 minutes at 4°C. After rinsing, slides were incubated with 50 μL of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) reaction mixture, containing TdT- and FITC-labeled dUTP, in a humidified atmosphere for 1 hour at 37°C in the dark. Rinsed slides were then coverslipped with Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) for nuclear counterstaining. TUNEL+ apoptotic cells, which fluoresce bright green, were viewed with a Nikon Eclipse E1000 fluorescent microscope (Nikon Corp., Tokyo, Japan) equipped with a FITC filter. The percentage of apoptotic cells on total cell number was determined for each sample in a blind fashion by counting the number of green fluorescent nuclei among a total of 300 or more DAPI-stained blue nuclei in randomly chosen fields, at a magnification of ×200/0.5 (objective). Photomicrographs were taken with a Zeiss camera. In some experiments, TUNEL labeling was carried out on cells in suspension (2 × 10⁶ per sample) fixed in ice-cold 70% EtOH. Total DNA was then counterstained by resuspending the cells in 0.5 mL PBS containing 5 μg/mL PI and 100 μg/mL DNase-free RNase for 30 minutes at room temperature in the dark. Cells were analyzed for fluoresceinlabeled dUTP incorporation (FITC) versus DNA content (PI) by two-color flow cytometry with a FACScan. FITC and PI fluorescences were carried out on cells fixed in 70% ice-cold EtOH. DNA was extracted sequentially with phenol/chloroform (1:1) and chloroform/isamyl alcohol (24:1). DNA was then precipitated with 3 mol/L sodium acetate (pH 5.2), 1 mol/L MgCl₂, and 100% isopropanol, and was resuspended in TE buffer. DNase-free RNase A (50 μg/mL; Roche Molecular Chemicals, Milan, Italy) was then added for 2 hours at 37°C for RNA digestion. DNA samples were electrophoresed on 1.2% agarose slab gel containing 0.1 μg/mL ethidium bromide. A 1-kb DNA ladder (New England Biolabs, Celebio, Milan) was run in parallel to provide molecular-sized markers. DNA fragments in a ladder pattern were visualized by UV light trans-illumination.

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cence intensities were recorded through a 520/530 nm and 575-nm filters, respectively. At least 20,000 events were collected in each plot. Data were analyzed with the CellQuest program.

**Analysis of DNA Synthesis by Bromodeoxyuridine Pulse-Labeling Technique.** Exponentially growing cells (2 × 10^6) were pulsed-labeled with 10 μmol/L bromodeoxyuridine (BrdUrd; Sigma), 4 or 18 hours before harvesting, were washed with 1% bovine serum albumin (BSA)/PBS, and fixed with 70% ice-cold EtOH for 30 minutes. Cells were then incubated for an additional 30 minutes with 2M HCl/0.5% Triton X-100, to partially denature the DNA, were washed, and were resuspended in PBS/1%BSA/0.5% Tween 20 solution. Incorporated BrdUrd was stained with 20 μL of FITC-conjugated anti-BrdUrd mouse monoclonal antibody (mAb; Becton Dickinson) for 30 minutes at room temperature. Samples were then washed and resuspended in PBS containing 5 μg/mL PI. Bivariate distributions of BrdUrd amounts (FITC) versus DNA content (PI) were analyzed by a FACScan flow cytometer with Cell Quest software. A total of 20,000 to 50,000 events were collected in each final plot.

**Evaluation of Cytochrome c Release.** CytC levels in cytoplasmic extracts were assessed with a specific ELISA (Bender MedSystems, Vienna, Austria), according to the manufacturer’s instructions. Briefly, 1.5 × 10^6 cells were washed in cold PBS and were lysed with lysis buffer. Cell lysates were cleared by centrifugation and were assessed for protein concentration with the Bradford assay (Bio-Rad, Richmond, CA). Equal amounts of proteins diluted in assay buffer were loaded onto a 96-well plate coated with an anti-CytC mAb and were subjected to ELISA. CytC concentrations were determined by measuring the absorbance values at 450 nm with a SPEC-TRAFluor Plus spectrophotometer and plotting them onto a standard CytC curve.

**Determination of Caspase Activity.** The protease activity of caspases 3, -8, -9, and -2 was determined simultaneously with the BD ApoAlert Caspase Assay plates (Becton Dickinson), according to the manufacturer’s instructions. Briefly, neuroblastoma cells (2 × 10^6 per sample) were washed with cold PBS and lysed with ice-cold cell lysis buffer. Cell lysates were cleared by centrifugation, and the supernatants were transferred to 96-well caspase assay plates, containing immobilized selective caspase substrates covalently linked to the fluorogenic dye AMC (7-amino-4-methyl coumarin), and incubated for 2 hours at 37°C. Caspase activity in each tested lysate was determined by measuring the intensity of the fluorescent signal generated by substrate cleavage and consequent AMC release with a SPEC-
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A. FLP

Gi-LI-N

LAN-5

B. Flp (nmol/L)

GI-LI-N

LAN-5

C. M

d

e

D. % TUNEL+ cells

IMR-32

HTLA-230

GI-ME-N

ACN

DMSO

Flp

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TRAFluor Plus spectrofluorometer with excitation at 388 nm and emission at 460 nm.

