Cancer Chemother Pharmacol (1997) 39: 467–472

SHORT COMMUNICATION

Connie L. Erickson-Miller · Richard D. May
Joseph Tomaszewski · Blaire Osborn
Martin J. Murphy · John G. Page · Ralph E. Parchment

Differential toxicity of camptothecin, topotecan and 9-aminocamptothecin to human, canine, and murine myeloid progenitors (CFU-GM) in vitro

Received: 20 October 1995 / Accepted: 5 September 1996

Abstract *Purpose*: 20(*S*)-Camptothecin (CAM), topotecan (TPT, active ingredient in Hycamtin) and 9-amino-20(*S*)-camptothecin (9AC) are topoisomerase I inhibitors that cause similar dose-limiting toxicities to rapidly renewing tissues, such as hematopoietic tissues, in humans, mice, and dogs. However, dose-limiting toxicity occurs at tenfold lower doses in humans than in mice. The purpose of the current study was to determine whether hematopoietic progenitors of the myeloid lineage from humans, mice, and dogs exhibit the differential sensitivity to these compounds that is evident in vivo. *Methods*: Drug-induced inhibition of in vitro colony formation by a myeloid progenitor in human, murine, and canine marrow colony-forming unit-granulocyte/macrophage (CFU-GM) provided the basis for interspecies comparisons at concentrations which inhibited colony formation by 50% (IC_{50}) and 90% (IC_{90}). *Results*: Murine IC_{90} values were 2.6-, 2.3-, 10-, 21-, 5.9-, and 11-fold higher than human values for CAM lactone (NSC-94600) and sodium salt (NSC-100880), TPT (NSC-609699), and racemic (NSC-629971), semisynthetic and synthetic preparations (NSC-603071) of 9AC, respectively. In contrast, canine IC_{90} values were the same as, or lower than, the human IC_{90} values for all six compounds. *Conclusions*: The greater susceptibility of humans and dogs to the myelotoxicity of camptothecins, compared to mice, was evident in vitro at the cellular level. Differential sensitivity between murine and human myeloid progenitors explains why the curative doses of TPT and 9AC in mice with human tumor xenografts are not achievable in patients. Realizing the curative potential of these compounds in humans will require the development of therapies to increase drug tolerance of human CFU-GM at least to a level equal to that of murine CFU-GM. Because these interspecies differences are complicated by species-specific effects of plasma proteins on drug stability, not all in vitro assay conditions will yield results which can contribute to the development of such therapies.

Key words Pharmacodynamics · Topoisomerase I · Hematopoiesis · Neutropenia · Myelosuppression

C.L. Erickson-Miller[1] · M.J. Murphy · R.E. Parchment
Hipple Cancer Research Center, 4100 South Kettering Blvd., Dayton, OH 45439, USA

R.D. May
Cell Biology and Immunology Group, Southern Research Institute, 2000 Ninth Avenue South, Birmingham, AL 35205, USA

J. Tomaszewski · B. Osborn
Toxicology and Pharmacology Branch, DTP, DCTDC National Cancer Institute, NIH, Executive Plaza North, Room 843, 9000 Rockville Pike, Bethesda, MD 20892, USA

J.G. Page
Preclinical Toxicology Division, Southern Research Institute, 2000 Ninth Avenue South, Birmingham, AL 35205, USA

R.E. Parchment (✉)
Division of Hematology-Oncology, Wayne State University, Hudson-5, 3990 John R, Detroit, MI 48201, USA
Fax 313–993–0559; Email parchment@oncgate.roc.wayne.edu

[1] *Present address*: SmithKline Beecham, Department of Molecular Virology and Host Defense, 1250 Collegeville Road, Collegeville, PA 19426, USA

Introduction

Camptothecin (CAM) is an alkaloid isolated from the plant *Camptotheca acuminata*, indigenous to China [39]. Although its development as an antineoplastic agent was halted originally because of life-threatening myelosuppression, diarrhea, and hemorraghic cystitis [15, 27], several recent events have generated renewed interest in developing the lactone form of the drug (in place of the hydroxy acid salt originally tested), as well as the lactone forms of CAM derivatives such as topotecan (TPT) and 9-aminocamptothecin (9AC) [23, 40]. First, the lactone form of CAM has been identified as the active species [9] and clinical use of lactone may reduce urological toxicity [33]. Second, these compounds exhibit a novel mechanism of action; they target the nuclear enzyme topoisomerase I [20, 21]. Third, resistance to CAMs usually involves alterations in topoisomerase I structure and/or expression, which does not affect chemosensitivity to topoisomerase II inhibi-

tors (e.g. VP-16), microtubule-targeted drugs (e.g. taxol), other MDR substrates, or alkylating agents [10, 11, 17].

