BCH-1868 [(-)-2-R-dihydroxyphosphinoyl-5-(S)-(guanin-9′-yl-methyl) tetrahydrofuran]: A Cyclic Nucleoside Phosphonate with Antitumor Activity

Lorraine Leblond,[1] Giorgio Attardo, Bettina Hamelin, David Y. Bouffard, Nghe Nguyen-Ba, and Henriette Gourdeau

Shire BioChem, Inc., Laval, Quebec, H7V 4A7 Canada

Abstract

Nucleoside phosphonates are widely used therapeutic agents with a broad spectrum of antiviral activity. However, only a few of them are reported to have antitumor activity. In this study, we show that a tetrahydrofuran phosphonate analogue of guanosine, (-)-2-R-dihydroxyphosphinoyl-5-(S)-(guanin-9′-yl-methyl) tetrahydrofuran (BCH-1868), previously reported as having antiviral activity, also displays antitumor activity. *In vitro*, BCH-1868 inhibited the proliferation of several murine and human cancer cell lines with IC_{50}s in the μM range independently of the tissue type or the presence of multidrug resistance protein MRP/gp190. *In vivo*, BCH-1868 was active against a variety of human tumor xenograft models (Caki-1, HT-29, DU 145, COLO 205, and CCRF-CEM). In all tumors tested, a significant tumor growth inhibition was noted at 40–50 mg/kg (daily × 5), but no tumor regression was observed in the settings used. To better understand these results, we partially characterized, at the cellular level, the mechanism of action of this new cyclic nucleoside phosphonate and investigated its pharmacokinetic characteristics in mice. We showed that BCH-1868 exerts its antitumor activity by an inhibitory mechanism at the level of DNA polymerase α, resulting in arrest of DNA synthesis and a block of cell division at the S phase of the cell cycle. Low-circulating plasma concentration (C_{max} = 87 μM; area under the curve = 1138 μmol·min/liters; after a bolus i.v. injection of 10 mg/kg) and rapid clearance of the drug (terminal half-life, $t_{1/2}$ = 16 min) may contribute to the modest antitumor efficacy observed *in vivo*.

Introduction

Nucleoside analogues constitute a major group of antimetabolite drugs in current clinical use for antiviral or cancer chemotherapy. They are generally prodrugs, which enter cells and are anabolized to their active diphosphate and triphosphate metabolites either by the viral thymidine kinase and/or cellular kinases. The diphosphate and triphosphate metabolites are incorporated into DNA during replication or repair, leading to termination of DNA chain elongation. Nucleoside analogues also compete with the cellular nucleotide pool for enzymes involved in the purine and pyrimidine metabolism (1). The most well-known representatives of this group are the dideoxynucleoside analogs 2′,3′-didehydro-2′,3′-dideoxythymidine and (-)-2′-deoxy-3′-thiacytidine and the acyclic nucleoside acyclovir and ganciclovir as antiviral agents (2). Nucleoside analogues with anticancer activity are 5-fluorouracil, pentastatin, fludarabine, cladribine, AraC,[2] and gemcitabine (3).

A significant limitation in the use of these nucleoside analogues is the development of viral or tumor resistance because of a loss of the key enzymes thymidine kinase or dCK. Acyclic nucleoside phosphonates are independent of this first phosphorylation step. They are nucleosides containing a catabolically stable phosphocarbon bond in a phosphonylmethylether group that simulates a phosphate moiety. Cidofovir and adefovir, representative members of this group, are potent and broad-spectrum antiviral agents (4). In addition to the antiviral activity, some of the acyclic nucleoside phosphonates are cytotoxic and possess antitumor potential (5–7). 9-(2-Phosphonylmethoxyethyl) adenine and, most particularly, the guanine derivative PMEG inhibit the growth of murine and human tumor cells. PMEG was shown to be a potent inhibitor of both human polymerase α and δ, two key enzymes involved in cellular DNA replication (8).

BCH-1868 is a representative of a new series of cyclic phosphonate nucleoside derivatives that was recently shown to have human cytomegalovirus activity (9). During the antiviral screening process, BCH-1868 showed reduced animal toxicity compared with cidofovir (10). In addition, we noted growth inhibitory activity on several human tumor cell lines, suggesting that in addition to its antiviral activity, BCH-1868 might also have antitumor activity. This molecule has structural features of the dideoxynucleoside analogue (didanosine), of the acyclic nucleoside analogue acyclovir and ganciclovir, and of the acyclic nucleoside phosphonate PMEG. It might also approximate an L-guanosine 5′ monophosphate. If this guanosine nucleoside analogue is processed intracellularly like acyclovir and ganciclovir, we may expect involvement of GMP kinase, a key enzyme for cancer

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[1] To whom requests for reprints should be addressed, at Shire BioChem Inc., 275 Armand-Frappier Boulevard, Laval, Quebec, H7V 4A7 Canada. Phone: (450) 978-7778; Fax: (450) 978-7946; E-mail: lleblond@ca.shire.com.

[2] The abbreviations used are: AraC, cytarabine, cytosine arabinoside; dCK, deoxycytidine kinase; PMEG, 9-(2-phosphonylmethoxyethyl) guanine; BCH-1868, (-)-2(R)-dihydroxyphosphinoyl-5-(S)-(guanin-9′-yl-methyl) tetrahydrofuran; HPLC, high-performance liquid chromatography; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HSF, human skin fibroblast; SCID, severe combined immunodeficient; q4dx3, 4-day interval × 3; q1dx5, daily × 5; ILS, increased life span; MRP/gp190, multidrug resistance protein; GMP, guanylate.

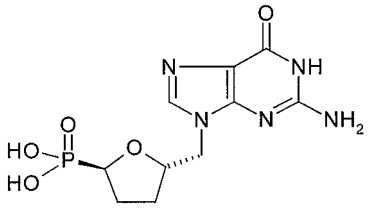

Fig. 1. Chemical structure of BCH-1868.

chemotherapy (11, 12). Furthermore, it might be a substrate for the polymerase α as reported for PMEG (8) and compete with the natural deoxynucleotide triphosphate pools. As evidenced by the literature, it has been very difficult to predict the mechanism of action, the metabolic fate, and the therapeutic activity of purine and pyrimidine nucleotide analogues just from their chemical composition.

This study was designed to evaluate the potential of BCH-1868 as an antitumor agent. To this end, we examined its *in vitro* antiproliferative activity against several murine and human tumor cells and its *in vivo* antitumor activity on a panel of human tumor xenografts. To gain additional insight into the mechanism of its antitumor activity, we also characterized the cellular mechanism involved in its toxicity. Finally, the pharmacokinetic profile of BCH-1868 was investigated.