**Southern and Northern Blot Analysis.** Genomic DNA was isolated from GI-LI-N, LAN-5, and ACN cells, cultured in 15-cm Costar plates at a density of 1 × 10^6 (LAN-5 and ACN) or 1.5 × 10^6 cells/mL (GI-LI-N) with the DNAzol reagent (Life Technologies, Cebio), according to the manufacturer’s instructions. Ten μg of DNA from each sample was digested with EcoRI restriction enzyme (New England Biolabs [NEB]), and the DNA fragments were electrophoresed on a 0.6% agarose gel, denatured by treatment with a solution of NaOH 0.4 M/NaCl 0.6 mol/L, and transferred to Nytran membranes (Schleichen & Schuell Inc., Keen, NH). A 1-kb marker was run in parallel as a molecular-sized standard. Total cellular RNA was purified from LAN-5 and GI-LI-N cells with the TriZOL RNA reagent (Life Technologies), according to the manufacturer’s instructions. Twenty μg of RNA from each sample was electrophoresed under denaturing conditions on a 1.2% agarose gel containing 2.2 mol/L formaldehyde and transferred to Nytran membranes. A RNA marker was run in parallel as a molecular-sized standard. Filter hybridization was done with 2 × 10^6 cpm/mL of 5'-[α-32P]dCTP-labeled human MYCN cDNA, kindly provided by Dr. Tonini (Advanced Biotechnology Center, Genoa, Italy), in Hybrisol I hybridization solution (Oncor, Gaithersburg, MD) as described previously (17). Blots were autoradiographed with Kodak XAR-5 film (Eastman Kodak, Rochester, NY), and quantitative assessment of the band intensity was carried out with the VersaDoc Image Analyzer (Bio-Rad Laboratories, Hercules, CA).

**Western Blot Analysis.** Neuroblastoma cells (1 × 10^7/ mL) were solubilized in TBS-E lysis buffer [20 mmol/L Tris (pH 8), 50 mmol/L NaCl, 5 mmol/L EDTA] containing 1% NP40, 10% glycerol, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 μg/mL pepstatin, 10 mmol/L DTT, and 1 mmol/L 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF; Roche). One hundred micrograms from each cleared cell lysate were resolved on 8% SDS-PAGE and electroblotted to Immobilon-P nitrocellulose membranes (Millipore Corp., Bedford, MA). A prestained protein marker (175–6.5 kDa; NEB) was run as a molecular-size standard. Membranes were immunoblotted overnight with biotin-conjugated antihuman MYCN mAb (OncoGene Research Products, Boston, MA), which recognizes two nuclear phosphoproteins of molecular mass 64,000 Daltons and 67,000 Daltons coded by the human MYCN gene, in blocking buffer (TBS supplemented with 0.5% Tween 20 and 5% BSA), washed in TBS supplemented with 0.05% Tween 20, and incubated for 30 minutes with horseradish peroxidase-conjugated-streptavidin (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) in washing buffer containing 2.5% BSA. An anti-β-actin mAb (Sigma) was used as an internal control for loading. Immunoreactivity was detected with enhanced chemoluminescence (ECL, Pierce, Cebio, Milan, Italy) and was autoradiographed.

**Statistical Analysis.** Data are the means ± SE of at least three independent experiments. The Student’s t test was used to determine the significance of the results (significance at P < 0.05).

**RESULTS**

**Flavopiridol Is Cytotoxic to Neuroblastoma Cells.** To study flavopiridol activity on advanced-stage neuroblastoma cells, the human neuroblastoma cell lines, GI-LI-N and LAN-5, which exhibit MYCN gene amplification (Fig. 1) and express MYCN protein (1), were exposed to increasing concentrations of flavopiridol (ranging from 0 to 300 nmol/L), and relative cell numbers were then assessed at different time points by MTT assay (Fig. 2A). Both cell lines displayed dose- and time-dependent decreases in viability, detectable as early as 24 hours after culture with 50 nmol/L of the drug. Seventy-two hours of continuous exposure to 100 nmol/L flavopiridol resulted in ~82 and 67% reduction in GI-LI-N and LAN-5 cell number, respectively, whereas 300 nmol/L were required to achieve total inhibition in both cell lines (Fig. 2A). Increased cell death was observed by trypan blue dye exclusion in flavopiridol-treated cells relative to control cultures (Fig. 2B), suggesting a cytotoxic activity of the drug.

**Flavopiridol Cytotoxicity Occurs by Programmed Cell Death.** Experiments were then carried out to establish whether neuroblastoma response to flavopiridol was apoptotic. As evidenced by phase-contrast microscopy of Giemsa-stained cysteine preparations (Fig. 3A), ~15% of GI-LI-N and LAN-5 cells, treated for 24 hours with 300 nmol/L flavopiridol, displayed typical morphologic hallmarks of apoptosis, including intense shrinkage, progressive cytoplasm and chromatin condensation, and nuclear fragmentation (22). Conversely, less than 4% of the control cells exhibited apoptotic features (Fig. 3A). Flavopiridol-treated neuroblastoma cells eventually rounded up and detached from the dish (data not shown).