CAM lactone, TPT and 9AC cure nude mice harboring xenografts from a variety of human cancers, producing cures even when therapy is delayed until tumors are 1 cm^3 in size [12–14]. However, humans can tolerate only 11% as much TPT per day as mice, as illustrated by the direct comparison of maximum tolerated doses (MTDs) in mice and humans on daily 5-day regimen (1.5 vs. 14 mg/m^2 per day), and the human MTDs from phase I trials of continuous infusion or multiconsecutive day schedules are well below the curative dosages in the nude mouse models [3, 18, 22, 30–34]. Obviously, the full curative potential of these drugs will not be realized without the development of approaches, in addition to cytokine support, to compensate for the dose-limiting neutropenia. The greater sensitivity of humans than of mice to myelotoxicity may be due to pharmacodynamic, rather than pharmacokinetic, differences. It cannot be a consequence of greater plasma drug exposure in humans at a specific dose, because the plasma clearance of TPT is no slower in humans than in mice [16]. It also cannot be a consequence of a greater proportion of drug circulating as lactone rather than hydrolyzed form, because the equilibrium is shifted more toward the hydroxy acid of CAM by human than by murine serum albumin [1, 2, 25, 26]. The failure to achieve dosages in patients which are curative in the murine models might be due to greater sensitivity of human than mouse myeloid progenitors, a difference which should be readily detectable using in vitro clonogenic assays.

The objective of the current study was to determine whether the difference in drug tolerance between humans and mice is evident at the level of myeloid progenitors directly exposed to drug, and if so, to find an alternative animal model that more closely approximates human sensitivity. Past assay results with a myeloid progenitor called colony-forming unit granulocyte/macrophage (CFU-GM) have provided qualitative correlations between preclinical data and clinical neutropenia [6–8, 36–38] and more recently a quantitative correlation after optimization [28, 29]. The quantitative in vitro-in vivo correlation with 9-methoxypyrazoloacridine has revealed that grade 3–4 neutropenia occurs at a drug exposure level equal to the IC_{90} concentration in vitro, i.e. a 1-log reduction of viable progenitors in the clonogenic assay [29]. Therefore, in the current study, we employed the same clonogenic assay for CFU-GM to quantitate CAM toxicity to myeloid progenitors from human, murine, and canine marrow, and we based the interspecies comparisons of drug sensitivity upon IC_{90} values.

Materials and methods

Materials

20(*S*)-Camptothecin lactone (CAM; NSC-94600), 20(*S*)-camptothecin sodium salt (Na+CAM; NSC-100880), topotecan (TPT; NSC-609699, SKF 104864), 9-amino-20(*R,S*)-camptothecin [(*R,S*)9AC; NSC-629971], synthetic 9-amino-20(*S*)-camptothecin (s9AC; NSC-603071, sample #7), and semisynthetic 9AC (ss9AC; NSC-603071, sample #9) were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, and stored desiccated at 4 °C. Recombinant human (rh) granulocyte-macrophage colony-stimulating factor (GM-CSF) and rhIL-3 were obtained from Immunex (Seattle, Wa.) and R&D Systems (Minneapolis, Min), respectively, prepared as 100× stock solutions in 1 mg/ml human serum albumin (HSA) phosphate-buffered saline, aliquoted, and stored at −70 °C. Cell culture medium was obtained from BioWhittaker (Walkersville, Md.), fetal bovine serum (FBS) from ICN (ICN Gold, Costa Mesa, Calif.), Trypsin/EDTA solution and α-MEM from GIBCO (Grand Island, NY), Seaplaque agarose from FMC Bioproducts (Rockland, Me.), preservative-free heparin from a local pharmacy and all other chemicals from Sigma (St. Louis, Mo.). Hematopoietic culture mix was obtained from the StemCell Technologies (Vancouver, BC).

