Species Differences in Troxacitabine Pharmacokinetics and Pharmacodynamics: Implications for Clinical Development

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ABSTRACT

Purpose: **Troxacitabine is the first unnatural L-nucleoside analog to show potent preclinical antitumor activity and is currently under clinical investigation. Significant differences in troxacitabine toxicity between mice, rats, monkeys, and humans were observed during preclinical and clinical evaluations. To better understand the different toxicity and efficacy results observed between the human xenograft mouse tumor models used for preclinical assessment and the clinical study results, the pharmacodynamics and pharmacokinetics of troxacitabine were reassessed in murine and human models.**

Experimental Design: **Clonal and thymidine incorporation assays were used to investigate the *in vitro* antiproliferative activity of troxacitabine on a selected panel of mouse and human tumor cell lines and normal hemapoietic cells. Analysis of the intracellular metabolites of [^{14}C]troxacitabine was determined in mouse and human T-lymphocytes obtained from peripheral blood. The antitumor efficacy of troxacitabine administered either as single or repeated high-dose bolus administrations or as low-dose continuous infusions was evaluated in the human colon HT-29 xenograft model. We also determined plasma concentrations of troxacitabine using the different administration schedules.**

Results: **Five to nine hundred-fold lower concentrations of troxacitabine were required to inhibit cell growth in human compared with murine tumor and normal hemapoietic cell lines. Furthermore, the sensitivity of cells of both species to troxacitabine was strongly time dependent, requiring >24 hours exposure for maximum activity. Analysis of the intracellular metabolites of [^{14}C]troxacitabine in T-lymphocytes obtained from peripheral blood revealed subsequently higher levels of mono-, di-, and triphosphates in human compared with mouse. Antitumor efficacy studies revealed that prolonged exposure schedules (up to 6 days) showed equivalent efficacy to repeated high-dose bolus administrations. Five-day continuous infusion of 20 mg/mL troxacitabine via subcutaneous implanted mini-osmotic pump maintained systemic concentrations of 262 ng/mL (1.2 μmol/L) for the duration of administration, which are clinically achievable plasma concentrations, and led to significant antitumor activity [treated *versus* control (T/C) of 27% and tumor regression during treatment].**

Conclusions: **These studies support the hypothesis that troxacitabine infusions might be the administration regimen with the greatest likelihood of fully exploiting clinically the potent preclinical antitumor activity of troxacitabine.**

INTRODUCTION

Troxacitabine (Troxatyl BCH-4556, (−)-2′-deoxy-3′-oxacytidine) is a novel deoxycytidine analog having an unnatural β-L-configuration and has been in clinical development since 1997. The unusual stereochemistry of the drug gives it unique mechanistic characteristics relative to araC (1-β-D-arabinofuranosylcytosine, cytarabine) and gemcitabine (dFdC, 2′,2′-difluorodeoxycytidine), the clinically used β-D-configuration deoxycytidine analogs. These include membrane permeation not mediated by nucleoside transporters (1), lack of susceptibility to deamination (2, 3), phosphorylation from di- to triphosphate by 3-phosphoglycerate kinase instead of nucleoside diphosphate kinase (4–6), complete DNA chain termination (7), and DNA excision by the apurinic/apyrimidinic endonuclease (APE1) instead of the 3′ to 5′ exonuclease associated with DNA polymerases (8).

Clinical development was instigated after preclinical evaluation, which showed the broad and potent antitumor activity against both solid and hematopoietic human tumor xenografts of troxacitabine when administered intraperitoneally twice daily for 5 days at 25 mg/kg (75 mg/m^2; summarized in Table 1; refs. 2, 3, 9–15). Schedule-dependency was evident preclinically with repeated injections being more effective than single administration regimens (16). Preclinical toxicology however revealed major species differences. In mice and rats, troxacitabine was generally well tolerated by intraperitoneal or intravenous administration.[1] In antitumor studies in mice, troxacitabine did not produce significant toxicity at doses up to 100 mg/kg when administered intraperitoneally once daily for 5 consecutive days (q1d×5) whereas treatment with 200 mg/kg (q1d×5) was toxic

Received 4/8/04; revised 7/16/04; accepted 7/16/04.
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[1] Gourdeau, H. unpublished observations.

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Table 1 Antitumor efficacy studies of troxacitabine against various human tumor xenografts in nude mice

Tumor type	Troxacitabine *%T/C †	Positive control drug (regimen)	%T/C †
Prostate (PC-3)	4	Doxorubicin (10 mg/kg/wk×3)	34
Prostate (DU-145)	7	ara-C (25 mg/kg twice a day×5)	100
Renal (CAKI-1)	16	Ccisplatin (6.7 mg/kg q4d×3)	108
Renal (A498)	27	Doxorubicin (8 mg/kg q4d×3)	41
Renal (RXF-393)	13	Cyclophosphamide (90 mg/kg q4d×3)	38
Renal (SN-12C)	8	Cyclophosphamide (90 mg/kg q4d×3)	122
Colon (HT-29)	5 ‡	5-FU (40 mg/kg qdx5 for 2 weeks)	84
Head & Neck (KBV)	19	Doxorubicin (10 mg/kg/wk×2)	32
NSC lung (NCI-H322M)	62	Mitomycin C (3 mg/kg/wk×3)	28
NSC lung (NCI-H460)	46	Mitomycin C (3 mg/kg q4d×3)	26
NSC lung (A549)	41	Mitomycin C (3 mg/kg q4d×3)	25
Liver (HepG2)	26 §	ara-C (25 mg/kg twice a day×5)	93
Pancreas (Panc-01)	16 ¶,‖	Gemcitabine (80 mg/kg q3d×4)	76
Pancreas (MiaPaCa)	77 ¶,‖	Gemcitabine (80 mg/kg q3dx4)	87
Leukemia (HL60)	422 ‖,**	ara-C (25 mg/kg qd×5)	106 **
Leukemia (CCRF-CEM)	152 ‖,**	ara-C (25 mg/kg qd×5)	146 **
Leukemia (KBM5)	171 ‖,**	Imatinib mesylate (50 mg/kg twice a day×10)	109 **

Data from Gourdeau and Jolivet (9).

* Troxacitabine was given intraperitoneally twice a day (6-hour interval) at a concentration of 25 mg/kg unless specified.

† Tumor measurements were taken twice weekly with callipers and were converted to tumor volumes with the following standard formula: $[\text{width (mm)}]^2 \times \text{length (mm)} \times 0.50$. T/C's were generated from the mean-treated tumor volume divided by the mean control tumor volume times 100. By National Cancer Institute criteria, %T/C < 42% indicates that the drug is active (32, 33)

‡ Two cycles of troxacitabine were given.

§ Troxacitabine was given orally instead of intraperitoneally.

¶ Troxacitabine was given intravenously instead of intraperitoneally.

‖ Once daily administration was used instead of twice daily.