Materials and Methods

Chemicals

BCH-1868 and its monophosphate and diphosphate forms were synthesized at Shire BioChem, Inc. (Laval, Québec, Canada), according to previously reported methods (9, 10). Its chemical structure is shown in Fig. 1. The [^{3}H]BCH-1868 was radiolabeled by Moravek Biochemicals, Inc. (Brea, CA), from material provided by Shire BioChem, Inc., with a radiochemical purity of 98.8% as analyzed by HPLC and was provided in ethanol:water solution (2:98 v/v). [4, 5-^{3}H]L-Leucine (63 Ci/mmol) and [5,6-^{3}H]uridine (43 Ci/mmol) were obtained from ICN Biomedicals (Toronto, Ontario, Canada). [methyl-^{3}H]Thymidine (2.0 Ci/mmol) was purchased from Amersham Pharmacia (Oakville, Ontario, Canada). Troxacitabine (BCH-4556 or Troxatyl) was synthesized at Shire BioChem, Inc. Doxorubicin was from Sigma (Oakville, Ontario, Canada) and AraC was from Bristol-Myers-Squibb (Montréal, Québec, Canada). All other reagents used were analytical grade and from commercial sources.

In Vitro Evaluation

Cell Lines and Cell Culture Conditions. Human renal carcinoma Caki-1 cells, human colon carcinoma COLO 205 cells, human colon carcinoma HT-29 cells, human breast carcinoma MCF-7 cells, human prostatic carcinoma DU 145 cells, human T lymphoblastic leukemia CCRF-CEM cells, and human promyelocytic leukemia HL-60 cells were obtained from the American Type Culture Collection (Manassas, VA). Human glioblastoma SF268 cells were obtained from the Frederick Cancer Research Tumor Repository (Frederick, MD). HL-60ADR-resistant cells, expressing the MRP/gp190 protein (13), were kindly provided by Dr. P. Genne (OncoDesign, Dijon, France). The CCRF-CEM/AraC (ARAC-8C), a human equilibrative nucleoside transport-deficient derivative of CCRF-CEM (14, 15), was a gift from Dr. B. Ullman (Portland, OR). The CCRF-CEM dCK$^-$, a dCK-deficient cell line, was obtained from Dr. A. Fridland (Memphis, TN). The murine melanoma B16F10 cells were obtained from Dr. I. Fidler (M. D. Anderson Cancer Center, Houston, TX), and the murine colon carcinoma MC38 cells were kindly provided by Dr. J. Young (National Cancer Institute, Bethesda, MD). HSF cells were prepared in-house according to a standard protocol (16).

Cells were grown in either RPMI 1640 (SF268, CCRF-CEM, CCRF-CEM dCK$^-$, CCRF-CEM/AraC, HL-60, HL-60ADR, MCF-7, COLO 205, and MC38), McCoy's 5 (Caki-1 and HT-29), or MEM (Eagle; HSF, DU 145, and B16F10). Media were supplemented with fetal bovine serum (10–20%; Life Technologies, Inc., Burlington, Ontario, Canada) and the supplements (nonessential amino acids, glutamine, sodium pyruvate, and vitamins; CellGro Mediatech, Inc., Herndon, VA) according to the cell supplier instructions. All cell lines were maintained in a humid chamber at 37°C in an atmosphere of 5% CO_2. Cells were grown in absence of antibiotics and *Mycoplasma* contamination was periodically checked with the Hoechst 33258 staining (17). Cell culture media were obtained from Life Technologies, Inc. (Grand Islands, NY), except for RPMI 1640 that was purchased from Wisent, Inc. (St. Bruno, Québec, Canada).

Cytoxicity Assays. Exponentially growing cells were seeded at appropriate density (1–2.5 $\times$ 10^3 cells/well) in 96-well plates (Falcon no. 353072) and allowed to attach overnight. Thereafter, test compounds, diluted in the appropriate vehicle, were added at a 1:10 serial dilution in the appropriate concentration range to a final volume of 200 μl. Cells were either continuously exposed to each drug for 72 h or exposed for a 4- or 24-h period. For the 4- or 24-h incubation period, drug-containing medium was removed, cells were washed, and fresh medium without drug was added for the remaining incubation period. At the end of the 72-h incubation period, cell viability was assessed both by measuring the NADH-dependent reduction of MTT to form a formazan product as described by Plumb *et al.* (18) and the [^{3}H]thymidine incorporation into DNA as described by Arnould *et al.* (19). For the [^{3}H]thymidine incorporation assay, 0.5 μCi/well [methyl-^{3}H]thymidine was added during the last 18 h of incubation. Cells were then trypsinized and harvested directly on a fiberglass filter using a Tomtec harvester (Tomtec, Orange, CT). Harvested plates were counted in a beta counter (Microbeta Counter no. 1450; Wallac, Turku, Finland). The MTT formazan was quantified by using an MR500 96-well microtiter plate reader (Dynatech MR5000 reader plate; Dynatech, Chantilly, VA) set at 570 nm. All measurements were performed in triplicate, and each experiment was repeated at least three times. The IC_{50}s were calculated using the PrismPad computer program (GraphPad Software, Inc., San Diego, CA) and are defined as concentration of drug causing 50% inhibition in absorbance or in radioactive material incorporation compared with control (vehicle) cells.

Inhibition of Macromolecular Synthesis and Uptake of Radiolabeled Precursor. HSF cells (10^4 cells/well) in exponential growth phase were incubated with BCH-1868 (0.1–100 μM). After a 1-h incubation period, 2 μCi/well of either [methyl-^{3}H]thymidine, [4, 5-^{3}H]L-leucine, or [5,6-^{3}H]uridine were added for either 3 h (4-h drug incubation period) or 23 h (24-h drug incubation period) to determine DNA, RNA, and protein synthesis, respectively. For the leucine incorporation assay, RPMI 1640 without leucine was used. The cells were trypsinized and harvested on a fiberglass filter using a Tomtec harvester. Radioactivity retained on the filter disk was determined by liquid scintillation counting (Microbeta counter no. 1450; Wallac).

Cell Cycle Analysis. HSF cells, exposed to 0.1–10 μM BCH-1868 for 24 h, were washed once with PBS, trypsinized, and collected by centrifugation. The pellet was washed and lysed with a 0.37% NP40 solution/0.1% solution of sodium citrate containing RNase A (0.02 mg/ml) and propidium iodide (0.05 mg/ml). The DNA content of the cells was determined by flow cytometry (Coulter Corporation's EPICS XL-MCL flow cytometer, Miami, FL), and the cell cycle analysis was performed with the Multicycle DNA content and cell cycle analysis software from Phoenix Flow Systems, Inc. (San Diego, CA). For each sample, 10,000 events were recorded.