Isolation of hematopoietic cells from human, canine, and murine bone marrow

Human and canine bone marrow cells were obtained with informed consent from remnant femoral canal reamings from patients undergoing orthopedic surgery in Dayton, Ohio, under an IRB-approved protocol [35] and from Beagle dogs at Battelle Institute in Columbus, Ohio, under IACUC-approved protocols, respectively. The marrow aspirate was placed in 20–30 ml α-MEM with preservative-free heparin at 10 U/ml. In the laboratory, the marrow cells were isolated by filtration through a sterile nylon mesh (# 60), centrifuged at 2000 *g* for 10 min, and harvested as a cell pellet. The cell pellet was resuspended in α-MEM and layered over 4 ml Ficoll-Hypaque in 100×17-mm tubes. After 30 min centrifugation at 2000 *g*, at 23 °C, the light density mononuclear cell fraction at the interface was harvested and washed twice in α-MEM. After resuspending in IMDM, the nucleated cells were counted in a hemocytometer (cell viability determined to be ≥85% by trypan blue dye exclusion). Canine mononuclear cells were prepared similarly after an initial culture period of 18 h in IMDM, 20% FBS, 25 ng/ml rhGM-CSF, 5 ng/ml rhIL-3, and harvesting for clonogenic assays by collection of all cells, including adherent cells with 0.05% trypsin/0.5 m*M* EDTA.

Murine marrow cells were obtained from femurs of male CD2F1 mice (from the National Cancer Institute) after euthanasia by carbon dioxide asphyxiation under an IACUC-approved protocol. The marrow was flushed from the femoral canal with sterile Hanks' balanced salt solution (HBSS) and a single-cell suspension was prepared by gentle pipetting. After 5 min on ice to allow bone, stromal tissue, and debris to settle, the cells in the supernatant were collected, washed twice in HBSS, and resuspended in α-MEM medium containing 1.25 m*M* HEPES and 5% fetal calf serum. Nucleated cell counts were obtained using a hemacytometer and 2% acetic acid and 0.04% trypan blue.

Human, canine and murine CFU-GM assays

After thorough mixing, 0.4 ml of a mixture of human or canine bone marrow mononuclear cells in IMDM at 2.5×10^5/ml, 20% FBS, 25 ng/ml rhGM-CSF (plus 5 ng/ml rhIL-3 for canine assays), 10% of a 10× drug solution or vehicle control, and 0.3% agarose (Seaplaque FMC BioProducts) was pipetted into microwells of a 24-well plate containing a 0.4 ml underlayer (IMDM, 20% FBS, 0.3% agarose). The cultures were allowed to gel at 4 °C for 15 min, and were then incubated at 37 °C in a fully humidified atmosphere of 5% CO_2 in air for 7 days (canine) or 14 days (human). CFU-GM colonies were counted under an inverted microscope as multicellular aggregates of 50 μm or greater in diameter.

For murine CFU-GM assays, each dish contained 1.1 ml composed of 10 parts of α-MEM-based hematopoietic culture mix and 1 part cells plus medium or drug solution at the following final concentrations: 5×10^4 cells/dish, 0.8% methylcellulose, 30% FBS, 1% bovine serum albumin, 1×10^{-4} *M* 2-mercaptoethanol, and 1% pokeweed mitogen-stimulated spleen cell conditioned medium (containing IL-3 and GM-CSF). After 12–14 days, CFU-GM colonies containing 50 or more cells were counted.