** For the leukemia studies, the results are expressed as the percentage of mean survival time of treated animals over the mean survival time of the control group (treated *versus* control, T/C%). By National Cancer Institute criteria, %T/C > 125% indicate that the drug has significant antitumor activity (35).

and mice died 3 days after the last injection (3). In rats, troxacitabine was relatively nontoxic when given as a single intraveneous injection (no effects up to 2,000 mg/kg). Repeated doses of 25 to 250 mg/kg administered intraveneously to rats over 5 days established the no observed adverse effect level at 25 mg/kg/day in male rats and 100 mg/kg/day in female rats. Cynomolgus monkeys were much more sensitive than rodents to single and multiple doses of troxacitabine. The maximum tolerated dose in the monkey after a single intraveneous dose was 1 mg/kg, and 0.20 mg/kg/day when administered for 5 days.[2]

The first phase I studies in patients with advanced solid tumors were thus undertaken at proportionally much lower doses than used for the mouse preclinical efficacy studies, and the final clinical recommended doses were over 15- to 125-fold lower than those used in the xenograft studies: 10 mg/m^2 as a 30-minute infusion every 3 weeks with granulocytopenia dose limiting toxicity (17); 1.2 mg/m^2/day for heavily pretreated patients and 1.5 mg/m^2/day for lightly pretreated patients as a 30-minute intravenous infusion daily for 5 days every 4 weeks with neutropenia and skin rash dose limiting toxicity (18) and 3.2 mg/m^2 as a 30-minute infusion weekly × 3 every 4 weeks as defined by prolonged myelosuppression (19). A phase I/II study of troxacitabine was also done in patients with acute leukemia using a 30-minute infusion daily × 5 days every 28 days. The dose-limiting toxicities were stomatitis and hand-foot syndrome, and the recommended dose was defined as 8 mg/m^2/day with activity observed in acute myelogenous leukemia (18% response rate in patients with relapsed/refractory disease) and chronic myelogenous leukemia in blastic phase (20). The pharmacokinetics of troxacitabine were consistent across the four phase I trials with urinary excretion of unchanged troxacitabine accounting for most of the drug elimination (17, 18). No metabolites were identified in the plasma or urine of treated patients.

Pilot troxacitabine phase II clinical trials were done at 10 mg/m^2 administered every 3 weeks in prostate, colorectal, pancreatic, renal cell (21), non–small-cell lung cancer (22), and malignant melanoma. Two partial responses were observed in 33 patients in the renal cancer trial with 21 patients having stable disease (median duration, 4.4 months) and 10 with progressive disease. Eight patients remained stable for >6 months, of whom six remain free of progression. The median survival was 18 months for patients classified as intermediate-risk and 8 months for the high-risk patients compared with 10 and 4 months, respectively, in Motzer's retrospective analysis (21, 23). Fifteen patients were enrolled in the pancreatic cancer study, nine previously treated with either a gemcitabine or 5-fluorouracil-containing regimen, and six chemotherapy naïve. Two patients with no prior chemotherapy met the criteria for attaining a clinical benefit response to troxacitabine, and a third patient had a partial response. Troxacitabine was then evaluated as first-line therapy in 54 patients with advanced adenocarcinoma of the pancreas but was administered at 1.5 mg/m^2 daily × 5 every 4 weeks (24). Median time-to–tumor progression was 3.5 months, median survival 5.6 months, and the

[2] Jolivet, J. unpublished observations.

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1-year survival rate 19%. A ≥50% decrease in CA 19-9 during therapy was seen in 16% of assessed patients. These results seem to be comparable overall to those reported with gemcitabine (25, 26).

Because the broad and potent antitumor activity of troxacitabine observed in the human xenograft mouse tumor models did not translate in the clinical setting, a reassessment and comparison of the pharmacodynamic and pharmacokinetic behaviors of troxacitabine *in vitro* and *in vivo* in murine and human tissues was undertaken. Our findings show that troxacitabine antitumor activity is dependent on total drug exposure (area under the curve, AUC) rather than on short elevated systemic drug concentrations (C_{max}). Furthermore, continuous administration of troxacitabine via subcutaneous implanted mini-osmotic pumps resulted in clinically achievable plasma concentrations (0.69–0.74 μmol/L) and led to comparable antitumor activity as when the compound was administered as bolus once a day for 5 consecutive days. With the bolus administration, C_{max} obtained in mice were ≥110 μmol/L that are not feasible in humans where we observed a C_{max} of 0.6 to 4 μmol/L at maximum-tolerated doses. Such sustained systemic concentrations would thus avoid elevated and toxic exposures in humans whereas maintaining clinically achievable and preclinically active drug concentrations.

MATERIALS AND METHODS

Materials. Troxacitabine (Troxatyl, (−)-2′-deoxy-3′-oxacytidine; Mr = 213) and its mono-, di-, and triphosphate forms were synthesized at Shire BioChem Inc. (Laval, QC, Canada) according to published procedures (27). Concentrated stock solution was prepared in saline 0.9%, aliquoted and stored at −20°C. At the time of testing, aliquots were thawed, and stock solution was further diluted in cell culture medium [thymidine incorporation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), clonogenic assay and *in vitro* metabolism studies] or in 0.9% saline (animal studies) and filter sterilized (Millex-GV, 0.22 μ, Millipore, Beford, MA). [^{14}C]Troxacitabine (50.4 mCi/mmol) was prepared by ViTrax Co. (Placentia, CA) from material provided by ShireBioChem Inc. [5-Methyl-^{3}H]thymidine (2.0 Ci/mmol) was purchased from Amersham Pharmacia (Oakville, ON, Canada). All other reagents used were of analytical grade and were obtained from commercial sources.

Cell Culture. Cell lines were obtained from the American Type Culture Collection (Manassas, VA), except for the murine colon carcinoma MC38 and the murine renal Renca cells that were kindly provided by National Cancer Institute (Bethesda, MD). Cells were grown in either RPMI (L1210, CCRF-CEM, Renca, and MC38), McCoy's 5 (Caki-1 and HT-29), Eagle's MEM (Calu-6) or DMEM (LLC1) supplemented with 10 to 20% fetal bovine serum (Life Technologies, Inc. Burlington, ON, Canada). When required, supplements (nonessential amino acids, glutamine, sodium pyruvate, vitamins from Wisent Inc., St-Bruno, QC, Canada; β-mercaptoethanol from Life Technologies, Inc.) were added according to the cell supplier instructions. Stock cell lines, which were shown to be free of *Mycoplasma* (routinely checked by Hoechst 33258 staining or PCR analysis; *Mycoplasma* PCR detection kit, Stratagene, La Jolla, CA), were maintained as suspension (L1210 and CCRF-CEM) or adherent (all others) cultures in the absence of antibiotics and incubated at 37°C in a humidified atmosphere (5% CO_2).

Chemosensitivity Testing. Exponentially growing cells were seeded in 96-well plates at a density of 2 to 3.5 × 10^3 cells/well (cell numbers were determined with a hemacytometer) and allowed to attach overnight. Thereafter, troxacitabine, diluted in media, was added at 1:10 serial dilutions in the appropriate media at final concentrations ranging from 10^{-10} to 10^{-5} mol/L (200 μL total volume). Cells were exposed to troxacitabine for 1, 4, 24, and 72 hours. For the 1, 4, or 24 hour(s) incubation period, drug-containing media was removed, cells were washed, and fresh media without drug was added for the remaining incubation period (72 hours total). As a measure of cell proliferation [methyl-^{3}H]thymidine (0.5 μCi/well) was added to the cultures during the final 18-hour incubation period. At the end of the incubation period (total 72 hours), cells were aspirated directly (L1210 and CEM-CCRF cells) or after trypsinization (adherent cell lines) onto glass fiber filters. Filters were dried and placed in plastic sample bags containing 6 mL of scintillation mixture. [5-Methyl-^{3}H]thymidine incorporation was measured with a Wallac 1450 MicroBeta Trilux (Perkin-Elmer, Wallac, Turku, Finland). For HT-29 cells, cell viability was also assessed with a standard MTT assay (28). After the addition of 50 μL of a 2 mg/mL MTT solution in PBS, the NADH-dependent reduction of MTT to form a formazan product was quantified with a MR500 96-well microtiter plate reader (MR5000 reader plate, Dynatech, Chantilly, VA) set at 570 nm. All measurements were done in triplicate, and each experiment was repeated two to three times. IC_{50} and GI_{50} values were calculated with the PrismPad computer program (GraphPad Software Inc., San Diego, CA). The IC_{50} was estimated from individual inhibition curves and represents the concentration of drug that inhibits 50% of cell proliferation. The 50% growth-inhibitory concentration (GI_{50}) is defined as the concentration of drug causing 50% inhibition in absorbance (minus the absorbance at day 0) compared with control (minus the absorbance at day 0; ref. 29).

For the clonal growth assay, subconfluent monolayers of HT-29 cells were trypsinized, plated as single cells onto 6-well flat-bottomed plates at a density of 200 to 400 cells/3 mL per well (cell numbers were determined with a hemacytometer) and allowed to attach overnight. Graded concentrations of troxacitabine were then added to plates for a period of 24, 72, and 144 hours. Thereafter, plates were washed twice, refed with fresh drug-free media, and incubated at 37°C in presence of CO_2 in a humidified atmosphere. At 10 days after seeding, cells were fixed and stained with crystal violet (0.5 in 20% methanol), and macroscopically visible colonies were counted. Drug effect is expressed in terms of the percentage of survival, obtained by comparison of the mean number of colonies formed on three drug-treated plates with the mean number of colonies formed on six untreated control plates.

Colony-Forming Assay of Bone Marrow Progenitor Cells. Hematopoietic progenitor cells (colony-forming unit granulocyte myeloid, CFU-GM) from human and mice were prepared for clonogenic assays according to the technique of Parchment and collaborators (30), and the assay was done at the

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Hipple Cancer Research Center (Dayton, Ohio). Murine bone marrow cells were obtained from male CD2F1 mice. The femurs were aseptically removed and the marrows flushed with Iscove's Modified Dulbecco's Medium. Human bone marrow cells were obtained from femoral canal reamings from patients undergoing orthopedic surgery with informed consent under an Institutional Review Board-approved protocol. The specimens were harvested by the surgeon and placed in a 50-mL tube containing 20 to 30 mL of α-MEM with preservative-free heparin. Mononuclear marrow cells were isolated by filtration through a sterile nylon mesh into a sterile centrifuge tube. The samples were centrifuged at 600 × *g* for 10 minutes. Cell pellets were resuspended in α-MEM and layered over 4 mL of Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) and centrifuged for 30 minutes at 600 × *g*. After centrifugation, the mononuclear cells were harvested from the interphase and washed twice in α-MEM. Mouse and human bone marrow cells were then suspended in Iscove's Modified Dulbecco's Medium that contained 20% fetal bovine serum, recombinant murine or human granulocyte-macrophage colony-stimulating factor (RDI, Flanders, NJ) and 0.3% agarose. Aliquots of 0.4 mL of this solution were plated into tissue culture plates over a preformed underlayer of agarose in Iscove's Modified Dulbecco's Medium. Bone marrow cells were incubated at 37°C in a humidified atmosphere flushed with 5% CO_2. For drug treatment, bone marrow cells from both species were exposed either continuously or for 1 hour to 0.001 to 100 μmol/L troxacitabine. At 7 (mouse) and 14 (human) days after seeding, the numbers of CFU-GM colonies were counted. The percentage of inhibition was calculated by comparing the number of colonies appearing in the troxacitabine groups with the number that grew in the untreated control group. The concentrations that caused 50% growth inhibition were calculated by regression analysis.

Intracellular Metabolism of [^{14}C]Troxacitabine. The intracellular metabolism of [^{14}C]troxacitabine was evaluated in mouse- and human-activated T-lymphocytes obtained from peripheral blood (PBLs). Heparinized blood was obtained from 10 adult inbred C57B/L6 female mice and three healthy human volunteers. Mononuclear cells were isolated from heparinized blood by density centrifugation with Lympholyte-M (mouse, CL-5035; Cedarlane, Labs Ltd., Hornby, ON, Canada) and Lympholyte-poly (humans, CL5070; Cedarlane, Labs Ltd.) to remove erythrocytes, granulocytes, and cellular debris. Mononuclear cells were washed three times in PBS, centrifuged at 600 × *g* for 10 minutes at 10°C and resuspended in RPMI 1640 supplemented with 10% fetal bovine serum. Cells were plated at a density of 5×10^6/mL in 100-mm culture dishes (Corning, Corning, NY) and placed in a 37°C humidified incubator conditioned with 5% CO_2 for 1 hour. Nonadherent cells, which are mostly PBLs (31, 32) were collected, resuspended in RPMI 1640 supplemented with 10%, plated in 12-well plates at a concentration of 5×10^6 cells per 2 mL per well and incubated at 37°C in the presence of Concavalin A (ConA; 0.5 μg/mL; Calbiochem, San Diego, CA) and 1μCi/mL [^{14}C]troxacitabine for up to 48 hours. After 4, 24, and 48 hours, cells were harvested (1 well per/time point/species), centrifuged at 600 × *g* for 7 minutes at 10°C, and the resulting cell pellets were resuspended in 60% ice-cold methanol (mice blood samples were pooled, but the human samples were treated individually). Analysis of samples and determination of troxacitabine, troxacitabine-monophosphate, troxacitabine-diphosphate, and troxacitabine-triphosphate were carried out by high-performance liquid chromatography with online radiodetection as described previously (1).

For viability studies, PBLs (obtained as described above) were seeded at 2×10^5/well in a 96-well round-bottomed tissue culture plates in RPMI plus 10% fetal bovine serum. The effect of drugs on quiescent PBLs was measured with the Live/Dead cell-mediated cytotoxicity kit according to the manufacturer protocol (Molecular Probe, Eugene, OR). ConA (0.5 μg/mL) was used to induce PBLs to proliferate. Before stimulation, appropriate dilutions of troxacitabine were added to the different wells. Cell proliferation was measured 72 hours later by adding [5-methyl-^{3}H]thymidine during the last 16 hours of incubation as described above.

Animal Studies. Animal studies were done in the animal facility at Shire BioChem Inc. with the prior approval of the local Institutional Animal Care Committee and in agreement with the guidelines provided by Canadian Council for Animal Care. Athymic *nu/nu* (CD-1) and inbred (C57BL/6) female mice, 6- to 8-week-old, were purchased from Charles Rivers Laboratories (St-Constant, Quebec, Canada). Animals were maintained under specific pathogen-free conditions and provided with sterile food and water *ad libitum*. The mice were allowed to acclimate for at least 5 days before the beginning of the study.

Antitumor Efficacy Studies. Female athymic CD-1(*nu/nu*) mice were injected subcutaneously with 2×10^6 human colon HT-29 tumor cells. Tumor-bearing animals were randomized (10–15 per group), and treatment started when tumor volumes reached 80 to 120 mm^3 (days 6 to 9, depending on the study). Tumor measurements, taken by calipers twice weekly, were converted to tumor volumes (in mm^3) with the following formula: $[\text{width (mm)}]^2 \times \text{length (mm)} \times 0.50$ (33). Troxacitabine treatments were done either by single or multiple bolus intraveneous injections (q1d×5 or q7d×3) or by continuous administration via Alzet osmotic minipumps (ALZA, Palo Alto. CA). The pumps were implanted subcutaneously under sterile conditions at the site opposite to the tumor. These pumps (internal volume, 200 μl) continuously deliver test agent at a rate of 1 μL/hour for 200 hours (8.3 days). Pumps were removed after 1, 3, 5, or 6 days, thus insuring continuous delivery of the test agent over the course of the experiment. The control groups received comparable bolus injection of saline (intraveneously) or subcutaneously implanted saline-loaded pumps. Compound efficacy was assessed at the end of the study by the percentage of T/C defined as the mean-treated tumor volume per mean control tumor volume × 100%, with a T/C of <42% being indicative of antitumor activity (34, 35). To monitor the drug-associated toxicity, mice were weighed at least twice a week and inspected daily for observable clinical signs. Statistical analysis was done by ANOVA or by Student's *t* test. Differences were considered to be significant at $P < 0.05$.

Pharmacokinetic Profiling. Female HT-29 tumor-bearing CD-1(*nu/nu*) mice were dosed either via the intraveneous bolus or subcutaneously continuous infusion route (as described above). For the pharmacokinetics of single bolus injections, mice ($n = 20$; $n = 4$ per time point) received 21 mg/kg (100

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μmol/L/kg) of troxacitabine in saline (administration volume was 10 mL/kg). Serial blood samples were collected by cardiac puncture into heparinized tubes at 5, 15, 30, 60, and 360 minutes after compound administration. To determine plasma concentrations of troxacitabine after continuous infusion, blood samples from mice receiving continuous administration via miniosmotic pumps containing 5, 10, and 20 mg/mL of troxacitabine (n = 5/time point) were collected at 3 hours, 1, 3, and 5 days after troxacitabine-loaded pump implantation. Blood was centrifuged at 10,000 × g for 10 minutes and plasma was collected and frozen at −20°C until further analysis. An aliquot of mouse plasma (100 μL) was precipitated with 300 μL of acetonitrile containing 3TC as internal standard. After centrifugation at 3,000 × g for 5 minutes, 300 μL of supernatant was collected and evaporated to dryness under a gentle stream of nitrogen. The residue was then reconstituted with 100 μL of deionized water. The calibration curve of troxacitabine was linear between 1 and 500 ng/mL. The chromatography was achieved on a Luna C18 column (150 × 2 mm, 5 μm, Phenomenix, Torrance, CA) with a flow rate of 0.25 mL/minutes. The aqueous mobile phase, consisting of 10 mmol/L ammonium acetate buffer (pH6.8), was held for 5 minutes and followed by a linear gradient of 0 to 50% acetonitrile over 8 minutes. Sample analysis was done on a liquid chromatography tandem mass spectrometry (TSQ7000, Thermo Finnigan, San Jose, CA) equipped with electrospray source. The signals of troxacitabine and the internal standard were monitored by m/z transitions at 214–112 and 230–112, respectively.

Pharmacokinetic parameters after intravenous administration were calculated by standard noncompartmental methods with Kinetica (Kinetica 2000, Innaphase Corp., Philadelphia, PA). C_{max} (defined as the concentration at 2 minutes) was read directly from the data. The AUC was calculated by the linear trapezoidal rule. The AUC was extrapolated to infinity by dividing the last measured concentration (C_n) by λ_z, where λ_z represents the terminal slope. Systemic clearance was calculated by dividing the dose by $AUC_{0\to\infty}$. The volume of distribution was estimated by the following formula: V_{ss} = dose · AUMC/AUC^2, where AUMC is the area under the first moment curve. The exposure ($AUC_{0\text{-}t}$) after continuous infusions via Alzet pumps was estimated assuming constant exposure to concentrations measured at the corresponding time points after pump implantation.

RESULTS

Antiproliferative Activity of Troxacitabine on Murine and Human Cells. The ability of troxacitabine to inhibit cell proliferation was examined on a panel of murine (leukemia, L1210; renal, Renca; colon, MC-38; and lung, LLC1) and human (leukemia, CCRF-CEM; renal, Caki-1; colon, HT-29; and lung, Calu-6) tumor cell lines. Troxacitabine-inhibitory activity increased with concentration and time exposure; the IC_{50} values for the 72-hour continuous exposure; were approximately 10-fold < IC_{50} values obtained after 1 and 4 hours (Table 2). Troxacitabine was also found to be significantly more potent toward human cell lines compared with murine cell lines (Table 2). The highest differential ratio was observed against the renal tumor cell lines (880-fold). In the leukemia, lung, and colon tumor cell lines, the ratios varied from 5- to 15-fold.

To further evaluate possible species-dependent toxicity, we evaluated the effect of troxacitabine on normal mouse and human hematopoietic cells. Troxacitabine was not toxic to quiescent PBLs (no effect was observed at 100 μmol/L, the highest drug concentration tested; results not shown). Cytotoxicity was apparent when the compound was evaluated on ConA-activated PBLs: IC_{50} values of 260 and 11 nmol/L were obtained for mouse and human cells, respectively (results not shown), indicating that human T-lymphocytes were 20 times more sensitive than mouse T-lymphocytes. Myelotoxicity of troxacitabine was evaluated on mouse and human granulocyte-macrophage (CFU-GM) progenitor cells. A dose-dependent inhibition of colony formation was observed in both species with human cells being the most sensitive. A 1 hour exposure resulted in IC_{50} values of 10 and 0.04 μmol/L for mouse and human CFU-GM progenitor cells, respectively, whereas these values were 0.05 and 0.001 μmol/L after continuous exposures (results not shown). Human cells were around 50 times more sensitive than mouse cells to continuous troxacitabine exposure.

Intracellular Metabolism. To understand the mechanism by which human cells were more sensitive toward troxacitabine compared with murine cells, we examined the formation of intracellular troxacitabine phosphate metabolites *in vitro* in T-lymphocytes obtained from peripheral blood of both species (as described above). As with all nucleoside analogs with antitumor activity, troxacitabine requires intracellular phosphorylation into its active triphos-

Table 2 Antiproliferative activity of troxacitabine on a panel of human and mouse tumor cell lines (IC_{50} μmol/L)

Cell lines	1 hour	4 hours	24 hours	72 hours	Ratio *
CCRF-CEM	0.38 ± 0.26	1.1 ± 0.6	0.185 ± 0.04	0.057 ± 0.05	
L1210	57 ± 47	12 ± 1	2.95 ± 0.5	0.825 ± 0.12	14.5
Caki-1	0.48 ± 0.2	0.13 ± 0.03	0.041 ± 0.02	0.017 ± 0.01	
Renca	>100	>100	76 ± 34	15 ± 1.4	882
Calu-6	2 ± 0.98	1.2 ± 0.4	1.0 ± 0.68	0.074 ± 0.01	
LLC	17.5 ± 0.7	12.5 ± 6.4	7.05 ± 0.9	0.77 ± 0.06	10.4
HT-29	0.55 ± 0.07	0.55 ± 0.06	0.24 ± 0.08	0.021 ± 0.001	
MC38	9.95 ± 7.14	3.55 ± 1.2	1 ± 0.28	0.106 ± 0.03	5.05

NOTE. IC_{50} was measured after 1, 4, 24, and 72 hours after troxacitabine addition. Antiproliferative activity was determined by adding [^{3}H]thymidine during the final 18 hours of a total incubation period of 72 hours. The values represent the means ± SD of two to three experiments where each data point is an average of three measurements.

* Ratio is the IC_{50} value of the mouse tumor cell line over its respective human tumor cell line at 72 hours.

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Table 3 [14C]Troxacitabine total incorporation (pmol × 10^6 cells)

Samples	4 hours	24 hours	48 hours
Mice (pool)	7.96	3.72	4.36
Human (1)	7.0	17.6	22.36
Human (2)	5.28	19.2	25.36
Human (3)	3.2	11.64	10.72

NOTE. PBLs were obtained from mice (pool of 10 animals) and human (3 healthy volunteers) heparinized blood as described in Material and Methods. Cells were incubated in the presence of ConA (to stimulate the T-lymphocyte population) and [^{14}C]troxacitabine (2 μCi/5 × 10^6 cells) for 4, 24, and 48 hours. Cells were harvested, centrifuged at 600 × *g* for 7 minutes at 10°C, and the resulting cell pellets were resuspended in 40 μl of 60% ice-cold methanol. Radioactivity present in the samples was determined with a β-scintillation counter.

phate form to exhibit its biological activity. The metabolism of [^{14}C]troxacitabine by mouse and human ConA-activated PBLs was compared after 4-, 24-, and 48-hour exposures. Uptake, as measured by the amount of pmol/10^6 cells, was similar in mouse and humans at the 4-hour time point (Table 3). Uptake in mouse PBLs seemed to have reached a saturation level by 4 hours because the radioactivity present in the samples was not significantly different after the 24- and 48-hour incubation periods. In humans, however, uptake had not reached saturation levels at 4 hours and continued to increase with incubation time (Table 3). We observed very little metabolism of troxacitabine in mice (Fig. 1*A*). At 4, 24, and 48 hours, 98.7, 88.9, and 97%, respectively, of total counts were coeluting with the parent compound. These values were 24.5, 12.8, and 16.6%, respectively, in human PBLs. In human PBLs, troxacitabine was converted into the mono-, di-, and triphosphate metabolites, with the mono- and di- forms each accounting for approximately 40% of the total counts at the different incubation periods (Fig. 1*B*). In contrast, the mono- and diphosphate metabolites represent only 10% of the total count in the mouse cells, and the levels of the triphosphate did not vary significantly as a function of time (0.42 to 0.69%; Fig. 1*A*). In human PBLs, the triphosphate metabolite continued to increase as a function of the incubation time, from 0.7 to 11% (Fig. 1*B*).

Time and Concentration-Dependent *In Vitro* Cytotoxicity in HT-29 Cells. To better define the concentration and exposure requirements of troxacitabine for optimal efficacy, we examined these parameters in detail with the HT-29 human colorectal carcinoma cell line. Two different methods were used, the MTT colometric assay and clonal growth. Troxacitabine showed weak-inhibitory activity after 1- and 4-hour exposures with GI_{50} values of 265 and 46 μmol/L, respectively (Fig. 2*A*), with 72-hour being 265-fold more potent than 1-hour exposures. Longer drug exposures were examined in the clonal growth assay (Fig. 2*B*). The 5-fold difference between IC_{50} values observed after 24- and 72-hour exposures (2.2 and 0.47 μmol/L, respectively) was similar to that observed in the MTT assay, in which we obtained GI_{50} values of 5 and 1 μmol/L, respectively. Increasing troxacitabine exposure from 72 to 144 hours had no further impact on *in vitro* cytotoxicity.

Schedule-Dependent *In Vivo* Antitumor Efficacy in Human Xenografts. The impact of the marked *in vitro* time dependency of troxacitabine on its *in vivo* activity was next examined with the same HT-29 human colorectal cancer cells grown as xenografts in nude mice. Troxacitabine was administered as a continuous infusion with Alzet minipumps at doses of 0.5, 5, 10, and 50 mg/mL for 6 consecutive days, when the tumors had reached 100 to 120 mm^3 (day 9; Fig. 3). The 50

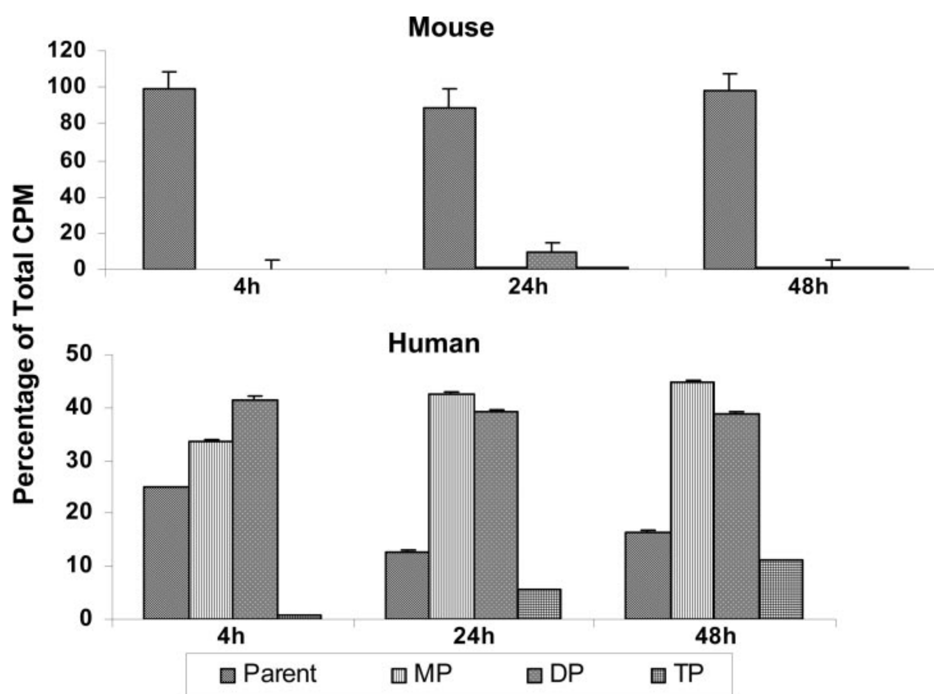

Fig. 1 Intracellular levels of troxacitabine and metabolites in mouse and human peripheral white blood cells. ConA-activated T-lymphocytes obtained from mouse and human peripheral blood (5 × 10^6 cells/time point) were incubated with 2μCi of ^{14}C-troxacitabine (specific activity, 50.4 mCi/mmol) for 4, 24, or 48 hours. Results are expressed as the relative percentage of each peak of the total counts loaded on the column, determined by high-performance liquid chromatography as described in Materials and Methods. Data points for troxacitabine metabolism studies represent the mean ± SD of three determinations. Parent, unchanged drug; MP, troxacitabine monophosphate; DP, troxacitabine diphosphate; TP, troxacitabine triphosphate.

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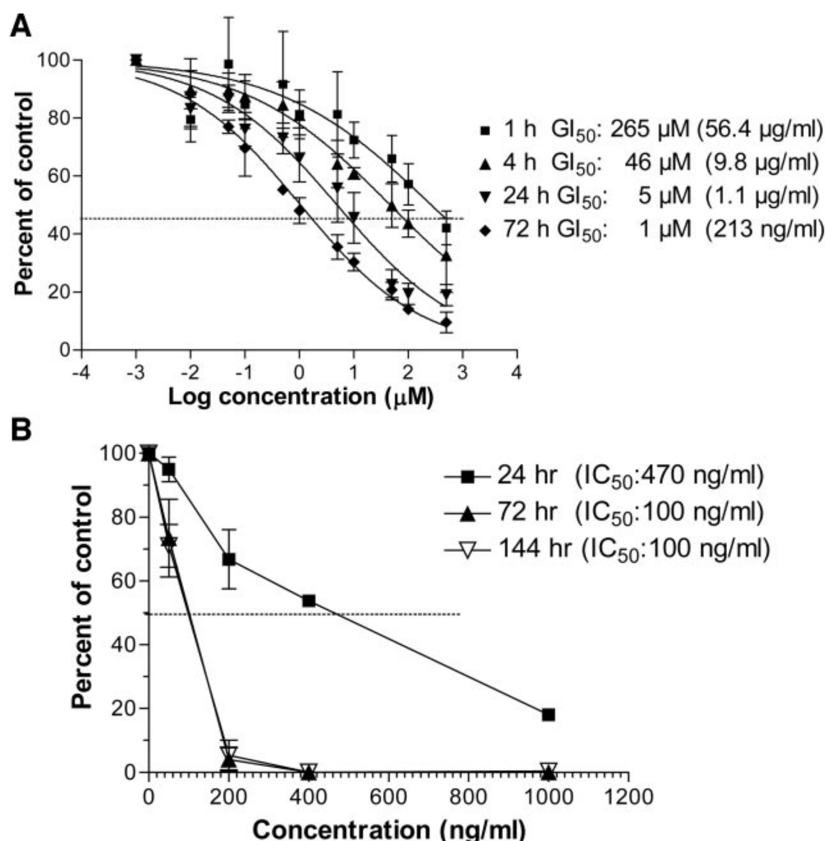

Fig. 2 Effect of drug exposure on the cytotoxicity of troxacitabine in human HT-29 colon carcinoma cells. *A*, cells were plated at a concentration of 3×10^3 cells/well and exposed to troxacitabine for 1, 4, 24, and 72 hours. At the end of the incubation period, cell viability was quantified by a standard MTT assay. *B*, clonal growth response of human HT-29 colon carcinoma cells. Cells were plated at 200 to 400 cells/dish and grown in the presence of increasing concentrations of troxacitabine for 24, 72, and 144 hours. At the end of the incubation period, cells were washed and refed with fresh drug-free media. Colonies were enumerated 10 days after seeding. Drug effect is expressed in terms of the percentage of survival, obtained by comparison of the mean number of colonies formed on three drug-treated plates with the mean number of colonies formed on six untreated control plates. Each point represents the average value from two separated experiments.

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mg/mL dose, equivalent to 45 to 50 mg/kg/day, was considered as the maximum tolerated dose as we observed ≥20% body weight loss 3 to 5 days after initiation of treatment. Furthermore, one mouse from this group had to be sacrificed because of significant weight loss. The nine remaining mice from this group started to gain weight at day 17 and by day 26 their body weight had reached those of control animals. Body weight gain of mice from the other treated groups were comparable with those from the saline-treated group. Animals were sacrificed on day 31, time at which tumor volumes in the saline-treated control group had reached 5 times their original volume (500% growth). T/Cs were 79, 42, 22, and 11% for the 0.5, 5, 10, and 50 mg/mL doses, respectively, indicating antitumor activity at all but the lower dose level. Moreover, tumor regressions were observed from days 29 and 21 in animals receiving 10 and 50 mg/mL troxacitabine, respectively.

A second study was done with 5, 10, and 20 mg/mL infused over 1, 3, and 5 days, when the tumors had reached 85 mm^3 (day 6). The 1 day infusion schedule had no effect, regardless of dose, and tumor growth was similar in all treated groups (data not shown). Antitumor activity was observed with 20 mg/mL when infused over 3 days (Fig. 4*A*). This dose and schedule resulted in tumor stasis during the 1st week after treatment followed by tumor regrowth. At the end of the study, when tumors in the control group had increased by 9- to 10-fold, the T/C was 44% (*P* value of 0.03 when compared with the saline-treated group). This dose resulted in significant antitumor activity when given over a 5-day infusion period (Fig. 4*B*). Tumor regressions were observed from day 18 till day 28 followed by regrowth, leading to a T/C of 27%.

We then compared the antitumor activity of troxacitabine given by continuous infusion to that of single and repeated bolus administration, using similar total doses (53–63 mg/kg). As illustrated in Fig. 5, a single bolus injection of 63 mg/kg showed little activity, inhibition of tumor growth was intermediate with 3-day administration regimens (q7d×3 or 3-day infusions) and optimal with 5 to 6 day regimens (q1d×5 or 6-day infusions). As predicted by the *in vitro* experiments, repeated or prolonged infusional drug administration regimens of smaller fractionated doses led to superior antitumor activity compared with larger drug doses administered less frequently.

Murine *versus* Human Pharmacokinetics. We next examined the pharmacokinetics of troxacitabine in nude mice to determine the pharmacokinetics parameters associated with activity and compared it with the same parameters achieved in humans. After a bolus intraveneous dose of 21 mg/kg, the troxacitabine plasma concentration taken at 5 minutes (earliest time point and considered as the C_{max}) was 115 μmol/L with an elimination $t_{1/2}$ of 13 minutes (Table 4). By 60 minutes, drug concentration was down to 4.5 μmol/L, which is well above the IC_{50} value for human cells (Table 2). By comparison, after intravenous administration of troxacitabine at 10 mg/m^2 (maximum tolerated dose in humans), the troxacitabine plasma C_{max} was 4.1 μmol/L with an elimination $t_{1/2}$ of 12 hours (Table 4;

ref. 17). Less than 30 minutes after the end of infusion, drug concentration was down to <1 μmol/L, but it remained above 0.1 μmol/L for approximately 12 hours. During continuous subcutaneous infusion with Alzet osmotic minipumps containing 10 or 20 mg/mL, mean troxacitabine steady state values of 152 ng/mL (0.7 μmol/L) and 304 ng/mL (1.4 μmol/L) were achieved by 3 hours after pump implantation (data not shown) and maintained over the experimental study (data for the 3 and 5 day exposure is presented in Fig. 4). Continuous administration of troxacitabine at 10 mg/mL resulted in a total exposure of 10.5 and 19 μg · hour/mL when administered over 3 and 5 days, respectively. These values were 24.9 and 31.4 μg · hour/mL after the 20 mg/mL administration.

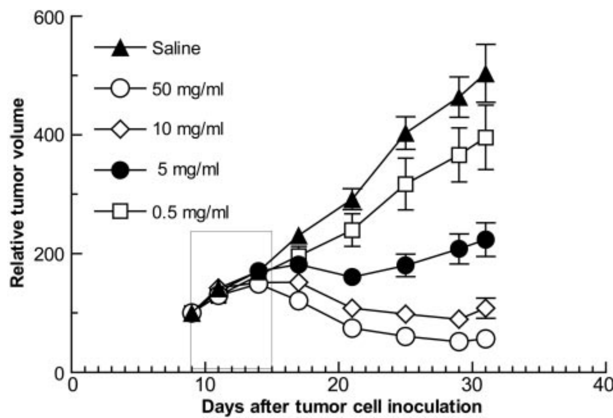


Fig. 3 Activity of troxacitabine against the HT-29 human colon tumor xenograft. Female athymic CD-1(*nu/nu*) mice were injected subcutaneously with 2×10^6 human colon HT-29 tumor cells. Tumor-bearing animals were randomized (10/group) and treatment started when tumor volumes reached an average size of 100 mm^3 (day 9). Troxacitabine treatments were done by continuous administration via Alzet osmotic minipumps for 6 days.

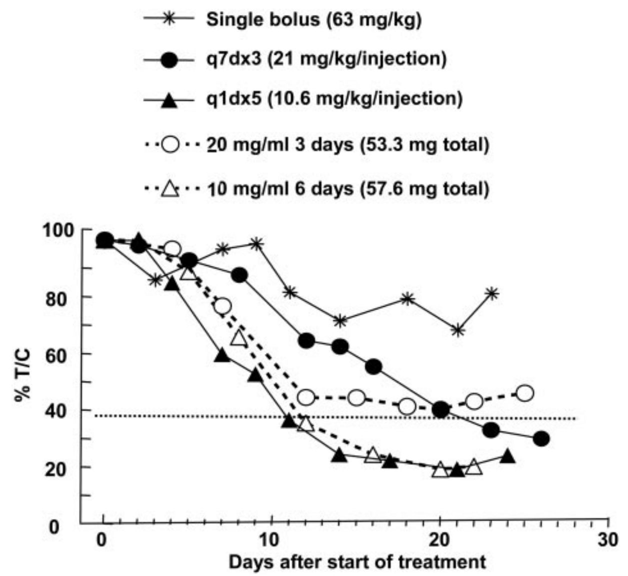
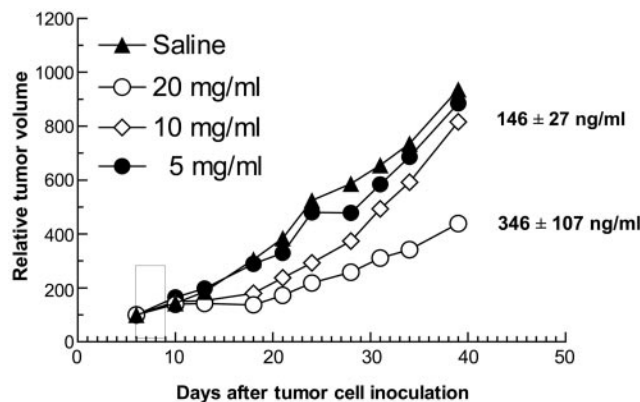


Fig. 5 Comparative antitumor activity of troxacitabine using different schedules but similar total dose (53–63 mg/kg). The curves generated for the treatment derived from separate experiments, in which the control tumors grew at a comparable rate. Female athymic CD-1(*nu/nu*) mice were injected subcutaneously with 2×10^6 human colon HT-29 tumor cells. Tumor-bearing animals were randomized (10–15/group) and treatment started when tumor volumes reached 80–120 mm^3 (day 6–9). Troxacitabine treatments were done either by single or multiple bolus intraveneous injections (q1d×5 or q7d×3) or by continuous administration via Alzet osmotic minipumps (3 and 6 days) at the doses indicated in the legend.

DISCUSSION

The preclinical and clinical safety evaluation of troxacitabine revealed the expected spectrum of target organ toxicities with the hematopoietic system being the common target in both rodents (mice and rats) and primates (monkeys and humans). Whereas the toxicity profile observed with troxacitabine was not



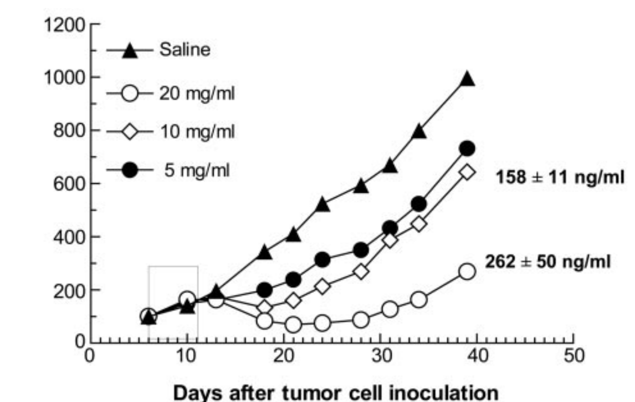


Fig. 4 Effect of infusion time on antitumor efficacy of troxacitabine against the HT-29 human colon tumor xenograft. Female athymic CD-1(*nu/nu*) mice were injected subcutaneously with 2×10^6 human colon HT-29 tumor cells. Tumor-bearing animals were randomized (15/group), and treatment started when tumor volumes reached an average size of 85 mm^3 (day 6). Troxacitabine treatments were done by continuous administration via Alzet osmotic minipumps for 3 days (*left*) or 5 days (*right*). At the end of each infusion treatment, 5 mice/group were bled for troxacitabine plasma concentration.

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Table 4 Pharmacokinetic parameters of troxacitabine for mice and humans after intravenous administration

Parameters	Mice	Humans *
Dose	21 mg/kg (60 mg/m^2)	10mg/m^2
C_{max} ng/mL †	24628	882 ± 128
μmol/L ‡	115	4.1 ± 0.6
AUC (μg · hour/mL)	7.44	1.89 ± 0.37
t (hour)	0.22	12 ± 3.5
Clearance mL/minute	1.27	159 ± 37
Clearance mL/m^2	192.4	99.4
V_{ss} (L)	0.0184	65 ± 20

NOTE. Data represents mean values obtained in three mice (per time point) and in seven patients.

* Data from Bélanger *et al.* (17).

† Measured concentration at 5 minutes.

‡ Troxacitabine has a Mr of 213.

unexpected, the species differences in sensitivity were remarkable and unexpected. On a mg/kg basis, troxacitabine was 500- and 2,500-fold more toxic to monkeys and humans, respectively compared with rats and mice after 5-daily doses. On a mg/m^2 basis, this difference was 120- and 200-fold, respectively when compared with mice and 240- and 400-fold when compared with rats [conversion factors used are from Freireich *et al.* (36)]. The purpose of this study was to gain a better understanding of the difference in species sensitivity and antitumor efficacy observed between the mouse models and the early clinical results in humans.

When assayed *in vitro* on a panel of mouse and human tumor cell lines and normal hematopoietic cells, we observed a 5- to 900-fold difference in sensitivity between mouse and human cells. Furthermore, troxacitabine activity was particularly vulnerable to the length of drug exposure with short exposures requiring up to 50-fold higher drug concentrations than longer exposure for activity. Lengthening exposures >72 hours was of no further benefit. The marked dependence of troxacitabine on the length of drug exposure can probably be explained by its low affinity for both the equilibrative and sodium-dependent nucleoside transporters. Indeed, the non-carrier-mediated membrane permeation of this hydrophilic drug ($\log P = -1.43$) is slow and probably requires very high extracellular drug concentrations or hours at lower concentrations for significant intracellular drug accumulation to occur (1).

Different phosphorylation rates were thought to be the most likely explanation for the different susceptibility of rodents and primates toward troxacitabine. To inhibit DNA synthesis and produce its cytotoxic effect, troxacitabine must, after entering the cell, undergo a series of phosphorylation reactions through a first rate-limiting step catalyzed by deoxycytidine kinase (dCK) to form the active triphosphate nucleotide (1, 2). Interspecies differences in the kinetic properties (37) and substrate specificity (38) of dCK have been reported previously. Furthermore, interspecies difference in toxicity and efficacy were reported for a few other purine and pyrimidine antimetabolites (39, 40). Indeed, we observed a significant difference in the phosphorylation profile of troxacitabine in mouse- and human-activated PBLs. There was a 12-fold increase in the amount of troxacitabine triphosphate, the active species, formed in human *versus* mouse-activated PBLs.

Pharmacokinetics differences between mice and humans are also likely to contribute to interspecies differences. In human xenograft models, troxacitabine was usually administered at 25 mg/kg once or twice daily for 5 consecutive days. This dosing strategy was consistently effective, not toxic, and was used in all of the reported preclinical *in vivo* troxacitabine efficacy studies (summarized in Table 1; refs. 2, 3, 9–15). These high-dose bolus drug administration yielded plasma concentrations of ≥110 μmol/L 5 minutes after drug administration. However, a similar bolus dose administration strategy was not achievable in humans (17–20). As shown in the current study, human tissues phosphorylate troxacitabine much more readily than murine tissues and are thus significantly more sensitive to the toxic effects of troxacitabine. Administered clinical doses have thus been proportionally over 15- to 125-fold lower than those used in nude mice yielding plasma concentrations of 0.6 to 4 μmol/L 5 minutes after drug administration. Mice can thus tolerate much higher drug systemic concentrations (>20-fold) and exposures (>6-fold) compared with humans. Consequently, the drug concentration profiles observed in human plasma seem to be suboptimal to achieve a maximal therapeutic effect based on the preclinical *in vitro* and *in vivo* data.

We thus examined in human xenografts if a different and potentially clinically applicable troxacitabine administration schedule could reproduce the potent antitumor profile achieved preclinically by repeated high-dose bolus administrations. We showed that prolonged exposure to low micromolar troxacitabine concentrations (0.7–1.4 μmol/L) leads to the same tumor growth inhibition as seen after high-dose bolus administration without the need to achieve peak plasma drug concentrations ≥110 μmol/L. Indeed, treatment with a continuous infusion of 10 mg/mL over 6 days resulted in the same antitumor activity as the bolus daily treatment for 5 consecutive days with 10.6 mg/kg (Fig. 5). Whereas both treatments resulted in similar total doses (57.6 and 53 mg/kg, respectively) they give very different pharmacokinetics profiles resulting in 100-fold differences in peak concentrations (0.7–1.4 μmol/L *versus* ≥110 μmol/L). Interestingly, total exposure of troxacitabine was similar with both regiments. Assuming linearity between drug and plasma concentrations and no accumulation, the 10.6 mg/kg daily treatment for 5 consecutive days would result in an AUC of 18.8 μg · hour/mL. In comparison, infusion of 10 mg/mL at a rate of 1 μL/hour over 5 consecutive days would result in a calculated AUC of 19 μg · hour/mL (for a 24g mouse). These data suggest that troxacitabine antitumor activity is more dependent on AUC rather than C_{max}. Consequently, clinical phase I studies of prolonged troxacitabine infusions have been initiated in patients with advanced solid tumors and acute leukemia. Early results from these studies suggest that prolonged (up to 6-day exposures) to low micromolar troxacitabine concentrations are clinically feasible (41). The preclinical evidence supports the hypothesis that troxacitabine infusions might be the regimen with the greatest likelihood of fully exploiting clinically the potent preclinical antitumor activity of troxacitabine.

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ACKNOWLEDGMENTS

We thank Lilianne Geerts for help with the animal studies and Jose E. Cardier, from the Hipple Cancer Research Center, for performing the myelotoxicity studies on mouse and human CFU-GM progenitor cells. We also thank Armand Girard and Roger Secker for helpful discussions and revisions and Johanne Cadieux for assistance in the preparation of the report.

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