Intracellular Metabolism of BCH-1868. CCRF-CEM cells (1–6 $\times$ 10^5/dish) were incubated with 1.5 μM [^{3}H]BCH-1868 in complete RPMI 1640 for 4, 24, or 48 h. Thereafter, the medium was removed and cells were collected, washed with ice-cold PBS, and counted. Cellular proteins were precipitated, and BCH-1868 and its metabolites were extracted overnight with 60% ice-cold methanol. Samples were centrifuged (15,000 $\times$ *g*; 15 min), and the supernatant was analyzed by HPLC. Reverse phase HPLC analysis was performed at an ambient temperature on a Waters system consisting of a 600 MS system controller, a 616 quaternary pump, a 996 photodiodearray detector, and a 717 autosampler Canberra Packard flow scintillation analyzer 150 TR. BCH-1868 and its phosphorylated metabolites were separated on a Zorbax Eclipse YMC ODS-A, XDB-C18 (5μ; 4.6-mm inside diameter $\times$ 250 mm) column. The mobile phase consisted of acetonitrile (A) and 50 mM phosphate buffer (50 mM Na_2HPO_4/NaH_2PO_4-50/50 v/v) containing 5 mM tetrabutyl ammonium dihydrogen phosphate (B) and was pumped at 1 ml/min. A gradient of 0–10% (A/B) over 30 min and 10–15% (A/B) over 15 min was applied before returning to initial conditions. Using these conditions, retention times of cold BCH-1868, BCH-1868-P, and BCH-1868-PP were 16.6, 29.0, and 43.0 min, respectively. The retention time of [^{3}H]BCH-1868 was 17.1 min. Data were processed using Millennium 3.05 software (Waters Corporate, Milford, MA).

DNA Polymerase α Inhibition Assay. DNA polymerase inhibition was measured by determining the incorporation level of a digoxigenin-labeled dUTP into a synthetic DNA template in the presence or absence of inhibitor. Briefly, a 57-mer synthetic oligonucleotide was annealed with a 19-mer 5′-biotin-labeled primer. The annealed (biotin-labeled) template (50 ng/well) was attached to a 96-well neutravidine-coated plate, and DNA polymerase α reaction was performed. Standard reactions were done in a 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 1 mM DTT, and 300 μg/ml BSA buffer containing 0.8 μM dATP, 1.4 μM dCTP, 3.0 μM dGTP, 4.8 μM dTTP, 0.3 μM dUTP-digoxigenin, 16 milliunits DNA polymerase α, and various dilutions of inhibitor in a final volume of 100 μl. The reaction was allowed to proceed for 1 h at 37°C and then was stopped by washing five times with TTBS buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20]. The level of incorporation of dUTP-digoxigenin within the template was measured by adding 15 milliunits of antidigoxigenin-POD Fab fragment (Roche, Laval, Québec, Canada)/well in a buffer consisting of 20 mM Tris (pH 7.5), 150 mM NaCl, and 10 mg/ml BSA and incubating for 1 h at room temperature. The antibody was washed off from the wells by five consecutive washes with TTBS buffer. The bound antibody was exposed to the TM Blue substrate (Intergen, Purchase, NY), and absorbance at 630 nm (Dynatech MR5000 plate reader) was determined after a 5-min exposure at room temperature. Percentage of inhibition was calculated by dividing the absorbance obtained with the inhibitor by that of the control (no inhibitor) and multiplying by 100. IC_{50} (concentration that inhibits dUTP-digoxigenin incorporation by 50%) was determined by standard regression analysis using the Prism software (GraphPad Software, Inc.).

***In Vitro* Stability in Whole Blood and Liver S9 Fractions.** BCH-1868 (10 μg/ml) was incubated with mouse, rat, and human whole blood at 37°C for 3 h. Aliquots were taken at 0, 10, 20, 30, 45, 60, 120, and 180 min after starting the incubation. Samples were diluted with water, centrifuged, and BCH-1868 was extracted at pH 1.5 by solid phase extraction (Oasis MCX cartridges; Waters Corporate). Cartridges were conditioned with methanol followed by water, samples were applied, and cartridges were washed with 2% methanol/0.1 N HCl. Samples were eluted with 5% NH_3 in methanol, dried under a gentle stream of nitrogen, reconstituted in water/0.05% trifluoroacetic acid, and analyzed by HPLC/UV. BCH-1868 was separated on a YMC ODS-A (5 μm, 120A; 4.6-mm inside diameter $\times$ 250 mm) column. The mobile phase consisted of acetonitrile (A) and 50 mM phosphate buffer containing 5 mM tetrabutyl ammonium dihydrogen phosphate (B) and was pumped at 1 ml/min. A gradient of 0–10% (A/B) and 10–100% (A/B) was applied for 30 and 3 min, respectively, before returning to initial conditions (100–0% A/B) over 3 min. Data were processed by Millennium 3.05 software (Waters Corporate) at 253 nm. The limit of detection of BCH-1868 was 0.1 μg/ml.

BCH-1868 (10 and 100 μM) was incubated with mouse, rat, or human liver S9 fractions (In Vitro Technology, Baltimore, MD) in the presence and absence of the Phase I and II reaction cofactors NADPH and UDP-glucoronic acid (UDPGA) (2 mM). A typical incubation reaction consisted of 10 or 100 μM BCH-1868, 2 mM NADPH and/or 2 mM UDPGA, 1.6 mg S9 protein/ml, and 100 mM phosphate buffer (Na_2HPO_4 $7H_20$ + NaH_2PO_4 − pH 7.4) in a final volume of 2 ml. Aliquots (250 μl) were collected at 30, 60, and 120 min. Controls included incubations containing S9 protein without cofactors or denatured S9 protein or only compound in buffer. Positive substrates of Phase I and II reactions were run in parallel to ensure proper functioning of the S9-mediated reactions. Ali-

quots were diluted with water (750 μl), and reactions were stopped by addition of phosphoric acid (30 μl). BCH-1868 and potential metabolites were extracted on Oasis MCX cartridges before HPLC analysis as described above.

In Vivo Evaluation

Animals. All animal studies were performed with the prior approval of an institutional animal care committee and in agreement with the guidelines provided by Canadian Council for Animal Care. Nude mice with CD-1 background and SCID mice were purchased from Charles Rivers Laboratories (St. Constant, Québec, Canada). They were maintained under specific pathogen-free conditions and provided with sterile food and water *ad libitum*. Six to eight-week-old mice, weighing 16–22 g, were used for these studies. The mice were allowed to acclimatize for at least 5 days before beginning the study.

Pharmacokinetics of BCH-1868. The disposition of BCH-1868 was evaluated in male CD-1 mice after a single dose of 10 mg/kg was administered i.v. or i.p. by the tail vein. Blood (1 ml) was collected into heparinized tubes by intracardiac puncture from control mice and from treated mice at 0, 5, 15, 30, 60, 120, and 240 min after administration of the drug (3 mice/time point). Urine was also collected over the 4-h study period. Plasma and urine were frozen at −20°C for later analysis by HPLC/UV.

Plasma concentration time data were analyzed using compartmental and noncompartmental analysis. Data were fitted to a two-compartmental model using WinNonlin 1.5 (Scientific Consulting, Inc., Cary, NC). The terminal phase rate constant (β) was obtained by extended least square regression analysis. A weighting factor of −1.3 was chosen for curve fitting. Maximum measured plasma concentrations (C_{max}) and time of C_{max} (T_{max}) were read directly from the data. Area under the curve ($AUC_{o\rightarrow\infty}$) was calculated using the trapezoidal rule up the last data point and extrapolated to infinity with the following formula: C_{last}/β, where C_{last} is the last measured concentration time point. The terminal half-life was calculated with the equation $t_{1/2} = 0.693/\beta$, where β is the terminal rate constant. In mice, the quantity of BCH-1868 in urine was determined, and the percentage of dose eliminated in urine unchanged (Ae) was calculated as Ae/dose × 100. BCH-1868 systemic clearance (Cl) was calculated as $dose_{i.v.}/AUC_{o\rightarrow\infty/i.v.}$ and its renal clearance (Cl_{renal}) as $Ae/AUC_{0\rightarrow\infty}$.

Antitumor Activity. BCH-1868 was evaluated for antitumor activity against human solid tumor xenografts growing on female athymic CD-1(nu/nu) mice (Caki-1, HT-29, and COLO 205) or on female SCID mice (DU 145). At day 0, mice were s.c. inoculated with 1–2 × 10^6 cells, and tumor growth was monitored by *in situ* caliper measurement to determine tumor volume. Tumor volumes were calculated from the length and width measurements using the formula: $(L \times W^2)/2$ (20). When mean tumor volumes of 60–100 mm^3 were reached, animals were divided randomly into test groups of 10 mice and treatment was started. Tumor volumes, at a given day, were expressed as relative tumor volumes (*RTV*s) according to the following formula: $RTV = TVn/TVo$, where *TVn* is the tumor volume on day *n* and *TVo* is the tumor volume on day 0. Compound efficacy was assessed by percentage of *T/C* defined as *RTV*tumor/*RTV*control × 100 with a percentage of *T/C* of <42, which is indicative of significant antitumor activity (21). Control mice received injections of the drug vehicle. To monitor the drug-associated toxicity, mice were weighed at least twice a week and daily inspected for abnormalities.

BCH-1868 was dissolved in 0.9% saline and sterilized by filtration (0.22 μm). Solutions were administered i.p. at variable doses (25–50 mg/kg) on either a q4dx3 or a q1dx5 (maximum volume, 10 ml/kg). In the Caki-1 and DU 145 models, a second round of injections (q1dx5) was done at the regrowth of the tumor.

The antitumor activity of BCH-1868 was also investigated in the CCRF-CEM dCK-deficient lymphoblastic leukemia model. Female SCID mice (10 mice/group) were given i.p. injections of 30 × 10^6 cells. BCH-1868 or Troxacitabine was administered i.p. starting 13 days after tumor inoculation when the first signs of leukemia appeared. Weight and survival were registered on a daily basis. Antitumor activity was evaluated by percentage of ILS relative to untreated controls. The percentage of ILS values was calculated from median survival time by the following formula: (median day to death of treated mice/median day to death of untreated control mice − 1) × 100.

Statistical Analysis. For the animal studies, results are expressed as the mean ± SE. Statistical analysis was performed by ANOVA or by Student's *t* test. Differences were considered to be significant at $P < 0.05$.

Results

In Vitro Evaluation

Antiproliferative/Cytotoxic Activity of BCH-1868. The antiproliferative/cytotoxic activity of BCH-1868 was evaluated on a panel of human leukemia and solid tumor cell lines, as well as on two murine solid tumor cell lines (Table 1). BCH-1868 showed potent inhibition of the proliferation of human leukemia, as well as proliferation of human and murine solid tumor cell lines. In the [^{3}H]thymidine incorporation assay, similar IC_{50}s were obtained on all cell lines tested ranging from 0.1 to 3.5 μM. In the MTT assay, leukemia (HL-60 and CCRF-CEM) and DU 145 were the most sensitive cell lines with IC_{50}s of 3–4 μM after a 72-h incubation. For the entire cell panel, the MTT IC_{50}s (72 h) for BCH-1868 ranged from 3 to 250 μM.

For some cell lines, a 4-, 24-, and 72-h incubation period in presence of the drug was performed. Similar differences between the 4- and 72-h exposures were observed for the MTT and [^{3}H]thymidine assay. IC_{50}s were 10–50 times higher after the 4-h exposure compared with the 72-h exposure. All cell lines used had doubling times of ≤36 h.

We also investigated the antiproliferative activity of BCH-1868 in cells expressing MRP/gp 190 protein (HL-60ADR cells) and cells genetically defective in dCK (CCRF-CEM dCK^-) or in their ability to transport nucleosides (CEM-CCRF/AraC8C; Table 2). BCH-1868 proved to be as active against the parental cell line as against the defective cell lines or the MRP/gp 190-expressing cell line (IC_{50} comparison) for both the MTT and the [^{3}H]thymidine assays. Doxorubicin and

Table 1 Growth inhibitory effect of BCH-1868 on a panel of murine and human cell lines.

	IC_{50} μM					
	MTT			[^{3}H]Thymidine		
	4 h	24 h	72 h	4 h	24 h	72 h
Normal cells						
HSF	50 ± 38[a]	14 ± 8	10 ± 2	3.7 ± 4.0	0.4 ± 0.3	0.1 ± 0.1
Human tumor cells						
SF268	539 ± 336	42 ± 27	36 ± 13	88 ± 58	8.0 ± 4.5	2.1 ± 1.5
MCF-7	227 ± 132	36 ± 5	20 ± 17	63 ± 40	2.4 ± 0.1	1.1 ± 0.5
Caki-1	580 ± 274	61 ± 27	67 ± 18	121 ± 51	13 ± 4	2.2 ± 0.9
DU 145			2.7 ± 2.4			0.3 ± 0.2
COLO 205			248 ± 158			2.0 ± 1.2
HT-29			45 ± 27			1.1 ± 0.6
CCRF-CEM			3.5 ± 2.2			1.5 ± 0.4
HL-60			3.9 ± 2.3			0.4 ± 0.2
Murine tumor cells						
B16F10			6.8 ± 3.7			0.8 ± 0.2
MC38			39 ± 29			3.5 ± 4.0

[a] Results are expressed as mean ± SD of three to five independent experiments.

Table 2 Growth inhibitory effect of BCH-1868 in HL-60 wild-type and doxorubicin-resistant cell lines and on CCRF-CEM cell lines defective in dCK or in nucleoside transporters.

	IC_{50} μM (72 h)					
	BCH-1868		Doxorubicin		AraC	
	MTT	3H-T[a]	MTT	3H-T	MTT	3H-T
HL-60	3.9 ± 2.3[b]	0.4 ± 2.3	0.02 ± 0.01	0.003 ± 0.002		
HL-60ADR	16 ± 9	2.5 ± 0.6	2.5 ± 1.5	0.32 ± 0.06		
RF	4.3	6.3	119	100		
CCRF-CEM	4.4 ± 3.7	1.4 ± 0.6			0.009 ± 0.004	0.005 ± 0.003
CCRF-CEM dck$^-$	1.7 ± 1.0	1.9 ± 0.9			87 ± 23	49 ± 20
RF	0.4	1.4			>2000	>2000
CCRF-CEM/AraC	19 ± 13	11 ± 5			65 ± 43	30 ± 18
RF	4.4	7.6			>2000	>2000

[a] 3H-T, [^{3}H]thymidine; *RF*, resistance factor expressed as IC_{50} of resistant cells/IC_{50} of parental cells.
[b] Mean ± SD of three independent experiments.

AraC were included in the assays to ensure the resistance factor of cell lines used. The resistance factor ratios were ~110 for HL-60:HL-60ADR and >2000 for CCRF-CEM dCK$^-$:CCRF-CEM or CCRF-CEM/AraC8C:CCRF-CEM.

Effect of BCH-1868 on DNA, RNA, and Protein Synthesis. To examine if BCH-1868 cytotoxicity involved only an inhibitory activity on DNA synthesis, we investigated its effects on RNA and protein by measuring the incorporation of radiolabeled uridine and leucine, respectively, in the acid insoluble fraction of BCH-1868-exposed HSF *versus* vehicle-exposed HSF (1, 10, and 100 μM for 4 and 24 h). No appreciable inhibition activity of BCH-1868 was noted under these experimental conditions for both protein and RNA synthesis after a 4- and 24-h period with the concentrations of BCH-1868 tested (Fig. 2). However, inhibition of [^{3}H]thymidine incorporation was observed to be both concentration and time dependent. After 24 h, the IC_{50} of BCH-1868 was 10 μM for thymidine incorporation and was >100 μM for uridine and leucine. Thus, it was concluded that in contrast to DNA synthesis, RNA and protein synthesis were not markedly inhibited in BCH-1868-treated cells.

Cell Cycle Arrest of BCH-1868-exposed HSF. Fig. 3 shows the cell cycle distribution of HSF cells incubated with increasing concentration of BCH-1868 for a 24-h exposure period. In the untreated control cell culture, G_1, S, and G_2-M phases represent 71, 19, and 10%, respectively, of the total

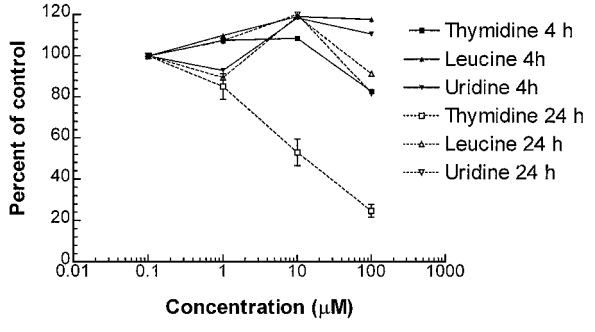

Fig. 2. Effect of BCH-1868 on macromolecular synthesis in HSF cells. Cells were incubated for 4 and 24 h with the indicated amount of BCH-1868, and then incorporation of [^{3}H]thymidine, [^{3}H]uridine, and [^{3}H]leucine was assessed as described in "Materials and Methods." Control incorporation were (cpm): 4 h, thymidine 2886 ± 30, uridine 1837 ± 184, and leucine 9944 ± 1893; and 24 h, thymidine 14392 ± 926, uridine 9132 ± 1026, and leucine 44100 ± 4674. For clarity of the graph, SD was added only for [^{3}H]thymidine incorporation at 24 h.

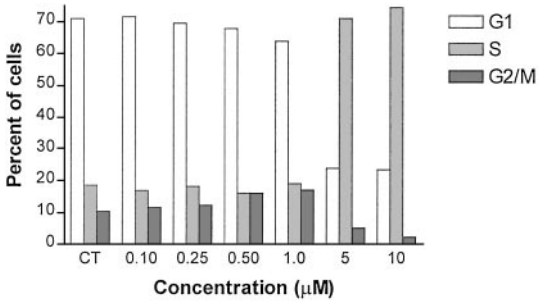

Fig. 3. Effect of BCH-1868 on the cell cycle distribution of HSF cells. HSF were exposed to BCH-1868 at varying concentrations for 24 h. Thereafter, DNA content of drug-treated and -untreated cells was analyzed by flow cytometry using propidium iodide staining.

cell population. In the presence of BCH-1868, the cell cycle distribution was still comparable with the control group with concentrations of ≤ 1 μM. In contrast, a marked accumulation of the cells in S phase (70%) was observed when the drug concentration was increased from 1 to 5 μM. No fractional DNA content (pre-G_1) was observed at the concentrations tested. Longer incubation period (48 h) did not lead to major increase in the pre-G_1 fraction.

Intracellular Metabolism of BCH-1868. Previous works had demonstrated that acyclic nucleoside phosphonates are transported into cells either by an active process or by endocytosis-like process and then phosphorylated by cellular kinases to their monophosphate and diphosphate derivatives (4, 22). To determine whether BCH-1868, a cyclic nucleoside phosphonate, was effectively phosphorylated by cellular enzymes, CCRF-CEM cells were incubated with 1.5 μM [^{3}H]BCH-1868 for up to 48 h, and cell extracts were analyzed by HPLC for BCH-1868 and its phosphorylated metabolites. Three peaks were observed: BCH-1868 itself (at 16–17 min); BCH-1868-P (at 29 min); and BCH-1868-PP (at 43 min). Total intracellular levels of BCH-1868 and phosphorylated metabolites were of 1.1, 1.2, and 1.8 pmol/10^6 cells at 4, 24, and 48 h, respectively. Over time, the monophosphorylated and diphosphorylated forms increased, whereas the parent compound decreased (Table 3). The monophosphate derivative (BCH-1868-P) was the major metabolite formed in cells and represented >75% of the radioactive material after 24 h.

Inhibition of DNA Polymerase α. To determine whether BCH-1868 and/or its metabolite inhibit DNA chain elongation, we used an *in vitro* polymerization assay for DNA polymerase α. Both monophosphate and diphosphate derivatives were potent inhibitors of DNA polymerase α with IC_{50}s of 2.2 $\pm$ 0.4 ($n = 3$) and 1.0 $\pm$ 0.1 μM ($n = 3$), respectively, whereas the phosphonate itself was not ($IC_{50} > 100$ μM).

Stability of BCH-1868 in Whole Blood and Liver S9 Fraction. BCH-1868 was stable in the presence of mouse, rat, and human plasma for a period up to 3 h (data not shown). In addition, no degradation or apparition of metabolites was observed over a 2-h period in the presence of rat or human liver S9 fractions (data not shown).

Table 3 Formation of intracellular metabolites of BCH-1868.

Time (h)	Level of metabolites (pmol/10^6 cells)		
	BCH-1868	BCH-1868-P	BCH-1868-PP
4	0.58 (53)[a]	0.53 (47)	
24	0.09 (8)	0.91 (80)	0.18 (12)
48	0.14 (8)	1.30 (74)	0.31 (18)

[a] Relative percentage of each metabolite. Data are from one representative experiment.

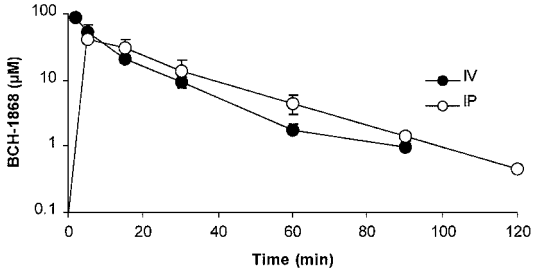

Fig. 4. Plasma concentration time profiles of BCH-1868 after a 10 mg/kg i.v. and i.p. dose administered to male CD-1 mice. Each point represents mean $\pm$ SD of 3 mice.

In Vivo Evaluation

Pharmacokinetic Disposition of BCH-1868. The plasma concentration time profile of BCH-1868 after i.v. and i.p. administration in mice is shown in Fig. 4. Pharmacokinetic parameters are summarized in Table 4. Maximum measured plasma concentrations (C_{max}) were respectively of 87 and 41 μM after i.v. and i.p. administration of a 10 mg/kg dose. Concentrations declined rapidly after a biphasic curve with an initial distributed half-life of ~3 min and a $t_{1/2}$ of 16 min. No drug was recovered at 120 min after the i.v. dose or at 240 min after the i.p. dose. Data on plasma concentration *versus* time indicate that concentration > 10 μM is maintained for ~30 min after administration of a 10 mg/kg dose. A similar amount of BCH-1868 was recovered from the i.p. and i.v. administration, indicating that the drug was completely absorbed after i.p. administration. BCH-1868 was eliminated unchanged in the urine with 70–80% of the dose recovered over the 4-h study period. The lack of full recovery is likely explained by the difficulty in collecting the total urine of mice.

Antitumor Activity. BCH-1868 antitumor activity was first evaluated in nude mice with human renal Caki-1 xenografts with concentrations of 25 and 50 mg/kg given either q1dx5 or q4dx3 (Fig. 5*A*). These doses and schedules were obtained from previous studies that had shown toxicity as indicated by poor health and a loss of body weight for concentrations > 50 mg/kg when administered for a period > 5 days (9). The results indicated that daily injections for 5 days was the most effective schedule. Mice treated with BCH-1868 (25 or 50 mg/kg) displayed a significant decrease in tumor volume compared with animals treated with vehicle alone (*T*/*C*: 48 and 37%, respectively, with $P < 0.05$). Tumor volume in mice treated q4dx3 was not statistically different

Table 4 Pharmacokinetic parameters for BCH-1868 in CD-1 mice after i.v. and i.p. administration of 10 mg/kg.

Pharmacokinetic parameter[a]	10 mg/kg i.v.	10 mg/kg i.p.
C_{max} (μM)	87.3	41.3
T_{max} (min)		5
$t_{1/2\alpha}$ distribution (min)	2.8	
$t_{1/2\lambda}$ elimination (min)	16.4	17.5
AUC_{0-n} (μmol·min/liters)	1120	1130
$AUC_{0-\infty}$ (μmol·min/liters)	1138	1142
F (%)		100
V_z (liters)	15.9	17.6
Cl (liters/min)	0.67	0.67
Ae (μg)	165.8 ± 8.6 (71.5% of dose)	202.9 ± 7.6 (79.6% of dose)
Cl_{renal} (liters/min)	0.40	0.51

[a] C_{max}, maximum measured plasma concentration; T_{max}, time to C_{max}; $t_{1/2}$, half-life; *AUC*, area under the curve; F, biovailability; V_z, volume of distribution; *Cl*, systemic clearance; Cl_{renal}, renal clearance; Ae, unchanged drug recovered in urine.

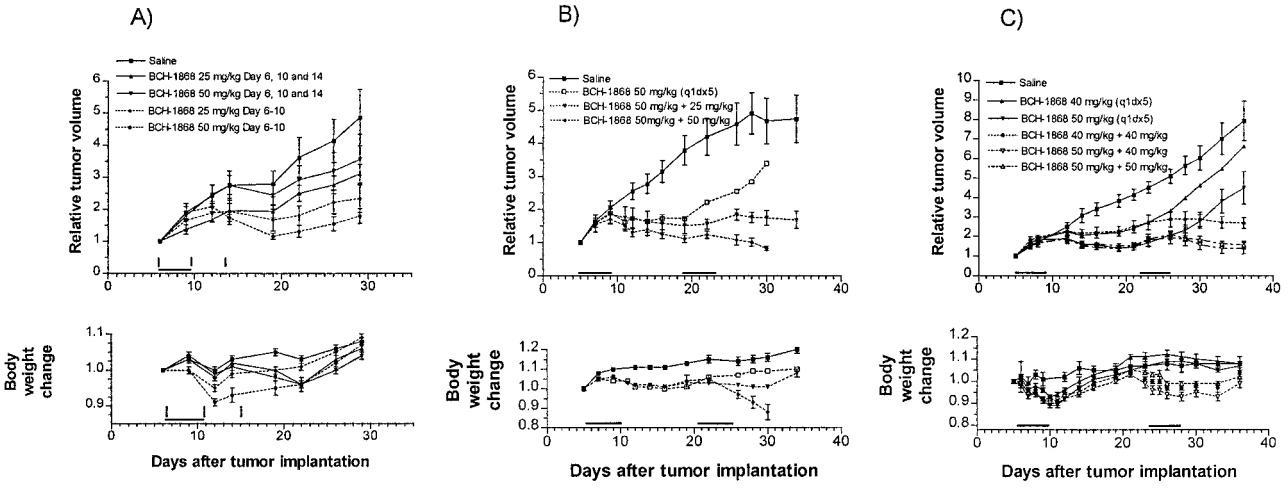

Fig. 5. Effect of BCH-1868 on tumor growth and body weight changes in nude mice bearing human renal Caki-1 cancer xenografts. Tumor inoculation was carried out as described in "Materials and Methods." BCH-1868 was administrated i.p. at indicated doses. *Arrows* and *lines* indicate days of treatment. Each *curve* represents the average of 10 mice ± SE. *A*, *B*, and *C* represent different doses and schedules of injection.

from control mice. Although BCH-1868 inhibited the growth of established Caki-1 tumors, no tumor regression was observed. Withdrawal of BCH-1868 resulted in growth of Caki-1 tumors with kinetics similar to the control group after a lag period of 1–2 weeks. A body weight decrease of 8% was associated with the highest dose, but mice were able to recover from it afterward. When a second 5-day course was performed at the regrowth of tumor, cells were still responsive to treatment (Fig. 5, *B* and *C*). Repeated doses of 40 mg/kg lead to minimal tumor growth with no associated toxicity. Repeated doses of 50 mg/kg were less well tolerated and caused some toxicity-related deaths (2 of 10 mice), indicating that this dose level was over the maximum tolerated dose (LD_{10}). Periods of treatment $>$ 5–7 days lead to severe weight loss (>20%; data not shown). These results demonstrated that BCH-1868 has cytostatic activity, with respect to tumor growth, in nude mice bearing a renal cancer xenograft.

We then examined BCH-1868 for activity in other xenograft models, including colon and prostate cancers (Fig. 6). Similar results were obtained in the three xenograft models. A dose-dependent growth delay was observed with a *T/C* of 40–45% ($P < 0.05$) at a dose of 50 mg/kg (q1dx5) on COLO 205 and HT-29 colon cancers (Fig. 6, *A* and *B*). For the DU 145 prostate carcinoma model (Fig. 6*C*), we observed some tumor regression at 40–50 mg/kg, followed by a rapid regrowth. A second injection, given at that time, produced only minimal growth delay.

The efficacy of BCH-1868 was also investigated in an i.p. CCRF-CEM/dCK$^-$ leukemia model (Fig. 7). The CCRF-CEM/dCK cells are deficient in dCK, a key enzyme required for the first phosphorylation step in activation of nucleoside analogues. Treatment (40 mg/kg; q1dx5) was initiated 13 days after the cells were injected, once animals had developed disseminated tumors. All of the saline control group mice died between days 28 and 35 (median survival time of 32 days) after cell inoculation. BCH-1868 was effective in that model with an ILS value of 41% ($P < 0.05$). Troxacitabine, a reference nucleoside drug that requires dCK activity for activation (23), was completely inactive in that tumor model with an ILS of only 1% (median survival time of 33 days).

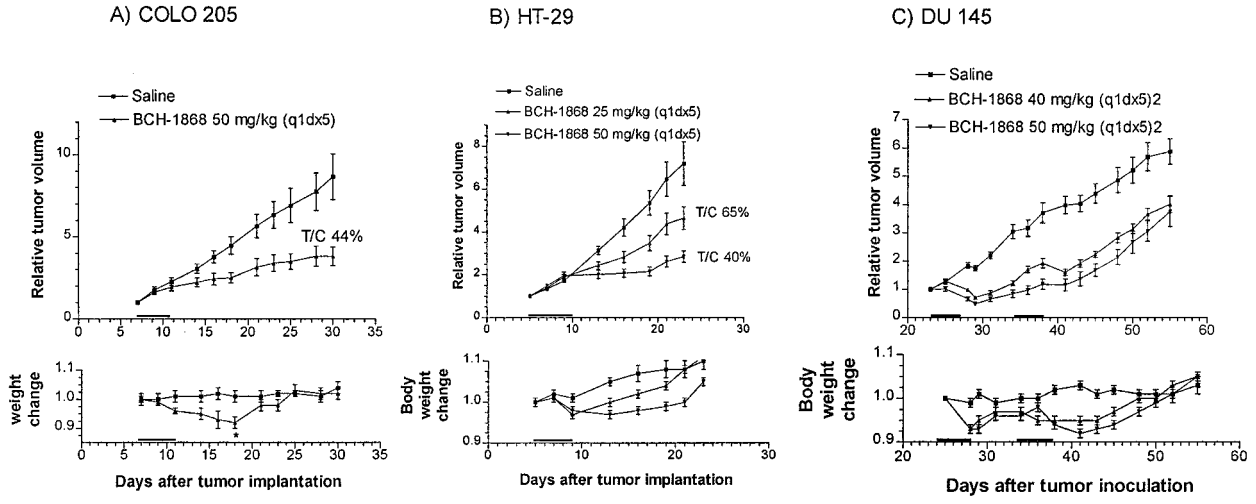

Fig. 6. Effect of BCH-1868 on tumor growth and body weight changes in nude (COLO 205 and HT-29) or SCID (DU 145) mice bearing human tumor xenografts. Tumor inoculation was carried out as described in "Materials and Methods." BCH-1868 was administrated i.p. at indicated doses. *Lines* indicate days of treatment. Each *curve* represents the average of 10 mice ± SE.

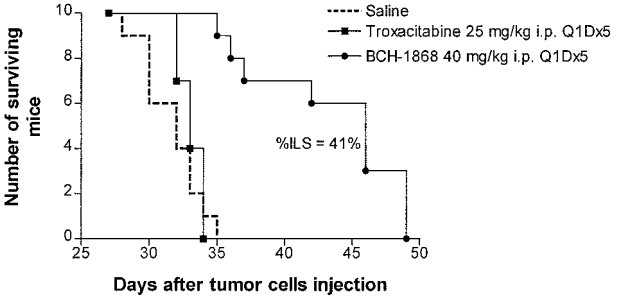

Fig. 7. Survival time of SCID mice injected i.p. with CCRF-CEM/dCK$^-$ lymphoblastoid human leukemia cells. Ascite tumors were established by injection of 30×10^6 cells. Thirteen days after tumor cell injection, when appeared the first signs of disease, animals were randomized into groups of 10 and received i.p. injections of either BCH-1868, Troxacitabine, or control saline (q1dx5).

Discussion

Nucleoside phosphonates are structural analogues of nucleotides consisting of a nucleoside moiety to which a phosphonate group has been attached through a stable phosphocarbon linkage (24). They are potent antiviral agents with a broad range of activity against DNA viruses (25). Recent findings indicate that in addition to their antiviral activity, some nucleoside phosphonates may potentially be developed as antitumor agents (6, 26–28). BCH-1868 is a tetrahydrofuran phosphonate analogue bearing a guanine base for which we have previously reported human cytomegalovirus antiviral activity (10, 29). The closely structurally related acyclic phosphonate nucleoside PMEG, displays significant antitumor activity in a number of *in vivo* animal models (5, 26). This study was undertaken to evaluate the potential of BCH-1868, a novel cyclic nucleoside phosphonate, as an antitumor agent.

Our data show that BCH-1868 efficiently stops, *in vitro*, the proliferation of cancer cells. BCH-1868 inhibited, in the μM range, the cell growth of a panel of human and murine cancer cell lines. Its *in vitro* antiproliferative potency is comparable with that of many antimetabolites used in antitumor chemotherapy such as 6-mercaptopurine, 6-thioguanine, and 5-fluorouracil (30, 31). Studies with human equilibrative nucleoside transport-deficient cells (hENT-1) and dCK mutant cells indicated that BCH-1868 enters cells by an hENT-1 independent route and does not require dCK activity for activation. Nucleoside phosphonate compounds were originally designed to circumvent the first phosphorylation step, which is necessary for activation of the classical nucleoside analogues and often represents a bottleneck in the intracellular metabolism of nucleoside analogues to their active 5′-triphosphate form. Antiproliferative efficacy of BCH-1868 was also found to be completely independent of the levels of the MRP/gp190. In addition, our data indicate that especially in tumor cells, a period time > 4 h is necessary to achieve maximum cytotoxicity, precluding a relatively slow cell penetration and conversion process before reaching the intracellular target.

Interestingly, IC_{50}'s measured by the [^{3}H]thymidine assay were much lower (10–125×) than those measured by the MTT assay, except for the two leukemia cell lines HL60 and CEM-CCRF. Similar observations were reported for drugs having an action on DNA synthesis (19). This study demonstrates that indeed the major inhibitory effect of BCH-1868 was through DNA synthesis inhibition rather than through inhibition of RNA or protein synthesis. This was previously observed with acyclic phosphonate nucleosides 9-(2-phosphonylmethoxyethyl) adenine and PMEG (32, 33) and with more conventional nucleotide analogues such as gemcitabine (34) and 2′,2′-difluorodeoxyguanosine (35).

Intracellularly, by an enzymatic pathway that still needs to be characterized, BCH-1868 undergoes conversion more effectively to its monophosphate analogue (BCH-1868-P) than to its diphosphate analogue (BCH-1868-PP). We know that it is not through the dCK because a CCRF-CEM cell line

defective in dCK was as sensitive to BCH-1868 as the wild-type cells were. One of the possible first phosphorylation enzymes is the GMP kinase. Guanine-derived acyclic nucleosides such as acyclovir and ganciclovir, as well as guanine-derived acyclic nucleoside phosphonates, were reported to be substrates for GMP kinase (11, 36). Because cellular kinases are necessary for activation of BCH-1868, our present results do not rule out the possibility that this acyclic nucleoside phosphonate may act as a competitor for the natural pool of nucleoside for phosphorylating enzymes. Upon its phosphorylation, BCH-1868 may act as inhibitor or substrate for the human DNA polymerase α and reduce the rate of DNA synthesis. DNA primer extension over a defined template revealed that BCH-1868-P and BCH-1868-PP were good inhibitors of DNA polymerase α. These results indicate that BCH-1868 arrests cell proliferation most likely through inhibition of the main replicative DNA polymerase, but interference with other cellular polymerases is not excluded as demonstrated with acyclic nucleotide phosphonates (37). In agreement with these previous observations, flow cytometric analysis of DNA content of HSF (24 h) revealed a marked retardation of S phase progression in the presence of BCH-1868, leading to the accumulation of cells in S phase and a severe perturbation of the normal cell cycle distribution pattern. The observation was more apparent at higher drug concentrations. The mechanism of polymerase inhibition by nucleoside analogues may involve either the incorporation of BCH-1868-phosphorylated derivatives into the growing DNA strand, or competition with the natural deoxynucleotide triphosphate's pool. The phosphorylated metabolites of nucleoside phosphonates are structural analogues of the natural deoxyribonucleoside 5′ triphosphates and thus may be recognized as alternative substrates for DNA synthesis by cellular DNA polymerases (8, 37, 38). Moreover, incorporation of nucleoside phosphonates, which does not provide a hydroxyl group, such as BCH-1868 for additional chain elongation would inevitably cause DNA chain termination.

We have also demonstrated that BCH-1868 has antitumor activity against a panel of human tumor xenografts (renal, colon, prostate, and leukemia). However, even if a statistically significant reduction in tumor growth was observed, no tumor regression occurred. The best response that was achieved was tumor stasis with repetitive schedules of injection. Various factors such as its mechanism of action, circulating blood concentrations, and distribution characteristics may potentially affect the ultimate response of tumors to BCH-1868. BCH-1868 was rapidly eliminated from the circulation (half-life of 17 min) such that concentration above the IC_{50} was maintained for a short period of time. This is not attributable to degradation because this compound has a high degree of stability in whole blood and does not undergo significative metabolism. Moreover, $>$70% of the injected doses were recovered unchanged in urine, indicating that BCH-1868 is eliminated renally as reported previously for acyclic phosphonates adefovir and cidofovir (39, 40). At this point, we do not know how efficiently BCH-1868 is distributed into tumor tissues. However, its volume of distribution indicates that the drug does not remain within the vasculature but does undergo some tissue distribution. Nucleotide analogues such as BCH-1868 are effective during the short S phase of the cell cycle, thus requiring continuous presence of the drug for better activity. Studies on the impact of plasma concentrations on production of therapeutically active nucleotide triphosphates in cell lines and patient-derived samples have indicated a strong correlation between efficacy and necessity to achieve continuous cytotoxic concentration of nucleotide triphosphates (3). In addition, *in vitro* studies done with PMEG, a closely BCH-1868-related acyclic nucleoside phosphonate, have shown a reversible slowdown in human leukemia cell growth at low concentrations because of continuous repairing of damaged DNA, whereas only high concentrations induced irreparable DNA damage (41). Because of the toxicity associated with higher doses or a longer schedule administration, it appears to be difficult to achieve circulating levels of BCH-1868 that would lead to tumor regression.

In conclusion, we have demonstrated that the phosphonate BCH-1868 has antitumor activity. We have shown that its suppressive effect on tumor growth does not persist for a long period of time after the end of treatment. This is probably related to the mechanism of action of this nucleoside phosphonate that consists of a slowdown of DNA synthesis and accumulation of cells in the S phase. The use of BCH-1868 as a single agent may not be adequate. However, frequent administration of a low dose, which might increase the proportion of cells in the S phases, and combination with S phase-specific antitumor drugs such as topoisomerase inhibitors may be a worthwhile approach.

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