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Preclinical pharmacology of bizelesin, a potent bifunctional analog of the DNA-binding antibiotic CC-1065

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Abstract. Bizelesin (NSC-615291), a potent, bifunctional analog of the cyclopropylpyrroloindole antitumor antibiotics CC-1065 and adozelesin, has been selected by the National Cancer Institute for evaluation as a potential chemotherapeutic agent. All three compounds bind to and alkylate DNA at the N-3 position of adenine in a sequence-selective manner. Bizelesin is unique among the analogs with bifunctional alkylating capability due to two chloromethyl moieties that are converted to the cyclopropyl alkylating species that interact with DNA. A reverse-phase high-performance liquid chromatography (HPLC) assay and an L1210 cell bioassay were developed for bizelesin and subsequently applied to stability and murine pharmacokinetics studies. Following 48 h of incubation with L1210 cells the 50% growth-inhibitory concentrations (IC_{50}) of bizelesin, adozelesin, and CC-1065 were 2.3, 3.4, and 88.1 p*M*, respectively. Bizelesin was stable in organic solvents but was less stable in aqueous solutions, with the half-life values obtained in buffers at pH 4, 7, and 10 being 9.6, 2.1, and <1 h, respectively. By HPLC analysis, bizelesin degradation was associated with the appearance of two peaks, the mono- and dicyclopropyl derivatives formed by base-catalyzed intramolecular alkylation of the chloromethyl groups. Bizelesin and the dicyclopropyl derivative were equipotent in the L1210 cell bioassay. Following i.v. administration of bizelesin (15 μg/kg) to male $CD2F_1$ mice, the plasma elimination of cytotoxic activity determined with the bioassay was described by a two compartment open model; the α-phase ($t_{1/2\alpha}$) and β-phase ($t_{1/2\beta}$) half-lives, steady-state volume of distribution (V_{SS}), and total body clearance (Cl_{TB}) were 3.5 min, 7.3 h, 7,641 ml/kg, and 16.3 ml min^{-1} kg^{-1}, respectively. The systemic drug exposure following i.p. administration was at least 10 times lower than that resulting from i.v. infusion. Following i.v. or i.p. administration the recovery of material in urine was $<0.1\%$ of the delivered dose.

Key words: Bizelesin – Murine – Pharmacology

Introduction

Bizelesin (NSC-615291, Fig. 1) is a potent experimental antitumor agent that binds to and subsequently alkylates DNA in a bifunctional manner [4]. Development of this novel agent followed earlier studies with CC-1065 (Fig. 1), a related molecule isolated as a fermentation product of *Streptomyces zelensis* [2, 7]. Although extremely potent [5], CC-1065 caused irreversible toxicity and delayed death in mice in preclinical toxicology studies [8], which precluded the clinical development of this agent. Among the analogs subsequently prepared and evaluated were adozelesin (Fig. 1), which was also active in murine model tumor systems [6], and bizelesin. Picomolar concentrations of bizelesin effectively inhibited the growth of several human tumor cell lines [4]. In murine screening studies, bizelesin was active against a variety of tumors, including the drug-resistant pancreatic 02 carcinoma and colon 38 carcinoma (NCI Decision Network Minutes, January 9, 1989). In addition, this agent did not produce the delayed irreversible toxicities observed in mice following the administration of CC-1065 [3].

CC-1065, adozelesin, and bizelesin bind to the minor groove of AT-rich regions of double-stranded DNA in a nonintercalative fashion. CC-1065 and adozelesin subsequently interact covalently with DNA at the N-3 position of adenine [4] via the monocyclopropyl moiety present in each of these molecules. Bizelesin is unique among the three analogs in its bifunctional alkylating capability due to the two chloromethyl moieties in each molecule that are converted (via intramolecular rearrangement) to the cy-

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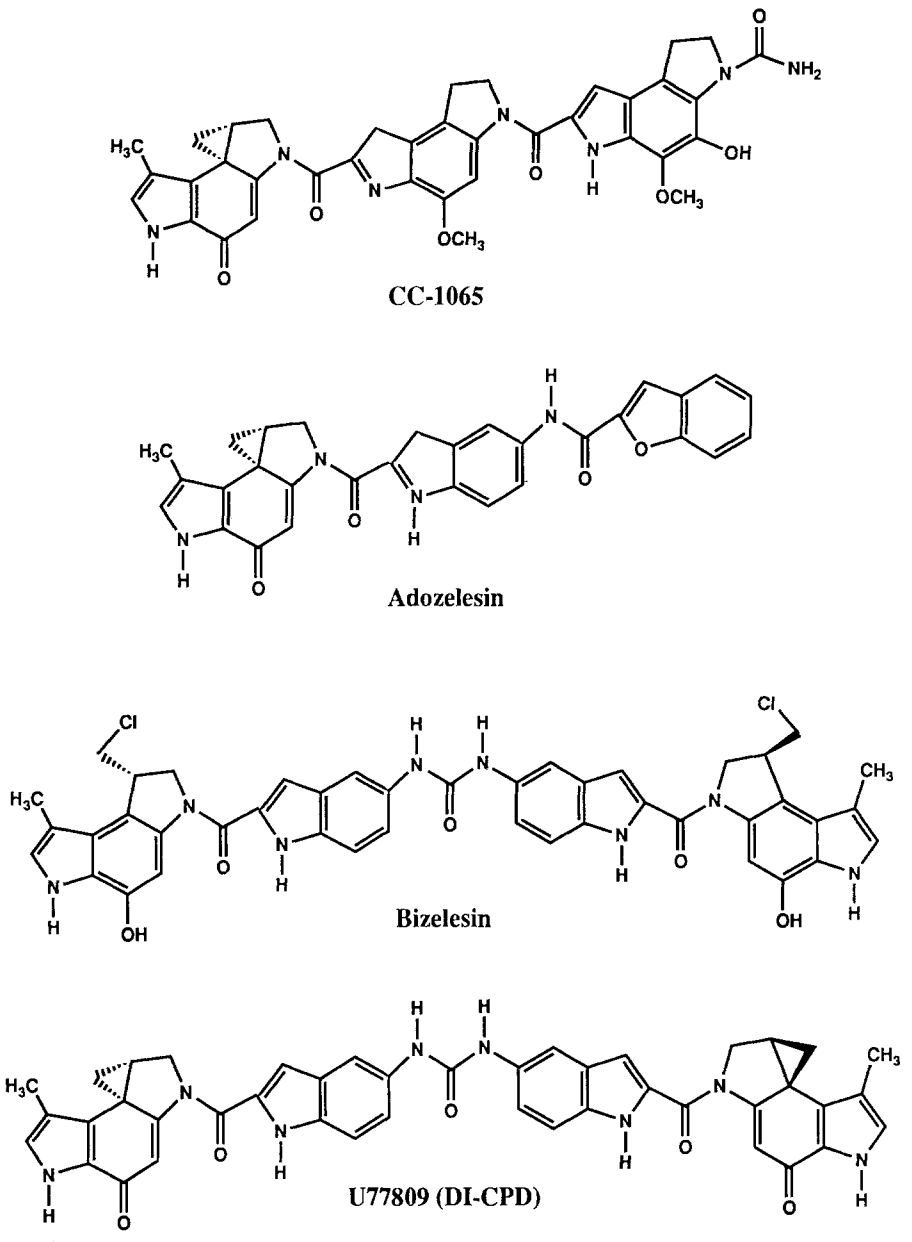

Fig. 1. Structures of CC-1065 and related analogs

clopropyl alkylating species that subsequently interact with DNA. DNA interstrand cross-links are formed after incubation of bizelesin with tumor cells [1, 4]. Sequence selectivity for adduct formation has been demonstrated for CC-1065 and bizelesin [1, 9].

On the basis of its potency, lack of delayed toxicity, and bifunctional alkylating activity, bizelesin was selected for further preclinical evaluation by the National Cancer Institute. A high-performance liquid chromatography (HPLC) assay and L1210 bioassay for bizelesin were developed and applied to preclinical pharmacology studies with this novel bifunctional agent. We specifically addressed the pharmacokinetics of bizelesin following its administration to mice and considered the role of cyclopropyl intermediates in the cytotoxicity of bizelesin to L1210 leukemia cells.

Materials and methods

Chemicals. Bizelesin was provided by the Pharmaceutical Resources Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, Md.). CC-1065, adozelesin, U-77809, and related analogs were generously provided by Upjohn Company (Kalamazoo, Mich.). Zorbax C_8 columns (4.6 mm inside diameter × 25 cm) were obtained from Mac Mod Analytical Inc. (Chadds Ford, Pa.), and Brownlee RP8 guard columns were obtained from Chromtech (Apple Valley, Minn.). All solvents were of HPLC grade. Fetal calf serum and RPMI 1640 media were obtained from Gibco (Gaithersburg, Md.).

Sample preparation and HPLC analysis. Aqueous samples (buffer, plasma, or cell-culture medium; 0.1–0.5 ml) were added to 5 ml ethyl acetate and shaken with a mechanical shaker for 15 min. Following centrifugation (1,000 *g*, 5 min), organic layers were transferred to conical centrifuge tubes and evaporated to dryness under nitrogen. The residues were reconstituted in mobile phase and kept on ice until HPLC analysis on a Hewlett-Packard 1090M ternary-gradient liquid chromatograph equipped with a diode-array detector (254 nm). Separation was achieved with a Zorbax C_8 column eluted with a mobile

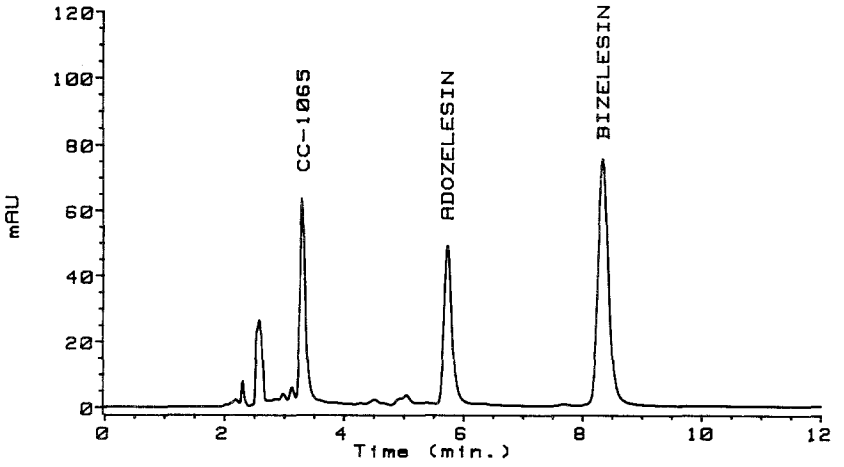

Fig. 2. Chromatogram of CC-1065, adozelesin, and bizelesin (20 μg/ml, prepared in mobile phase) separated on a Zorbax C_8 column eluted with a mobile phase of acetonitrile:tetrahydrofuran:40 m*M* ammonium phosphate, pH 3 (50:5:45, by vol)

phase of acetonitrile:tetrahydrofuran:40 m*M* ammonium phosphate, pH 3.0 (50:5:45, by vol.). Concentrations of drugs were determined by fitting unknown samples to the linear regression equations of standard curves prepared by adding known amounts of the compound of interest to appropriate solutions.

Growth-inhibition assay. Exponentially growing mouse leukemia L1210 cells were maintained in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS). Cultures were maintained at 37° C in an incubator under a humidified atmosphere (100% relative humidity) of 5% CO_2:95% air. On the day prior to each experiment, approximately half of the cells were aspirated from each flask and fresh medium was added to reduce cell numbers to $5-8\times10^5$/ml. Samples containing known amounts of drugs were prepared by diluting stock solutions [1 mg/ml in dimethylsulfoxide (DMSO)] to concentrations of 2.39–612.7 p*M* with culture medium (RPMI 1640 supplemented with 5% FCS) or dilutions of plasma (see below). Aliquots of standard or unknown samples (0.15 ml) were added to flasks containing 2.85 ml medium and 1×10^5 L1210 cells. The resulting incubation mixture contained 0.5% DMSO. Cells and drugs were incubated (5% CO_2:95% air at 37° C) for 48–72 h. Cell numbers were determined by Coulter counter. All samples were assayed in duplicate. Growth inhibition was expressed as the percentage of cells in drug-treated flasks relative to the number of cell in control flasks. Graphs of the percentage of growth inhibition versus ln [drug concentration] were used to determine 50% growth-inhibitory concentrations (IC_{50}) values as follows; the linear portion of dose-response curves were fitted by least-squares linear regression to the equation $y = m \ln x + b$, where y is the percentage of inhibition, x is the concentration of drug in the linear portion of the dose-response curve, and b is a constant. IC_{50} values were calculated from these data as the concentration of drug required to produce 50% inhibition of cell growth in flasks incubated with drug during a 48-h period.

Murine pharmacokinetics. Stock solutions of bizelesin (1 mg/ml in DMSO) were diluted to a concentration of 2.5 ng/ml with 5% dextrose in water (D_5W). Drug (15 μg/kg) was injected i.v. with a 1-cc tuberculin syringe fitted with a 27-gauge needle via the tail vein of male $CD2F_1$ mice immobilized in standard Broome restraints. Drug was also given i.p. to mice at a dose of 20 μg/kg. Samples were collected at 3, 6, 9, 15, 30, 60, 120, 240, 480 (some experiments), and 1,440 min following the administration of bizelesin. Blood samples were obtained from mice anesthetized under ether vapors by cardiac puncture using citrate phosphate dextrose adenine (CPDA) anticoagulant syringes. Whole blood was transferred to silanized microcentrifuge tubes and the plasma was separated by centrifugation (10,000 rpm, 3 min). The plasma was transferred to silanized microcentrifuge tubes and immediately frozen (–20° C) until analysis.

Plasma concentrations of bizelesin were determined with the L1210 bioassay described above, utilizing the linear portion (10–40 p*M*) of the percentage of growth inhibition-ln (drug concentration) graphs. Following i.v. and i.p. administration of bizelesin, plasma concentrations frequently exceeded those that were linear in the bioassay. Therefore, plasma samples were routinely subjected to 2- to 400-fold dilution (i.v. – 10-, 100-, and 400-fold; i.p. – 2- and 10-fold) with cell-culture medium prior to their assay. Pharmacokinetic parameters were obtained by fitting plasma concentration-time data by nonlinear least-squares regression analysis using the program PCNONLIN.

To determine urinary excretion following i.p. or i.v. injection of bizelesin, treated mice were placed in glass metabolism cages (four per cage) and urine was collected in Erlenmeyer flasks placed on dry ice at the base of each cage. Urine was filtered with Acrodisc (0.45 μm) filters prior to determination of drug concentrations with the growth-inhibition assay.

Results

Analytical methodology

HPLC conditions afforded simultaneous separation and quantitation of bizelesin, CC-1065, and adozelesin as shown in Fig. 2. Isolation of bizelesin from aqueous samples including plasma was best accomplished by ethyl acetate extraction, with the recovery in phosphate-buffered saline (PBS), RPMI 1640, and murine plasma being 92%–98%, 70%–76%, and 63%–72%, respectively. The recovery of adozelesin and CC-1065 with ethyl acetate was comparable with that obtained for bizelesin.

Although HPLC methodology was useful for studies of bizelesin stability and conversion to cyclopropyl intermediates, the extraordinary potency of bizelesin precluded the utilization of HPLC methodology for quantitation of plasma cencentrations following the administration of tolerable doses to mice. However, modification of the L1210 growth-inhibition assay developed by Li et al. [6] for quantitation of CC-1065 afforded quantitation of bizelesin and related analogs. It should be emphasized that the bioassay does not distinguish between the parent drug and cytotoxic chemical degradation products and/or metabolites. This distinction is relevant for bizelesin since, as noted, the chloroethyl moieties of the parent drug molecule undergo cyclization to form the cyclopropyl species, which are believed to be responsible for interactions with DNA and the resultant cytotoxicity. Thus, whereas the bioassay

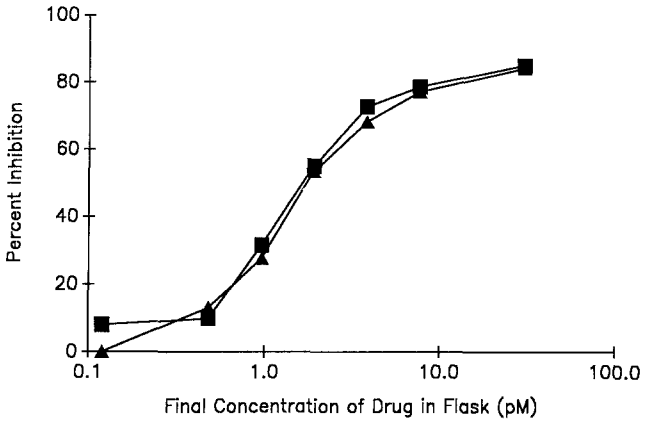

Fig. 3. Growth-inhibitory activity curves generated for bizelesin prepared in cell-culture medium (*squares*) and a 1:2 dilution of murine plasma (*triangles*). Standards (0.15 ml) were added to flask containing 2.85 ml medium and 1×10^5 L1210 cells and the mixture was incubated for 48 h

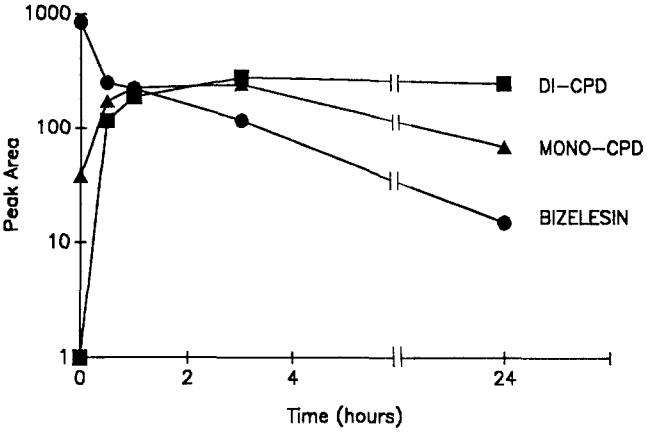

Fig. 5. Time course of the appearance and disappearance of bizelesin and the presumed monocyclopropyl derivative (*MONO-CPD*) and dicyclopropyl derivative (*DI-CPD*) of bizelesin following the incubation of bizelesin (12.25 μ*M*) in pH 7.4 at 37° C for 24 h as measured by HPLC

precludes direct quantitation of the parent drug, it allows the determination of cytotoxic activity in the plasma. A representitive growth-inhibition curve generated for bizelesin incubated with L1210 cells in cell-culture medium is shown in Fig. 3. Following 48-h periods of incubation, the IC_{50} values obtained for bizelesin (Fig. 3), adozelesin, and CC-1065 (data not shown) were 2.3, 3.4, and 88.1 p*M*, respectively.

The L1210 bioassay was modified for analysis of plasma samples. Following the administration of bizelesin to mice, the plasma concentrations exceeded those that were in the linear range of the bioassay. Addition of diluted murine plasma (1:2–1:400) to cultures did not alter the growth-inhibitory activity of bizelesin (Fig. 3). Interassay variability was assessed at one of the lower (10 m*M*) and higher (40 p*M*) concentrations in the linear range of the plasma standard curves. The coefficients of variation for 10- and 40-p*M* plasma concentrations of bizelesin were 20% and 12%, respectively. The slope of the standard curves varied by $<10\%$.

Stability studies

Initial stability studies with bizelesin, adozelesin, and CC-1065 were conducted at concentrations suitable for HPLC analysis to assess the disappearance of the parent drug and the appearance of degradation products. Bizelesin was stable in organic solvents such as methanol and acetonitrile ($<11\%$ degradation over a 24-h period). Bizelesin was less stable in aqueous solutions, with the degradation half-life values obtained in buffer solutions (25° C) at pH 4, 7 and 10 being 9.6, 2.1, and <1 h, respectively. The half-life of bizelesin in cell-culture medium (37° C, pH 7.4) was 1.5 h. In marked contrast, $>90\%$ of the adozelesin and CC-1065 remained in the cell-culture medium following a 24-h period of incubation (data not shown).

Degradation of bizelesin at pH 7.4 was associated with the appearance of two peaks in HPLC chromatograms (Fig. 4). Literature data suggest that the cytotoxic and antitumor activities of bizelesin are mediated by conversion of the parent-drug chloromethyl moieties to the dicyclopropyl product (U-77809, Fig. 1), which subsequently cross-links DNA [1, 4]. The conversion in aqueous solution

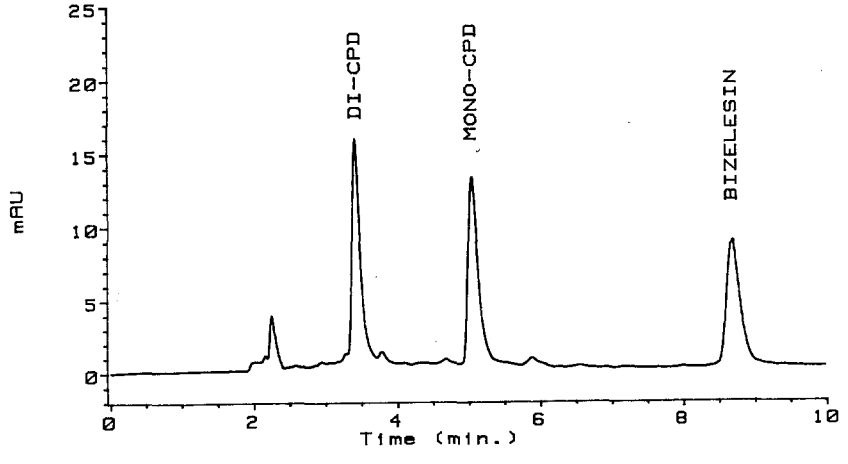

Fig. 4. Chromatogram of bizelesin, the dicyclopropyl derivative (*DI-CPD*, U-77809) and the presumed monocyclopropyl derivative (*MONO-CPD*) following a 60-min period of incubation of bizelesin (12.25 μ*M*) in pH 7.4 buffer at 37° C

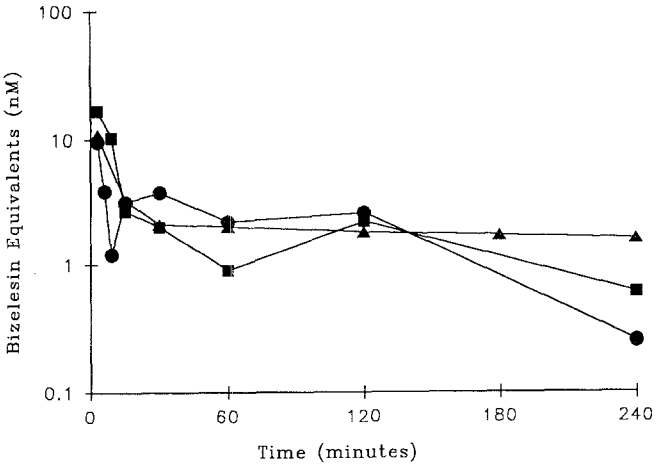

Fig. 6. Plasma profile of the growth-inhibitory activity of bizelesin in the L1210 bioassay following i.v. administration (15 μg/kg) to male $CD2F_1$ mice. Activity in the bioassay was not detected in 8- or 24-h plasma samples

occurs via base-catalyzed intramolecular alkylation of the two chloromethyl groups [10]. The HPLC retention time of the authentic dicyclopropyl intermediate (i.e., U-77809) was identical to that of one of the two degradation products, and the ultraviolet absorbance spectrum of that degradation product was essentially identical to that of the authentic U-77809 (data not shown). The other degradation product may well be the monocyclopropyl intermediate, since it was detected prior to the dicyclopropyl product, and the peak area declined as that of the dicyclopropyl product accumulated in buffer solutions. The time course of the appearance and disappearance of all three species in pH 7.4 buffer is shown in Fig. 5. The dicyclopropyl product was relatively stable in buffer at pH 7.4, with less than 10% decomposition occurring during the 24-h periods of incubation. Bizelesin and U-77809 were equipotent in the L1210 bioassay after 48 h of exposure (data not shown).

Pharmacokinetics

The pharmacokinetics of bizelesin were determined following i.v. and i.p. administration to $CD2F_1$ mice at doses of 15 and 20 μg/kg, respectively. Plasma concentrations were determined with the L1210 bioassay for all experiments. