Cancer Chemother Pharmacol (1996) 39: 143–149

ORIGINAL ARTICLE

Donna A. Volpe · Joseph E. Tomaszewski
Ralph E. Parchment · Achal Garg · Karl P. Flora
Martin J. Murphy · Charles K. Grieshaber

Myelotoxic effects of the bifunctional alkylating agent bizelesin on human, canine and murine myeloid progenitor cells

Received: 27 July 1995/Accepted: 1 February 1996

Abstract Bizelesin is a potent synthetic derivative of the anticancer agent CC-1065 that preferentially alkylates and binds the minor grove of DNA. Preclinical animal studies have found bizelesin to be more toxic to beagle dogs than to rodents and that myelosuppression was the dose-limiting toxicity. This toxicity was dose- and time-dependent in all species. Due to the significant difference in the in vivo myelotoxicity between species, it was important to determine which one most closely resembles humans on a pharmacodynamic basis. Therefore, hematopoietic clonal assays were utilized to evaluate the effects of bizelesin on granulocyte-macrophage (CFU-gm) colony formation. Marrow cells were exposed in vitro to bizelesin (0.001–1000 n*M*) for 1 or 8 h and then assayed for colony formation. There was a 3-log difference in drug concentration at which 100% colony inhibition occurred (1 or 8 h) for murine CFU-gm versus human or canine CFU-gm. The IC_{70} value after an 8-h bizelesin exposure for human CFU-gm (0.006 ± 0.002 n*M*) was 2220-times lower than for murine CFU-gm (13.32 ± 8.31 n*M*). At any given concentration, an 8 h drug exposure resulted in greater colony inhibition than a 1 h exposure for all species ($P < 0.05$). Increasing exposure time from 1 to 8 h increased toxicity to human and canine CFU-gm much more than to murine CFU-gm. The clinically formulated drug solution was a more potent inhibitor of human colony formation than drug dissolved in DMSO. The IC_{70} value after a 1-h exposure was 1.7 times lower for human CFU-gm with formulated bizelesin (0.106 ± 0.105 n*M*) than bulk drug in DMSO (0.184 ± 0.044 n*M*). The results of these in vitro clonal assays were qualitatively consistent with those seen in whole animal studies, suggesting that bizelesin will be a potent myelosuppressive agent in the clinic. Since the dose-limiting toxicity in preclinical models is myelosuppression and the in vitro sensitivity of human and canine CFU-gm is similar, the canine maximum tolerated dose (MTD) is better than the murine MTD to determine a safe starting dose for phase I clinical trials.

Key words Comparative · CFU-gm · In vitro · Bizelesin · Clonal assays

D.A. Volpe (✉)
Food and Drug Administration, MOD-1 Building, Room 2009, 8301 Muirkirk Road, Laurel, MD 20708-2476, USA
Telephone (301) 594–5878; Fax (301) 594–3037

R.E. Parchment · K.P. Flora · C.K. Grieshaber
Division of Research and Testing, Food and Drug Administration, Laurel, MD, USA

J.E. Tomaszewski
Toxicology and Pharmacology Branch, National Cancer Institute, Bethesda, MD, USA

A. Garg · M.J. Murphy
Hipple Cancer Research Center, Dayton, OH, USA

Introduction

Bizelesin (NSC-615291, U-77,779) is a highly potent antitumor agent that preferentially alkylates and binds to DNA. Bizelesin serves effectively as a prodrug which can form two DNA-reactive cyclopropylpyrroloindole subunits connected by a rigid indole-ureido-indole segment [16]. Bizelesin is a synthetic analog of the antibiotic CC-1065 (U-56,314) which was isolated from the fermentation broth of *Streptomyces zelenis* cultures [8]. CC-1065 binds in the minor groove of DNA and induces sequence-specific alkylation [2]. Although CC-1065 is highly potent in vitro and modestly active in several murine tumor models [13], preliminary toxicity studies found that CC-1065 causes a delayed, lethal hepatotoxicity in non-tumor-bearing mice [15]. Analogs of CC-1065, such as bizelesin and adozelesin, that display in vivo antitumor activity without these delayed deaths

were then synthesized [16, 24]. As a symmetrical dimer, bizelesin forms interstrand crosslinks (ISC), of which one links two adenine N^3 positions within an A/T-rich sequence and another has a preference for contiguous runs of adenines [12]. Bizelesin has an increased sequence specificity over monoalkylating CC-1065 analogs since a monomeric species can react at more than one DNA site while the dimer reacts only where there are two suitably positioned alkylation sites [4].

Bizelesin is effective in vitro against a number of human carcinoma cell lines in a tumor colony assay, with concentrations required for a 1-log cell kill ranging from 1.0 to 10 p*M* [11]. Human colon (HT-29, BE), lung (A549, A427) and ileocecal (HCT8) cell lines were exposed to bizelesin for 2 or 6 hours prior to addition in clonal assays. For HT-29 and BE lines, toxicity increases with longer drug exposure. Concentrations required to reduce colony formation by 50% (EC_{50}) for a 6-h exposure range from 0.4 p*M* (A549) to 8 p*M* (HCT8) [11]. In an ATP-chemosensitivity assay on a number of gynecologic cancer cell lines, bizelesin has a mean EC_{50} value of 0.63 ng/mL (0.77 n*M*) with a range of 0.21 to 0.89 ng/mL (0.26 to 1.09 n*M*) [9]. Against the same cell lines, bizelesin is 7000 and 370 times more cytotoxic than cisplatin and doxorubicin, respectively [9].

Bizelesin is very effective in vivo against human Caki-1 renal carcinoma cell xenografts in mice [3]. A single intravenous (i.v.) injection 2 days after tumor implantation results in 97–100% inhibition of tumor growth (TGI). Multiple i.v. injections resulted in greater than 90% TGI for CX-1 colon and LX-1 lung carcinoma cell xenografts [3]. Following i.v. administration of 15 μg/kg bizelesin in DMSO to CD_2F_1 mice, plasma elimination can be described by a two-compartment open model with half-lives of 3.5 ± 1.4 min (α) and 7.3 ± 2.4 h (β). Peak bizelesin plasma levels in the mice are approximately 10 n*M* and 1–2 n*M* at steady-state [23].

Preclinical toxicology studies have found bizelesin to be more toxic to beagle dogs than to rodents regardless of administration schedule. Toxicity has been shown to be time-dependent in the animal studies [1]. For all species, a larger total dose can be administered on a divided dose ($D \times 5$) than on a single dose ($D \times 1$) schedule (Table 1). However, for mice and rats, the total dose for a $D \times 5$ schedule is only 50% more than that for a single dose schedule, not five-times more. Morbidity results when beagle dogs are given single i.v. doses of 0.5 to 2.0 μg/kg (10–40 $\mu g/m^2$) bizelesin with leukopenia observed in all dogs [18]. Beagle dogs are able to tolerate 0.1 μg/kg (2 $\mu g/m^2$) with reversible myelosuppression (leukopenia, anemia, thrombocytopenia) [18]. A single dose of 2.5–7.5 μg/kg (15–45 $\mu g/m^2$) in rats results in moderate leukopenia [5]. Myelosuppression and gastrointestinal toxicity are the dose-limiting adverse effects of bizelesin in mice, rats and dogs, with dogs being the most sensitive species [1].

Table 1 Bizelesin toxicity in vivo (from reference 1) (*MTD* maximum tolerated doze)

Species	Schedule[a]	MTD ($\mu g/m^2$/day)	Total dose ($\mu g/m^2$)
Mouse	$D \times 1$	30	30
	$D \times 5$	9	45
Rat	$D \times 1$	45	45
	$D \times 5$	12	60
Beagle dog	$D \times 1$	2	2
	$D \times 5$	0.8	4

[a] $D \times 1$, single i.v. dose; $D \times 5$, daily i.v. dose for 5 days

A preliminary study on the in vitro myelotoxicity of bizelesin found over 130,000-fold difference in 50% inhibition concentration (IC_{50}) for human (< 0.01 n*M*) and murine (1300 n*M*) myeloid precursors [14]. This significant difference between species in vitro, coupled with the dose differential in the preclinical studies, prompted a more thorough evaluation of the comparative in vitro myelotoxicity of bizelesin towards human, canine and murine myeloid progenitor cells (colony-forming unit granulocyte-macrophage, CFU-gm) using biologically relevant drug concentrations, exposure times, and culture conditions to better judge how an appropriate starting dose might be derived for phase I studies.

Methods

Reagents and materials

Carrier-free recombinant (r), human (h) and murine (m) granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) were purchased from R&D Systems (Minneapolis, Minn.), Amgen Biologicals (Thousand Oaks, Calif.) or Immunex Corp. (Seattle, Wash.). Human recombinant cytokines were used for the canine marrow cells. Iscove's modified Dulbecco's medium (IMDM), alpha-minimum essential medium (alpha-MEM), trypan blue and gentamicin were obtained from Life Technologies (Grand Island, N.Y.) or BioWhittaker, (Walkersville, Md.). IMDM used in hematopoietic clonal assays contained 10 μg/ml gentamicin. Dimethyl sulfoxide (DMSO) was from Sigma Chemical Company (St. Louis, Mo.). Ficoll-Paque was procured from Pharmacia, (Piscataway, N.J.), heat-inactivated fetal bovine serum (FBS) from BioWhittaker or ICN Biomedicals, (Irvine, Calif.), and SeaPlaque agarose from FMC BioProducts (Rockland, Me.). Tissue culture 24-well plates were obtained from Falcon Labware (Becton Dickinson and Co., Lincoln Park, N.J.).

Cell preparation

Human bone marrow cells were obtained from discarded femoral canal reamings or epiphyseal and diaphyseal fragments from orthopedic surgery patients after obtaining informed consent [21]. Canine marrow cells were aspirated from the femoral canal of beagle dogs following an IACUC-approved protocol at Battelle Memorial Institute (Columbus, Ohio). Canine and human mononuclear cells

(MNC) were collected after centrifugation on a Ficoll-Paque gradient, washed twice and resuspended in IMDM. The cells were enumerated and viability determined in trypan blue with a hemacytometer. CD_2F_1 (BALB/c × DB_2A) female or male mice (Charles River Laboratories, Wilmington, Mass.) were sacrificed by cervical dislocation according to an IACUC-approved protocol. Femoral and tibial marrow cells were flushed from the bone with IMDM, and the cells counted as above. Marrow cells were stored at 4 °C in IMDM with 20% FBS, 1 ng/ml rGM-CSF, 1 ng/ml rIL-3 and 10 μg/ml gentamicin until exposure to bizelesin.

Drug preparation

Frozen (–20°C) vials of bizelesin (Division Cancer Treatment, NCI, lot ♯93–905) were formulated as 1 μg drug in 200 μl vehicle solution consisting of 20 μL of PET (PEG 400/Ethanol/Tween 80, 60 : 30 : 10) and 180 μl 0.9% aqueous saline with 1 mg/mL citric acid [10]. Bizelesin was diluted 1 : 61.2 in IMDM to yield a stock solution of 100 n*M*. This stock was serially diluted 1 : 10 into IMDM containing 1.6% (v/v) diluent (vehicle). The time from initial dilution of bizelesin to completion of drug addition to the cell suspensions was less than 15 min. Bulk bizelesin was dissolved in DMSO and then diluted in IMDM containing the appropriate concentration of DMSO.

Drug exposure

Human, canine and murine marrow cells (2.5×10^5/ml) were exposed to bizelesin in IMDM with 20% FBS, 10 ng/ml rGM-CSF and 5 ng/ml rIL-3 in a total volume of 10 ml. The cell suspensions were incubated with unfiltered bizelesin dilutions at 37 °C for 1 h (0.001 –100 n*M*) or 8 hr (0.0001–10 n*M*). Murine cells were exposed to additional concentrations up to 1000 n*M*. Negative controls contained medium alone or medium with vehicle (0.16% v/v) to duplicate the amount in the drug-treated groups. Following incubation, the cells were centrifuged, the supernatant removed, and the cells washed with fresh IMDM followed by resuspension in IMDM plus 20% FBS for plating in the soft agarose assays.

Hematopoietic clonal assays

CFU-gm were cultured in a double-layer agarose assay in 24-well plates and incubated at 37 °C in a fully humidified atmosphere of 5% CO_2 in air [17]. The bottom layer (0.5 ml) consisted of 0.5% agarose in IMDM. The top layer (0.4 ml) for human and canine CFU-gm contained IMDM with 20% FBS, 10 ng/ml rhGM-CSF, 5 ng/ml rhIL-3, 2.5×10^5/ml MNC, and 0.3% agarose. For murine CFU-gm, the top layer (0.4 ml) contained IMDM with 20% FBS, 10 ng/ml rmGM-CSF, 5 ng/ml rmIL-3, 2.5×10^5/ml marrow cells, and 0.3% agarose. Each treated or control group consisted of three or four wells. The cultures were incubated for 7 (murine) or 10 (human, canine) days. CFU-gm colonies contained at least 40 cells when examined under an inverted microscope.

Data analysis

Colony counts were expressed as plating efficiency (PE), i.e. the number of colonies per 10^5 nucleated marrow cells. Neither the PET nor DMSO vehicle inhibited CFU-gm colony formation in the three species at the concentrations used in the drug-treated groups. Percent colony inhibition was determined by comparing the PE of drug-treated groups (T) to that of negative (untreated) controls (C):

$$\%\text{Inhibition} = \frac{C - T}{C} \times 100$$

The concentration-inhibition curve was analysed with a linear regression equation utilizing SigmaPlot software (Jandel Scientific, Corte Madera, Calif.). This equation correlated effect (colony inhibition) to drug concentration:

$$Y = m \times \log X + b$$

where Y is percent inhibition, X is drug concentration (n*M*), m is slope and b is the y-axis intercept. After fitting the data to this equation, IC_{70} and IC_{90} values were calculated for each individual experiment (i.e. the concentrations at which 70% and 90% colony inhibition occurred, respectively). Although the IC_{50} value is the normal standard for tumor cell lines, the IC_{70} and IC_{90} values may be more relevant for myelotoxic comparisons of anticancer agents [17]. Levels of significance between two sample means were computed in a *t*-test with QuattroPro software (Novell, Orem, Utah). Differences were considered statistically significant if P was less than 0.05. All results are expressed as means ± SE.

Results

The effect of bizelesin on human, canine and murine CFU-gm colony formation was both concentration- and time-dependent. Incubation of marrow cells for 1 and 8 h reflects the half-life of bizelesin in cell culture medium (1.5 h) and mice (7.3 h) [23]. A 1-h exposure (Fig. 1) inhibited CFU-gm colony formation less than an 8 h exposure (Fig. 2) at any given concentration. For example, to achieve 90% inhibition of human CFU-gm, 0.1 n*M* bizelesin was required after 1 h exposure, but only 0.01 n*M* for an 8-h exposure. The greater toxicity of the longer drug exposure was made evident when comparing inhibitory concentrations (Table 2). The IC_{70} values for the 8 h exposure were 20, 36 and 5 times lower than for the 1-h

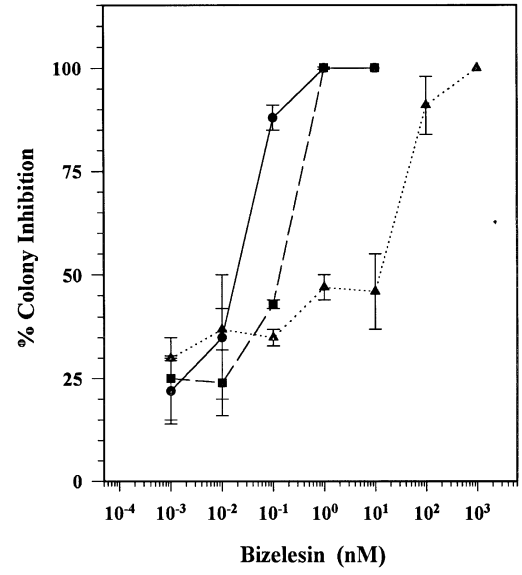

Fig. 1 Effect of bizelesin on CFU-gm following a 1-h exposure. Values are means ± SE from four human (●) four canine (■) and seven murine (▲) individual experiments. Control plating efficiencies for human, canine, and murine cells were 58 ± 16, 84 ± 37, and 88 ± 37, respectively (mean ± SE)

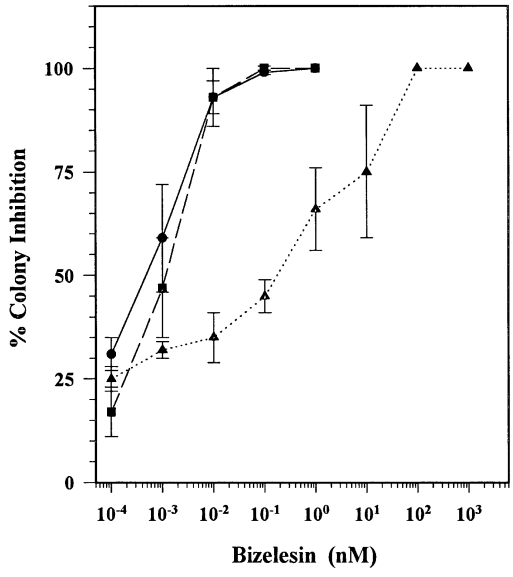

Fig. 2 Effect of bizelesin on CFU-gm following an 8-h exposure. Values are means ± SE from five human, (●) four canine (■) and six murine (▲) individual experiments. Control plating efficiencies for human, canine, and murine cells were 49 ± 12, 66 ± 14, and 57 ± 17, respectively (mean ± SE)

Table 2 Effects of 1-h and 8-h bizelesin exposure on to CFU-gm. Values are means ± SE (range) from *n* individual experiments calculated from the linear regression equation

Species	Exposure	IC_{70} (n*M*)	IC_{90}(n*M*)	*n*
Human	1-h	0.119 ± 0.039 (0.056 – 0.218)	0.906 ± 0.126 (0.690 – 1.198)	4
	8-h	0.006 ± 0.002 (0.001 – 0.218)	0.061 ± 0.016 (0.034 – 0.099)	5
Canine	1-h	0.327 ± 0.023 (0.268 – 0.377)	2.659 ± 0.455 (1.914 – 3.942)	4
	8-h	0.009 ± 0.003 (0.005 – 0.018)	0.075 ± 0.012 (0.049 – 0.098)	4
Murine	1-h	69.28 ± 24.46 (6.20 – 175.60)	659.77 ± 109.34 (198.38 – 942.87)	7
	8-h	13.32 ± 8.314 (0.148 – 53.60)	172.14 ± 75.01 (3.632 – 502.73)	6

exposure for human, canine and murine CFU-gm, respectively ($P < 0.03$). The difference in colony suppression between the exposure times was greater for the canine and human cells than for the murine cells (Table 2).

Human CFU-gm were more sensitive to bizelesin than their canine counterparts, and dramatically more sensitive than murine CFU-gm, regardless of drug exposure time (Figs. 1, 2). Importantly, canine CFU-gm were almost as sensitive as human CFU-gm, especially at concentrations > 1.0 n*M* for the 1 h and > 0.01 n*M* for 8 h exposures. There was a 3-log difference in bizelesin concentrations at which colony formation was completely suppressed (100% inhibition) with either a 1- or an 8-h exposure between murine CFU-gm and

Table 3 Effects of diluent on bizelesin toxicity to human CFU-gm. Values are means ± SE (range) from *n* individual experiments with a 1-h drug exposure, calculated from the linear regression equation

Diluent	IC_{70} (n*M*)	IC_{90} (n*M*)	*n*
PET	0.106 ± 0.105 (0.0002 – 0.316)	3.105 ± 2.897 (0.121 – 8.898)	3
DMSO	0.184 ± 0.044 (0.117 – 0.268)	3.715 ± 1.741 (1.944 – 7.197)	3

human or canine CFU-gm. For example, human and canine CFU-gm were completely inhibited at 1.0 n*M* after 1 h exposure, but it took 1000 n*M* to achieve the same level of suppression for murine CFU-gm. Comparing IC_{70} values from the linear regression equation for a 1 h drug exposure, the value for human CFU-gm was 0.119 ± 0.039 n*M* which was 2.7 times lower ($P = 0.003$) than that for canine CFU-gm (0.327 ± 0.023 n*M*) and 582-times lower ($P = 0.015$) than murine CFU-gm (69.28 ± 24.46 n*M*) (Table 2). After an 8-h exposure to bizelesin, the human IC_{70} value was 2220-times lower than that for murine CFU-gm ($P > 0.05$). The IC_{90} values demonstrated the greater resistance of the murine myeloid precursors to bizelesin with values 728-times (1 h, $P = 0.0002$) or 2822-times (8 h, $P = 0.035$) higher than human CFU-gm.

The bizelesin used in these experiments was formulated for clinical use in a vehicle consisting of PEG 400, ethanol and Tween 80 (PET) in aqueous 0.9% saline solution buffered with citric acid [10]. A preliminary study of bizelesin's in vitro myelotoxicity showed higher IC_{70} values [14] than those reported here, but the previous studies were performed before formulated drug was available. To determine if the vehicle potentiated the toxicity of a bizelesin to human CFU-gm, we compared the toxicity of a 1 h drug exposure (0.01 to 1.0 n*M*) in the PET vehicle versus bulk drug in a DMSO vehicle. Greater toxicity was observed with PET-formulated than DMSO-dissolved bizelesin at 0.01 and 0.1 n*M*. This difference was not statistically significant when comparisons were made between bulk and formulated drug owing to the variability of sensitivity between individual marrow samples (Table 3).

Discussion

We utilized hematopoietic clonal assays to compare the human, canine and murine myelotoxicity of the synthetic alkylating agent bizelesin in an effort to predict the clinical safety of this new drug. Human CFU-gm proved to be the most sensitive to the toxicity of bizelesin, followed closely by canine and then murine CFU-gm. There was a 1000-fold difference in the concentration required for complete colony inhibition

between murine CFU-gm and human or canine CFU-gm, suggesting a similar relationship in vivo based on murine and canine preclinical toxicity data. For all species, a 1-h bizelesin exposure was less toxic than an 8-h exposure at a given concentration. The contribution of time to overall toxicity was not unexpected since bizelesin has been previously shown to be more inhibitory to colon carcinoma cell lines following a 6 h than a 2 h exposure [11]. Since bizelesin is a DNA-reactive drug [4, 12], it can be assumed that a longer exposure results in greater alkylation and crosslinking of DNA and consequently greater inhibition of proliferation and/or cell death. The variation in colony suppression between 1- and 8-h exposure periods was more prominent in the canine and human precursors than in murine precursors, suggesting that schedules of drug administration and differences in pharmacology will be important for extrapolating from preclinical models to clinical trials. For example, if bizelesin's half-life is longer in humans than mice or dogs, myelotoxicity would be greater in humans at a comparative dose (mg/m^2).

Differential in vivo toxicity occurred when bizelesin was administered with the same schedule to different species. The preclinical data from two rodent species shows their relative insensitivity compared to beagle dogs for this drug and may not make them an accurate model for predicting toxicity in humans [1]. The maximum tolerated dose (MTD, mg/m^2) for a single bizelesin injection is 15-times lower for beagle dogs than for mice and on a daily times five schedule the difference is 11-fold with myelosuppression as the dose-limiting toxicity for both species and schedules (Table 1) [1]. The ranking of species based in in vitro myelotoxicity assays correlates with in vivo ranking, i.e. mice are more resistant to bizelesin than dogs. However, the quantitative difference in MTD between dogs and mice (11-fold) is less than the difference in IC_{70} values in vitro ($>$ 100-fold). Since the in vitro toxicity of bizelesin (1 h) to human CFU-gm was three-times greater than canine CFU-gm ($P < 0.02$), but over 500-times greater than murine CFU-gm ($P < 0.02$), the canine MTD would be more appropriate than murine MTD to determine a safe starting clinical dose in phase I trials. The vast difference in sensitivity of myeloid progenitors between mice and humans makes calculations of a clinical starting dose based on murine MTD potentially dangerous.

Another example of an antitumor agent that displays a large difference between species is the DNA-binding agent tallimustine (FCE 24517). Preclinical toxicity studies demonstrated a much greater toxicity to dogs ($LD_{50} = 0.12$–$0.24\ mg/m^2$) than to rodents ($LD_{50} = 12$–$24\ mg/m^2$) [22]. After a 4-h exposure to tallimustine, canine CFU-gm ($IC_{70}0.001\ nM$) is much more sensitive to the drug's cytotoxic effect than human (0.173 n*M*) or murine (0.221 n*M*) CFU-gm [22]. As with the present drug, bizelesin, the in vitro canine and murine hematopoietic assays correlated with preclinical animal toxicity studies. In contrast to bizelesin, tallimustine is equally toxic to human and murine myeloid progenitors and more toxic to canine cells. In a phase I study, tallimustine given as a bolus every 3 weeks resulted in grade 4 neutropenia in more than 75% of patients at a dose of 750 $\mu g/m^2$ or more [19]. This dose is approximately 16- to 32-times lower than the LD_{50} for rodents and about 5-times higher than the canine LD_{50}.

Although bizelesin exhibits formidable in vitro activity against a number of cancer cell lines, a comparison of published data with the present CFU-gm data indicates little, if any, selectivity for malignant cells. Hightower et al. exposed gynecological cell lines to bizelesin (in DMSO) for 90 min and calculated a mean EC_{50} value of 0.77 n*M* [9] while the IC_{50} value for human CFU-gm to DMSO-dissolved bizelesin after a 1-h exposure was 0.010 ± 0.002 n*M*. Lee and Gibson performed colony-forming assays with colon carcinoma cell lines following 2- or 6-h exposures to DMSO-dissolved bizelesin with EC_{50} values ranging from 5 to 40 p*M* [11]. Comparing these results with human CFU-gm data after an 8-h exposure ($IC_{50} = 0.7 \pm 0.4$ p*M*), another low therapeutic index is derived. However, IC_{70} or IC_{90} values may be more appropriate in predicting drug levels which will result in clinical neutropenia [17]. Even if we calculated the therapeutic index as IC_{70}/EC_{50} with the above values for tumor cells, the in vitro ratio is still considerably less than one.

Bizelesin is a highly potent, but insoluble and unstable molecule. Early myelotoxicity studies were conducted with bizelesin dissolved in DMSO [14]. In preparation for clinical use, bizelesin has since been formulated as a stable solution in 10% PET with 90% saline containing 1 mg/ml citric acid [10]. Formulated bizelesin was approximately twice as toxic to human CFU-gm than bulk drug in DMSO. As bizelesin is insoluble and unstable in aqueous media, vehicle-attenuated differences in either solubility or stability after dilution could explain these differences. The IC_{50} values from a 1-h exposure in PET-solubilized drug for human (0.018 ± 0.010 n*M*) and murine (8.188 ± 4.627 n*M*) cells found here were much lower than those reported by May et al. (< 0.10 n*M* and 1300 n*M*, respectively) using bizelesin in DMSO [14]. This discrepancy could be due to either an enhancement of cellular uptake of bizelesin by the Tween-containing PET vehicle, or the difficulties associated with solubilization of bulk drug in DMSO and filter-sterilization. It could have also resulted from extended handling times before adding drug to marrow cell suspensions. Bizelesin degrades in culture medium (1.5 h half-life) to mono- (MONO-CAD) and di-cyclopropyl (DI-CAD) derivatives that are analogous to CC-1065 and adozelesin [23]. DI-CAD (U-77, 809) is relatively stable in buffer at pH 7.4, with less than 10% decomposition after 24 h. Bizelesin and DI-CAD have been shown to be

equitoxic in an 8-h bioassay [23]. Therefore, we can assume bizelesin, MONO-CAD and DI-CAD are all present in the incubation mixture in differing concentrations during the 1- and 8-h drug exposures and for the different vehicles as well.

Others have shown that bizelesin is generally more potent in vitro than other synthetic CC-1065 analogs. In a tumor colony assay with colon carcinoma cell lines, bizelesin has IC_{50} values 4- to 6-times lower than adozelesin [11]. After a 48 h exposure of murine L1210 leukemia cells to bizelesin, adozelesin or CC-1065, the resultant IC_{50} values are 2.3 p*M*, 3.4 p*M* and 88.1 p*M*, respectively [23]. However, adozelesin (IC_{50} = 0.34 n*M*) is more potent than bizelesin (0.80 n*M*) and carzelesin (4.15 n*M*) against a number of gynecologic carcinoma cell lines [9]. Both adozelesin (U-73,975) and carzelesin (U-80,244) are currently undergoing clinical trials. Adozelesin in a PET vehicle (30–100 $\mu g/m^2$), given as a 24-h infusion every 3 to 6 weeks results in prolonged leukopenia [6]. Leukopenia and thrombocytopenia are also the dose-limiting toxicities for bolus adozelesin (30–188 $\mu g/m^2$ in PET) every 3 weeks [20]. Reversible grade 3 leukopenia and grade 3/4 thrombocytopenia follow bolus injections of 96–130 $\mu g/m^2$ carzelesin [7].

The observations of in vitro inhibition of human CFU-gm at biologically active concentrations and the in vivo cytopenias in preclinical studies [1, 5, 18] indicate that bone marrow suppression will likely be a dose-limiting toxicity of bizelesin in patients. Comparing its effect on myeloid colony formation, we found that human and canine CFU-gm were significantly more sensitive than murine CFU-gm. However, the sensitivity of the bone marrow assay does not quantitatively translate into the in vivo setting since the murine/canine ratio of IC_{70} is greater than 200, while it is only 15 for a single i.v. MTD in whole-animal studies. Obviously there are other factors, such as metabolism, protein binding and cellular uptake, involved in the animal that are not necessarily taking place in vitro and it has not yet been possible to determine in vivo drug levels due to lack of sensitive analytical methods. Therefore, while the in vitro bone marrow data indicate that beagle dogs more closely resemble humans, these data must be used cautiously, as well as judiciously, in setting the clinical starting dose. The data suggest two reasonable approaches for deriving a safe starting dose that will not prolong phase I testing unnecessarily: (a) from the canine MTD on an equivalent dosing schedule; or (b) from the relative in vitro potencies of bizelesin, adozelesin and carzelesin to human CFU-gm if the relative in vitro potencies against murine and canine CFU-gm match the relative MTDs in these preclinical models.

Acknowledgements The authors wish to thank Dr. Michael Placke, Dr. Kenneth Pohl and Ms. Michelle Klein for assistance in collection of canine, human and murine bone marrow cells, respectively. Technical assistance by Ms. Melissa Mogle and Ms. Jenny Kwak was greatly appreciated. This study was Supported in part by NCI contract N01-CM-37834.

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