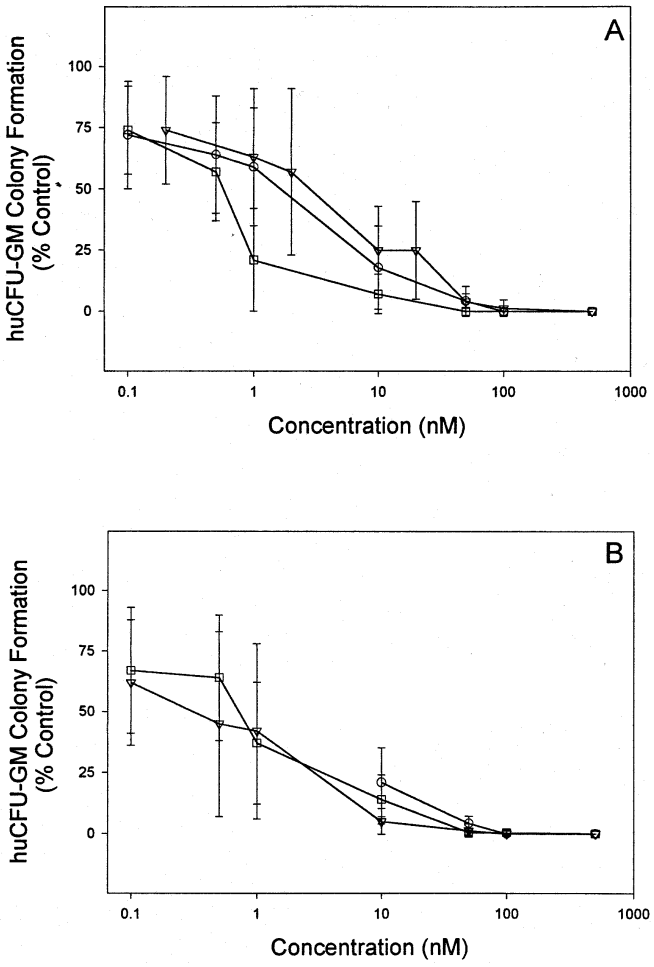

Fig. 1A Direct comparison of the toxicity of CAM lactone (○), TPT (△), and s9AC (□) to human CFU-GM. *B* Toxicity of Na^+CAM (○), ss9AC (△), and racemic 9-AC (□) to human CFU-GM. Error bars represent one $n-1$ standard deviation from the mean value of the assay results from at least six marrow specimens

Preparation of drug test solutions

On the day of each assay, stock solutions that were 10,000 times the highest concentrations to be tested were prepared by dissolving the drugs in an appropriate volume of dimethylsulfoxide (DMSO). Immediately before use, the DMSO drug stock was diluted 1:100 into culture medium, then 1:10 into medium plus 100 μg/ml gentamicin sulfate. Serial dilutions were made with IMDM containing DMSO and gentamicin sulfate, so that only the concentration of the drug changed. The resulting 10× solutions were then added to a final concentration of 10% in a cell suspension plus agarose, and the cell/drug/agarose mix was plated into tissue culture plates. For murine marrow, appropriate drug dilutions were added to tubes containing hematopoietic culture mix and cells and then plated as described above. Drug solutions were not filtered. The vehicle control was DMSO in medium containing gentamicin sulfate. Stock solutions were discarded at the end of the day.

Data analysis

As in past studies [29], IC_{50} and IC_{90} values were determined from the log-linear regression line through flanking data points of the concentration-inhibition curve generated from multiple marrow specimens. This average regression line relating drug concentration and colony inhibition was derived from CFU-GM data from 12 human, 6 canine and 3 murine marrow specimens, respectively.

Table 1 The concentrations of drug (in n*M*) inhibiting CFU-GM colony formation from human, canine and murine femoral marrow by 50% (IC_{50}) and 90% (IC_{90}). These values were obtained from the inhibition curves shown in Fig. 2 and reflect the average percent inhibition of CFU-GM from 12, 6, and 3 human, canine, and murine marrows, respectively

Compound	IC_{50} (n*M*)			IC_{90} (n*M*)		
	Human	Murine	Canine	Human	Murine	Canine
CAM lactone	1.7	18	0.5	16	42	7.6
Na^+-CAM	N/A	17	N/A	29	67	6.9
TPT	2.8	128	1.7	39	381	7.6
s9AC	0.6	20	0.3	6.2	66	7.6
ss9AC	0.3	16	0.6	7.3	43	7.4
(*R.S*)9AC	0.7	75	0.6	16	331	6.9

Results

The toxicity of the CAMs to human myeloid progenitors was quantified from the inhibition of marrow CFU-GM colony formation resulting from continuous drug exposure. The CAMs in clinical trials (CAM lactone, TPT, and s9AC) inhibited colony formation in a concentration-dependent manner (Fig. 1A). Other CAMs showed a similar type of inhibition of colony formation (Fig. 1B).

Mild inhibition of colony formation by CAM lactone was apparent at the lowest concentration tested (0.1 n*M*), while 10 n*M* inhibited colony formation by 82%. The inhibition was characterized by IC_{50} and IC_{90} values of 1.7 n*M* and 16 n*M*, respectively (Table 1). The toxicity of CAM lactone to murine and canine progenitors was quantified in similar assays and compared with the toxicity to human CFU-GM (Fig. 2A). Colony formation by CFU-GM from both species was inhibited in a concentration-dependent manner. Canine CFU-GM were slightly more sensitive than their human counterpart, with an IC_{50} of 0.5 n*M* and IC_{90} of 7.6 n*M* (Table 1). In contrast, murine CFU-GM tolerated higher drug concentrations than human CFU-GM, exhibiting an 11-fold higher IC_{50} of 18 n*M* and 2.6-fold higher IC_{90} of 42 n*M* (Table 1).

Na+-CAM, the hydroxy acid form of CAM, was also tested for toxicity to CFU-GM at the higher concentrations (Figs. 1B, 2B). The IC_{90} values for inhibition of human, canine, and murine CFU-GM were 29 n*M*, 6.9 n*M* and 67 n*M* (Table 1), only a 0.9- to 1.8-fold difference in IC_{90} values from CAM lactone.

Both TPT and all three preparations of 9AC inhibited CFU-GM from the three species in a similar concentration-dependent manner (Fig. 2C–F), so the relative toxicity of TPT, 9AC and CAM lactone to CFU-GM from the three test species was compared using IC_{50} values (Table 1). In murine CFU-GM assays, TPT was 7-fold less potent than CAM lactone in inhibiting colony formation, while Na+-CAM, s9AC and ss9AC were equally potent inhibitors. Racemic (*R,S*)9AC was 4-fold less potent than CAM lactone. In human CFU-GM assays, TPT was only a 2-fold less potent inhibitor than CAM lactone, while s9AC, ss9AC, and (*R,S*)9AC were consistently more potent than CAM lactone by 2.5- to 6-fold, and the potency of pure

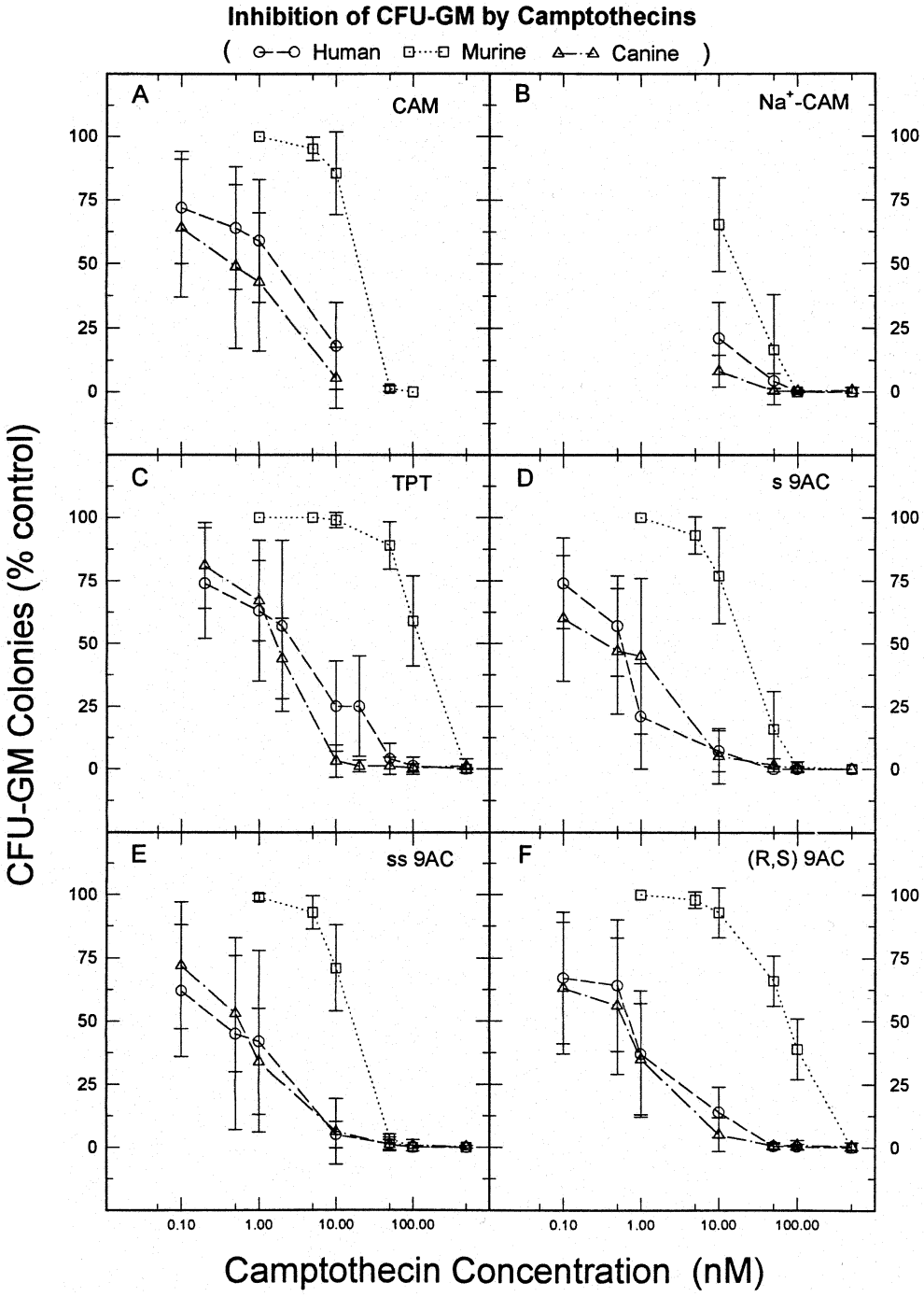

Fig. 2A–F Direct comparison of the toxicity of (*A*) CAM lactone, (*B*) Na^+CAM, (*C*) TPT, (*D*) s9AC, (*E*) ss9AC, and (*F*) (*R*,*S*) 9AC to human (○), canine (△) and murine (□) CFU-GM. Error bars represent one *n*-1 standard deviation from the mean value of the assay results from 6–12, 6, and 3 marrow specimens, respectively. Note that murine CFU-GM is much more resistant to the toxicity of all six CAMs than either human or canine CFU-GM

(S)9AC was similar to that of racemic (*R*,*S*) 9AC. In canine CFU-GM assays, TPT was 3-fold less potent than CAM lactone, while s9AC, ss9AC, and (*R*,*S*)9AC showed similar toxicity to CAM lactone. In all three species, TPT was consistently a less potent inhibitor of CFU-GM than the parent CAM. The 7-fold difference in potency between TPT and CAM lactone on murine CFU-GM was the greatest potency difference between CAM lactone and any of the analogs in the three species based on IC_{50} values. The difference in potency between (*R*,*S*)9AC and CAM lactone to murine CFU-GM was consistently observed across the entire concentration-response range (i.e. both IC_{50} and IC_{90} values), while the difference between ss9AC and CAM lactone to human CFU-GM narrowed as the IC_{90} values were approached.

Of greater importance for comparative toxicology are the relative toxicities of these drugs to CFU-GM from human, murine, and canine marrow were also compared using IC_{90} values (Table 1). The IC_{90} values from canine and human CFU-GM assays differed by 5.1-fold with TPT, 4.2-fold with Na^+-CAM, 2.1-fold with CAM lactone, 1.2-fold with s9AC, and 2.3-fold with racemic (*R*,*S*)9AC, but they did not differ with ss9AC. Differences in IC_{50} values were similar. Canine CFU-GM was always at least

as sensitive as human CFU-GM to drug toxicity, and in some cases more sensitive, regardless of whether comparisons were IC_{90}- or IC_{50}-based. There was a greater difference between the IC_{90} values from murine and human CFU-GM assays: 10-fold with TPT, 2.5-fold with CAM lactone, 2.3-fold with Na^+-CAM, 11-fold with s9AC, 21-fold with racemic (*R*,*S*)9AC, and 5.9-fold with ss9AC. Excepting TPT, these differences were even larger when IC_{50} values were used to compare the two species. Therefore, the susceptibility of human CFU-GM to drug toxicity is more closely approximated by canine than by murine CFU-GM.

Discussion

The results of the current study suggest that humans cannot tolerate the doses of CAMs which are curative in murine models because of greater susceptibility of myelopoietic tissue to drug toxicity. This finding explains why even subcurative doses of camptothecins are severely myelotoxic in patients, even though pharmacokinetics and the circulating levels of the corresponding lactones are similar between mice and humans. Based on the IC_{90} values (Table 1), murine myeloid progenitors tolerated higher drug concentrations than did their human counterparts for all the compounds examined. The differences between mice and humans were large with TPT and 9AC.

An important aspect of the pharmacology of the CAM compounds is that each exists as an equilibrium of hydroxy acid (hydrolyzed A ring) and lactone, the lactone form being responsible for cytotoxicity [9]. Mi and Burke have shown that 13–18% of total TPT exists in the lactone form at equilibrium regardless of the protein concentration or the species of origin of the plasma [1, 2, 25, 26]. Since murine and human CFU-GM assays are performed in similar plasma protein concentrations, the level of TPT lactone should be identical, and therefore the difference in IC_{90} values determined in the present study reflected intrinsic differences in the sensitivity of murine and human marrow progenitors to topoisomerase I inhibition. In addition, the ratio of human and mouse IC_{90} values should approximate the ratio of murine and human MTDs on similar schedules because there are no differences between mice and humans in the plasma equilibrium levels of TPT lactone [26] or in drug clearance [16]. The human MTD is 1.5 mg/m^2 per day on a daily 5-day schedule without cytokine support [31, 32, 34], and the mouse LD_{10} on the same schedule is 14 mg/m^2 per day [16]. The LD_{10}:MTD ratio of 9.3 is consistent with the murine to human IC_{90} ratio of 9.8 observed in this study with TPT (Table 1). The ratio of murine to human IC_{90} values of 9.8 is also close to the ratio of murine to human Cpmax values of 22 for TPT [12, 16, 19, 24].

For s9AC, the human IC_{90} of 6.2 n*M* is consistent with the reported neutropenic Cpss value of 8.5 n*M* [3], while the canine to human IC_{90} ratio of 1.2 compares favorably with the canine to human MTD ratio of 1.1 using 72-h continuous infusions [3–5]. However, 9AC shows large species differences in percent lactone at equilibrium in the presence of plasma protein: 25% for dogs compared with $<0.5\%$ for humans [26]. The similar sensitivity between canine and human CFU-GM to s9AC in combination with the species difference in lactone plasma stability should have resulted in a significantly lower MTD in dogs than in humans. The MTD ratio of 1.1 using 72-h continuous infusions indicates a discrepancy between the in vitro and the in vivo data that might be due to differences between dogs and humans in (1) lactone clearance rates, (2) sensitivity to the total level of s9AC or to more potent marrow toxins than lactone or hydroxy acid, or (3) the concentration of free (unbound) lactone. Like 9AC, CAM lactone shows marked species differences in lactone stability and percent lactone at equilibrium in plasma [26], so the IC_{90} ratios for this drug do not necessarily reflect its MTD ratios either.

The greater sensitivity of human than of murine progenitors must be overcome to achieve in patients the doses that cure human tumor xenografts in nude mice. One way to resolve the major clinical obstacle for the use of CAM, TPT, and 9AC is to develop therapies that increase the drug tolerance of human progenitors to a level similar to that of their murine counterparts. Human progenitor drug tolerance might need to be increased even more, if differences in growth fraction and cell cycle time between tumors in patients and human tumor xenografts in mice increase the required clinical dosages. Using in vitro assays to develop these therapies to compensate for the neutropenia of 9AC or CAM chemotherapy may be much more complicated than for TPT, because of the interspecies differences in plasma stability of CAM and 9AC lactones (but not TPT). Therefore, in vitro evaluations of CAM or 9AC toxicity will be most meaningful when they employ human plasma (or serum) for human CFU-GM assays and murine plasma for murine CFU-GM assays to accommodate the species differences in percent lactone at equilibrium. The in vitro evaluation of TPT toxicity to find ways to achieve equal drug tolerance between murine and human progenitors is simpler, because identical in vitro conditions can be employed since there are not marked species differences in percent TPT lactone at equilibrium [26]. These considerations illustrate the complexity of correlating in vitro and in vivo data with this family of antineoplastic agents.

Acknowledgements The authors would like to thank Drs. Charles K. Grieshaber and Karl Flora for their scientific critique of this work, and Marsha Harting, Maria Kitchens, Jenny Kwak, Karen Long, Melissa Mogle, and Stephanie Redwine for expert technical assistance. This work was supported in part by the National Cancer Institute under contract CM-07361, a subcontract under contract to NCI contract N01-CM-87259, and a Sigma Beta Sorority International Cancer Research Fellowship to CLE-M.

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