Internucleosomal cleavage of genomic DNA is typical of
Fig. 4 Dose- and time-dependent induction of hypodiploid DNA in GI-LI-N and LAN-5 cells by flavopiridol (Flp). GI-LI-N cells were stimulated: (A) for 24 hours with increasing concentrations of Flp (50–300 nmol/L) or the vehicle alone (−); (B) with 300 nmol/L Flp for 48 hours (panel b) and 72 hours (panel c) or with DMSO for 72 hours (panel a). In C, LAN-5 cells were exposed for the indicated time points to different doses of Flp. Hypodiploid changes were evaluated by flow cytometric analysis after PI staining. A and B, histograms of total DNA content, representative of one of three different determinations. Horizontal axis, linear fluorescence intensity. Vertical axis, relative cell number. In each panel (A and B), the percentage of cells with sub-G1 DNA content is indicated. In C, the percentages of cells containing hypodiploid DNA are plotted on a bar graph (h, hours). Bars are the mean ± SEM of three independent experiments. 2N, diploid DNA content; 4N, tetraploid DNA content.
cells undergoing apoptosis (22). As shown in Fig. 3B, a characteristic pattern of nucleosome-sized ladder of DNA fragments was detectable by agarose gel electrophoresis in both cell lines after a 24-hour flavopiridol treatment, with as little as 100 nmol/L able to induce DNA fragmentation in GI-LI-N and 200 nmol/L effective in LAN-5. Almost no DNA fragmentation was observed in control cells (Fig. 3B).

Induction of apoptosis was confirmed by the TUNEL assay (Fig. 3C), which measures DNA strand breaks in individual cells (22). We detected a time-dependent increase in the number of cells displaying small, bright green fluorescent condensed nuclei after exposure to 300 nmol/L flavopiridol (Fig. 3C). The percentages of TUNEL+ cells were ~17 and 15% in GI-LI-N and LAN-5 cells, respectively, after 24 hours (Fig. 3C, c and d), correlating with cell death evaluated by trypan blue exclusion, and increased progressively with time in culture, reaching ~35–40% and 50–60% after 48 (Fig. 3C, e and f) and 72 hours (Fig. 3C, g and h). Apoptotic bodies were also observed (Fig. 3C, d, inset). Conversely, only a few control cells exhibited a faint fluorescent signal after 72 hours of culture (Fig. 3C, a and b). No TUNEL+ cells were detectable in cultures treated with flavopiridol for 12 hours or at earlier time points (data not shown). Induction of apoptosis was also triggered by flavopiridol in another four human neuroblastoma cell lines derived from metastatic, stage IV neuroblastoma tumors, two exhibiting high levels of MYCN gene amplification and MYCN protein expression (IMR-32 and HTLA-230; ref. 1, 45) and two without amplified MYCN gene (ACN and GI-ME-N; ref. 1), ranging from 30 to 72% after 48 hours of treatment (Fig. 3D), thus providing clear evidence that programmed cell death is a general response of neuroblastoma cells to flavopiridol.

To better quantify the percentage of cells dying of apoptosis, GI-LI-N and LAN-5 cells were exposed to increasing drug concentrations for various time points and were analyzed by flow cytometry after PI staining (Fig. 4), which allowed us to identify cells with a sub-G1 DNA content (22). A dose-dependent increase in the percentage of cells containing hypodiploid DNA was detected in GI-LI-N cells after 24 hours of exposure to the drug, with ~17% of cells undergoing apoptosis in response to 300 nmol/L (Fig. 4A). Longer exposure to flavopiridol further increased the apoptotic fraction of cells, causing up to 56% cell death after 72 hours (Fig. 4B), consistent with the TUNEL results. Conversely, less than 6% apoptotic cells were detected in control cultures (Fig. 4A and B). Comparable results were obtained in flavopiridol-treated LAN-5 cells (Fig. 4C), with only slight differences in the extent of cell death.

We conclude that flavopiridol triggers apoptotic cell death in neuroblastoma cells.

**Flavopiridol-Induced Apoptosis Is Preceded by Cell Cycle Arrest.** Given the ability of flavopiridol to impose a block to cell cycle progression in several tumor types (36, 39, 40), we were interested in determining whether induction of neuroblastoma apoptosis was coupled with growth arrest. To test this possibility, we exposed exponentially growing LAN-5 (Fig. 5A) and GI-LI-N (Fig. 5B and C) cells to flavopiridol and pulse-labeled them with BrdUrd for 4 (Fig. 5A and B) or 18 hours (Fig. 5C). The amount of BrdUrd incorporated into DNA was then evaluated by fluorescence-activated cell sorting as a measure of DNA synthesis. As depicted in Fig. 5A and B, which shows BrdUrd uptake (FITC) versus total cellular DNA content (PI), ~40% of control cells actively incorporated BrdUrd during the last 4 hours of a 24-hour incubation period. BrdUrd incorporation rate showed a dose-dependent decline on treatment with flavopiridol, with the concentrations required for a 50% reduction (IC$_{50}$) ranging between 100 and 200 nmol/L (Fig. 5A) and almost complete blockage occurring at 300 nmol/L (Fig. 5A and B). DNA synthesis inhibition was an early response to flavopiridol, because a 12-hour treatment with 300 nmol/L was sufficient to inhibit BrdUrd uptake by 80% (Fig. 5B). The decrease in BrdUrd-incorporation rate was paralleled by a 3- to 5-fold increase in the percentage of cells with a G$_2$ DNA content, suggesting flavopiridol-mediated arrest of cell cycle progression at the G$_2$ checkpoint. Although no significant change in the G$_1$ cell fraction was apparent within the 4-hour pulse-labeling period (Fig. 5A and B), we detected cell accumulation in G$_1$ during a 18-hour BrdUrd pulse (Fig. 5C), suggestive of G$_1$ block from entry into S phase. Two-color flow cytometric measurement of dUTP incorporation (FITC) versus cellular DNA content (PI) evidenced apoptotic cell death in both G$_1$ and G$_2$ populations in response to longer drug exposure (Fig. 5D). A progressive increase in the fraction of TUNEL+ G$_1$ and G$_2$ cells was, in fact, detectable during a 24- to 72-hour treatment with 300 nmol/L flavopiridol, which, accordingly, decreased the number of viable cells in each population and increased the percentage of sub-G$_1$ cells relative to control cultures.

These results suggest that flavopiridol-treated neuroblastoma cells arrest in G$_1$ and G$_2$ before undergoing apoptotic cell death.

**Hypoxia Enhances Flavopiridol-Induced Apoptosis.** Next, we investigated the effects of O$_2$ deprivation on neuroblastoma response to flavopiridol. GI-LI-N and LAN-5 cells were cultured for various time points under normoxic (20% O$_2$) or hypoxic conditions (1% O$_2$), in the presence or absence of flavopiridol, and relative cell numbers were assessed by MTT. Hypoxia led to a time-dependent increase in neuroblastoma cell number, which was 1.5- and 2-fold higher than that of normoxic controls by 48 and 72 hours of culture, respectively (data not shown). In accordance to what was shown under normoxia, flavopiridol caused dose- and time-dependent decreases in hypoxic cell viability, with almost complete inhibition achieved after 72 hours’ exposure to 300 nmol/L. The drug IC$_{50}$ under hypoxia and normoxia are compared in Table 1 for each of the time points studied. Under normal oxygen tension, the IC$_{50}$ values were within the 50- to 120-nmol/L and the 75- to 175-nmol/L range in GI-LI-N and LAN-5 cells, respectively, depending on the length of exposure, which suggests a greater sensitivity of GI-LI-N cells to flavopiridol. Exposure to 1% O$_2$ caused some decrease of the IC$_{50}$ values, although this effect did not appear to be statistically significant (P > 0.05).

The effects of hypoxia on apoptosis induction by flavopiridol were then evaluated. As shown in Fig. 6A, which depicts electrophoretic analysis of genomic DNA in flavopiridol-treated GI-LI-N and LAN-5 cells cultured under 1% O$_2$ tension, DNA fragmentation was effectively elicited in both cell lines by as low a concentration as 100 nmol/L flavopiridol, suggesting a higher sensitivity of hypoxic than normoxic LAN-5 cells to flavopiridol, in agreement with the MTT assay. Moreover, as determined by TUNEL reaction, combined exposure to fla-
vopiridol and hypoxia resulted in a 1.6-fold increase in the percentage of cells undergoing apoptosis relative to flavopiridol alone (Fig. 6B). As expected, no DNA laddering was detectable in cells exposed to hypoxia alone, which, accordingly, did not substantially modulate the number of TUNEL+ cells (Fig. 6A and B). Essentially equivalent results were obtained when apoptosis was evaluated by PI staining. The time course of appearance of apoptotic LAN-5 cells is depicted in Fig. 6C, which shows a representative experiment of three experiments performed. Combined exposure of LAN-5 cell to hypoxia and flavopiridol increased the number of cells with a sub-G₀ DNA content relative to cells treated with flavopiridol alone. Apoptosis enhancement by hypoxia was detectable after 24 hours culture and was still evident at later time points, with the percentage of apoptotic cells augmented from 50% to almost 80% after 72 hours. Similar results were observed in GI-LI-N cells (data not shown).

These results demonstrate that hypoxia enhances flavopiridol cytotoxic effects on neuroblastoma cells.

**Flavopiridol Induces Caspase Activation and Cytochrome c Release.** Experiments were carried out to elucidate the role of caspases (46) in apoptosis induction by flavopiridol. Figure 7A depicts the kinetics of caspase 2, -3, -8, and -9 activity in GI-LI-N cells. Exposure to flavopiridol resulted in a marked and rapid activation of caspase 3, which was already evident after 3 hours and was maximal after 6 hours, decreasing but remaining elevated at later time points. Induction of caspase 2 activity was also triggered by flavopiridol with a similar kinetics, although the extent of activation was lower than that of caspase 3. In contrast, under the same experimental conditions flavopiridol failed to induce caspase 9 and -8 enzymatic activity.

The effects of hypoxia on caspase activation by flavopiridol were evaluated in parallel experiments. Although hypoxia alone did not affect protease activity at any of the time points analyzed, it enhanced flavopiridol effects on caspase 3, leading to approximately a 1.5 increase in its activation over that induced by flavopiridol alone after 6 hours of stimulation (data not shown). A similar pattern of results was observed in LAN-5 cells (data not shown), suggesting the involvement of caspases 3 and -2 in apoptosis induction by flavopiridol.

We next analyzed the effects of the cell-permeable pan-caspase inhibitor, zVAD-fmk. We first determined the concentration of zVAD-fmk necessary to inhibit caspase activation by flavopiridol. As shown in Fig. 7A, caspases 3 and -2 activity was suppressed by preincubation for 2 hours with 50 μmol/L zVAD-fmk. Accordingly, both flavopiridol-mediated cell killing (Fig. 7B) and internucleosomal DNA fragmentation (Fig. 7C) were
blocked by zVAD-fmk, suggesting that caspases 3 and -2 activation was instrumental in these processes.

The possibility that mitochondrial events were also involved in apoptosis induction by flavopiridol was next addressed by determining flavopiridol effects on CytC. CytC translocation from mitochondria to the cytosol occurs at an early step of programmed cell death and is causally related to this process (47). CytC cytoplasmic content was determined at different time points in both LAN-5 (Fig. 7D) and GI-LI-N cells (data not shown) by a specific ELISA. Flavopiridol did not significantly affect CytC cytosolic concentration during the first 3 hours of treatment but led to a 4.5- and 6-fold increase after 6 and 12 hours, respectively, relative to control cells. Although hypoxia alone was ineffective in augmenting CytC content, it enhanced the effects of flavopiridol (Fig. 7D) up-regulating CytC basal levels after 3 hours’ exposure. Similar results were obtained in the GI-LI-N cell line (data not shown), suggesting that CytC is released in response to flavopiridol and that hypoxia can cooperatively interact with flavopiridol in eliciting this effect.

Taken together, these results indicate that caspase 3 and -2 activation associated with CytC release is involved in apoptosis induction by flavopiridol in neuroblastoma.

**Flavopiridol Down-Regulates MYCN Expression in Neuroblastoma Cells.** MYCN down-regulation was found to correlate with drug-induced apoptosis and growth inhibition in neuroblastoma (48–51). We were, thus, interested in determining whether flavopiridol modulated MYCN expression. To address this question, we examined the changes in MYCN mRNA and protein in GI-LI-N (Fig. 8) and LAN-5 (data not shown)
after exposure to increasing concentrations of flavopiridol for different time points. GI-LI-N cells constitutively expressed high levels of MYCN transcript, which remained essentially constant for up to 24 hours (Fig. 8A and B). Flavopiridol induced a rapid and dose-dependent mRNA down-regulation, detectable with as low a concentration as 100 nmol/L flavopiridol (Fig. 8A) and within the first hour of culture (Fig. 8B). mRNA levels continued to decrease with time in culture, until they became barely detectable. Conversely, culture under hypoxia alone only marginally affected MYCN expression (Fig. 8A and B). Interestingly, however, hypoxia further enhanced flavopiridol inhibitory effects, inducing complete inhibition of mRNA expression after 24 hours’ exposure to 300 nmol/L flavopiridol (Fig. 8A and B). Comparable down-regulation was detected in LAN-5 cells (data not shown). mRNA reduction was paralleled by decreased protein expression, which was more pronounced in hypoxic versus normoxic cells (Fig. 8C).

These data indicate that flavopiridol down-regulates MYCN expression in MYCN-amplified neuroblastoma cells.

**DISCUSSION**

Impaired apoptosis is involved in the pathogenesis of cancer, and triggering this process in tumor cells is an important target of therapy (52, 53). Advanced-stage, MYCN-amplified neuroblastoma are often resistant to conventional chemotherapeutic drugs because of aberrations/dysfunctions in their apop-

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**Table 1** Comparison of Flp effects on neuroblastoma viability under normoxia and hypoxia

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>IC$_{50}$ (nmol/L)</th>
<th>24 hours</th>
<th>48 hours</th>
<th>72 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>GI-LI-N</td>
<td></td>
<td>120 ± 25</td>
<td>80 ± 10</td>
<td>50 ± 7</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>110 ± 30</td>
<td>72 ± 11</td>
<td>49 ± 5</td>
</tr>
<tr>
<td>LAN-5</td>
<td></td>
<td>175 ± 45</td>
<td>110 ± 20</td>
<td>75 ± 11</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>160 ± 25</td>
<td>80 ± 16</td>
<td>50 ± 6</td>
</tr>
</tbody>
</table>

NOTE. Exponentially growing GI-LI-N and LAN-5 cells were seeded into 96-well plates and treated for the indicated time points with increasing concentrations of Flp (0 to 300 nmol/L) under normoxic (20% O$_2$) or hypoxic (1% O$_2$) conditions. MTT assay was then used to assess relative cell numbers. Concentrations of Flp required to reduce by 50% viable cell numbers (IC$_{50}$) at the different time points are presented as the mean ± SE of four independent experiments, each performed in triplicate.

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**Fig. 6** Enhancement by hypoxia of flavopiridol (Flp) cytotoxic activity on neuroblastoma cells. A, dose-dependent DNA fragmentation. GI-LI-N and LAN-5 cells were treated for 24 hours under hypoxia alone (−) or in the presence of the indicated concentrations of Flp, and DNA fragmentation assay was carried out. The 1-kb ladder is shown as molecular-sized marker (M). B, detection of apoptosis by TUNEL assay. TUNEL assay was carried out on cells treated for 24 hours with 300 nmol/L Flp (+), or with the vehicle alone (−), under hypoxic (Hy, +) or normoxic conditions (Hy, −), as described in the legend to Fig. 3C and D. Results (mean ± SEM of three independent experiments) are presented as a bar graph and expressed as percentages of TUNEL+ cells. C, determination of hypodiploid DNA content. LAN-5 cells were cultured for 24 hours (panels b, f, 48 hours (panels c, g), and 72 hours (panels d, h) with 300 nmol/L Flp, or for 72 hours with DMSO alone (panels a, e), under normoxic (Normo, a, b, c, d) and hypoxic (Hy, e, f, g, h) conditions. Representative DNA histograms generated from flow cytometric analysis of PI-stained cells are shown. % in each panel, the percentages of cells containing hypodiploid DNA.
Fig. 7 Flavopiridol (Flp) triggers caspase activation and CytC release in neuroblastoma. A, time course of caspase activation by Flp in GI-LI-N cells. Cells were treated with 300 nmol/L Flp in the presence or absence of 50 µmol/L zVAD-fmk, and the proteolytic activity of caspases-3, -8, -9, and -2 was assessed at the indicated time points [1h (1 hour), 3h, 6h, 12h, 18h] against specific fluorogenic substrates, as detailed in Materials and Methods. Values are the mean of duplicate reactions run in parallel and are expressed as fold induction relative to protease activity in control samples (equal to 1) at each time point. One representative experiment of two performed is shown. B and C, inhibition of Flp-induced apoptosis by the pancaspase inhibitor, zVAD-fmk. GI-LI-N and LAN-5 cells were left untreated (−) or treated (+) with 50 µmol/L zVAD-fmk for 2 hours before the addition of 300 nmol/L Flp (+) or DMSO alone (−) for 24 hours, and (B) cell killing was determined by counting live (trypan blue excluded) and dead cells (trypan blue stained) on a hemacytometer. Results are presented as a bar graph and expressed as percentage of cell death. The values are the mean ± SEM of three independent experiments with SEM less than 10% of the mean. In C, DNA fragmentation assay was carried out as described in the legend to Fig. 3B. The 1-kb ladder is shown (M). D, assessment of CytC cytosolic content. LAN-5 cells were treated for the indicated time points (3h, 6h, 12h) with 300 nmol/L Flp (+), or the vehicle alone (−) under hypoxic (Hy, +) or normoxic (Hy, −) conditions, and cytosolic extracts were tested for CytC (Cytochrome) concentration by ELISA, as described in Materials and Methods. Results are presented as a bar graph and are the mean ± SEM of two different experiments, each done in triplicate.
totic machinery (22–25), making the search for novel agents capable of inducing programmed cell death in this type of tumor mandatory. The data presented in this paper provide the first evidence that the synthetic flavone, flavopiridol, causes apoptosis in neuroblastoma cell lines derived from metastatic, MYCN-amplified, stage IV tumors.

Flavopiridol activity against a variety of tumor types, both in vitro and in vivo in experimental xenografts, is well documented (26–33, 39, 41), and recent findings have reported promising therapeutic results of this agent in patients with refractory neoplasms (36–38). To our knowledge, studies of flavopiridol antitumor effects have been limited to adult malignancies, and this is the first report showing that this compound is also active on a childhood tumor. We demonstrate that flavopiridol activity on neuroblastoma cells is strictly dependent on the concentration of the drug and the duration of exposure. GI-LI-N cells appeared to be slightly more susceptible than LAN-5 cells to flavopiridol, in agreement with previous observations showing that the LAN-5 cell line is endowed with a more aggressive phenotype (1, 17). It is noteworthy that the range of effective doses of flavopiridol (50–300 nmol/L) is comparable with that shown to be active in other tumor systems (27, 30, 41), indicating a similar sensitivity of neuroblastoma and other cancer cell types to flavopiridol. The observation that these doses correspond to clinically achievable pharmacological concentrations of the drug (26, 36, 38) suggest potential therapeutic implications of these findings. However, clinical trials of flavopiridol are limited to adults, and studies should be conducted in children to determine its safe doses in pediatric patients.

Flavopiridol was initially characterized as a cytostatic agent, capable of blocking cell cycle progression through the inhibition of multiple CDK activity (36, 39, 40, 43). It was later found to be an efficient inducer of apoptosis in a variety of cancer cell lines (27, 29–31, 33, 41, 43), suggesting the engagement of different growth-inhibitory pathways depending on the histotype of malignant cells. We demonstrate that flavopiridol is cytotoxic to neuroblastoma cells and that cytotoxicity is the result of programmed cell death. A time- and dose-dependent decrease in viable cell counts occurred in response to the drug, with complete inhibition achieved after a 72-hour exposure to a 300 nmol/L concentration, and was associated with the appearance of morphologic hallmarks of apoptosis. Activation of an apoptotic pathway was confirmed by accumulation of cells with a sub-G1 DNA content and by the demonstration of DNA fragmentation, caspase activation, and CytC release. Importantly, flavopiridol cytotoxicity was not unique to GI-LI-N and LAN-5 cells but was also exerted on another four cell lines derived from advanced-stage neuroblastoma tumors, two exhibiting high levels of MYCN amplification and two without amplified MYCN gene, indicating that apoptosis is a general response of neuroblastoma cells to flavopiridol. Interestingly, short exposure (i.e., 12–24 hours) to the drug resulted in growth arrest, manifested by DNA synthesis inhibition and G1-G2 block of cell cycle progression, which preceded the time at which extensive cell death occurred (i.e., 48–72 hours). These find-
ings, along with the observation that both G1 and G2 cell populations underwent apoptosis during flavopiridol treatment, indicate that cell death was not restricted to one particular phase of the cell cycle and confirm the ability of flavopiridol to induce apoptosis in noncycling cells (29–31, 33, 40), thus raising the possibility that this agent can be combined with other chemotherapeutics causing growth arrest in neuroblastoma to potentiate their therapeutic activity, as shown in other tumor systems (34, 35). The mechanisms underlying flavopiridol-induced cell cycle arrest in neuroblastoma cells still remains to be elucidated, and we are currently investigating the possibility that the inhibition of CDKs, which are the primary targets of the drug at the dose range studied (36, 39), may account for this effect.

Apoptosis involves activation of members of the caspase family of cysteine proteases in a hierarchical cascade, with “initiator caspsases” functioning upstream as triggers of the apoptotic process and “executioner caspsases” acting downstream as effector elements (46, 54), and may be regulated by various mitochondrial apoptogenic mediators including CytC (47). Flavopiridol can trigger multiple apoptotic pathways depending on the type and stage of the target cell, thus circumventing drug-resistance mechanisms (42–44). Initial studies demonstrated that flavopiridol induced apoptosis via caspase 8 activation in some tumor cell types (42), whereas more recent evidence indicated that flavopiridol cytotoxic effects involved CytC release in others (33, 55, 56). Moreover, reports by Alonso et al. (44) and Achenbach et al. (42) demonstrated that flavopiridol apoptotic activity was exerted independently of both caspase 8 activation and CytC release in a panel of glioma and small-cell lung carcinoma cell lines, suggesting the implication of alternative, unidentified apoptotic mechanisms. In this study, we demonstrate that flavopiridol-mediated apoptosis in neuroblastoma occurred through the engagement of the mitochondrial-dependent pathway, as indicated by the marked increase in CytC cytosolic levels after short (i.e., 6–12 hours) drug treatment. Moreover, flavopiridol-treated neuroblastoma cells showed extensive activation of caspases-3 and -2. The inhibition of which by the pancaspase inhibitor zVAD-fmk completely reversed induction of cell death and DNA fragmentation, pointing toward a critical role for these proteases in regulating this process. The implication of caspase 3 in the death mechanism used by flavopiridol has been described previously in other systems (31, 33, 35, 42), whereas this is the first evidence that flavopiridol can trigger caspase 2 activation. Caspases 3 and -2 are known downstream effectors of apoptosis (46, 54). However, their potential involvement in the initiation of the caspase cascade has been proposed in recent studies that demonstrate their colocalization with various apoptogenic proteins within the mitochondrial intermembrane space in nonapoptotic cells and their rapid release and activation on apoptotic challenge (57, 58). Thus, the observation that flavopiridol induced early caspase 3 and -2 activation, before the occurrence of extensive cell death and concomitant to CytC release, raises the possibility that these molecules play an upstream regulatory role in flavopiridol-induced neuroblastoma apoptosis. Moreover, given the ability of caspase 2 to induce CytC release (57) and consequent activation of caspase 3 (47, 58), which in turn can activate caspase 2 (57), the temporal correlation between increased caspase-2 and -3 activity and CytC release suggests the existence of a circular positive feedback amplification loop between these apoptotic proteins in flavopiridol-treated neuroblastoma cells. In contrast, the lack of caspase-8 and caspase-9 activation in response to flavopiridol argues against the requirement for these molecules in the regulatory phase of flavopiridol apoptotic cascade in neuroblastoma, despite their well-recognized importance as initiator caspsases in apoptosis induced by other stimuli (46, 54, 59, 60).

Recent studies have documented an important role for hypoxia in neuroblastoma cell acquisition of an immature, angiogenic, and more aggressive neural-crest-like phenotype (14, 15, 17). Accordingly, xenografted hypoxia-pretreated neuroblastoma cells were reported to form palpable tumors early and to grow faster than grafted normoxia-treated cells (14). Because hypoxia is present at common sites of neuroblastoma relapse (7) and represents a major challenge to therapy (8–10), a critical question was whether flavopiridol retained its activity in a reduced O2 environment. Our data demonstrate that not only was flavopiridol active on hypoxic neuroblastoma cells but that hypoxia potentiated its cytotoxic activity by ~1.6-fold, with up to 80% hypoxic cells undergoing apoptosis after a 72-hour treatment. The increased sensitivity of hypoxic cells to flavopiridol was also indicated by the finding that lower IC_{50,O2} of the drug were required under hypoxia than under normoxia to achieve comparable decreases in viable cell counts and that a 100 nmol/L concentration of flavopiridol was sufficient to induce DNA fragmentation in LAN-5 cells cultured in 1% O2, whereas a 200 nmol/L concentration was necessary in normal O2 tension. The observation that ~10% flavopiridol-treated cells were TUNEL+ after 12 hours of culture under hypoxia, whereas they were undistinguishable from control samples when treated under normoxic conditions (data not shown), further supports this conclusion. These results were unexpected because no cell death was detectable in GI-LI-N and LAN-5 cells exposed to 1% O2. Conversely and in accordance with the results by Jogi et al. (14), the hypoxic stress resulted in increased proliferation over that seen at normal O2 tension, further supporting the hypothesis of a hypoxia-induced shift toward a more aggressive behavior.

The mechanisms accounting for the increased apoptotic response of hypoxic neuroblastoma cells to flavopiridol are unclear, although our results point toward the involvement of caspase-3 and CytC in this process. Indeed, we found that hypoxia enhanced flavopiridol effects on caspase-3 activation, and a higher and earlier increase in cytosolic CytC levels over that induced by flavopiridol alone was observed in response to the combined treatment. CytC release during apoptosis may depend on different mechanisms, including mitochondria swelling and physical disruption of the outer membrane, loss of mitochondrial transmembrane potential, or formation of permeability transition pores at the site of contact between mitochondrial inner and outer membrane (47). Whether any of these mechanisms could explain the synergistic effect of flavopiridol and hypoxia on CytC still remains to be elucidated. Because both flavopiridol and hypoxia can modulate the expression of some Bcl-2 family members, which regulate mitochondrial membrane integrity and the formation of permeability transition...
pores (10, 36, 43, 61), it is possible that their interaction may modify the ratio between pro- and antiapoptotic proteins, thus increasing mitochondrial permeability and CytC release. Loss of mitochondrial membrane potential is also known to result from changes in the expression of Bcl-2 family members and was previously shown to occur in response to flavopiridol (33, 42), indicating another potential mechanism for CytC increase. Detailed studies will be required to elucidate this issue.

Flavopiridol-treated neuroblastoma cells rapidly and substantially down-regulated MYCN expression. This finding is relevant, given the correlation between MYCN overexpression and neuroblastoma aggressive phenotype. Accumulation of MYCN protein is known to alter neuroblastoma cell cycle progression by increasing the levels of Id2 protein, with consequent inactivation of the Rb-dependent antiproliferative pathway (16), and by enhancing DNA-synthesis through the decrease of CDK inhibitor p27 expression and consequent induction of cyclin E-dependent kinase activity (50). Moreover, MYCN overexpression was found associated with neuroblasta-
oma angiogenesis, metastatic dissemination, and poor prognosis (4–6). Recent studies demonstrated that MYCN-amplified cell lines are tumorigenic when xenografted into immunodeficient mice and that MYCN-targeted expression in transgenic mice leads to neuroblastoma tumor development (62). Furthermore, growing evidence supports a link between MYCN amplification, impairment of neuroblastoma apoptotic response (23–25), and consequent resistance to therapy both in vitro (22, 49) and in vivo in neuroblastoma patients (63). Inhibition of MYCN expression has, thus, been proposed as a strategy for neuroblasto-
toma treatment. In this regard, down-regulation of MYCN expression by chemotherapeutics or antisense cDNA was found to correlate with the suppression of neuroblastoma cells growth (50, 51) and the induction of differentiation/apoptosis (48, 49, 51). Whether MYCN inhibition plays a role in neuroblastoma growth arrest and/or apoptosis induction by flavopiridol is controversial. The observation that hypoxia enhanced both MYCN down-regulation and the extent of flavopiridol-triggered cell death raises the possibility of a causal correlation between these two events. However, the data showing that flavopiridol cytotoxic activity was not restricted to MYCN-amplified cell lines argue against this hypothesis. This issue is intriguing and is currently under investigation.

In conclusion, our data demonstrate that MYCN-amplified neuroblastoma cells can be induced to undergo apoptosis by pharmacologically relevant concentrations of flavopiridol and that this response is preceded by cell cycle arrest, enhanced by hypoxia, and associated with caspase activation, CytC release, and MYCN down-regulation. These findings, together with the recently reported ability of flavopiridol to inhibit constitutive and hypoxia-induced VEGF production by aggressive neuroblastoma cell lines (17), make this compound a promising candidate for the treatment of advanced-stage neuroblastoma.

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Induction of Apoptosis by Flavopiridol in Human Neuroblastoma Cells Is Enhanced under Hypoxia and Associated With N-myc Proto-oncogene Down-Regulation

Maura Puppo, Sandra Pastorino, Giovanni Melillo, et al.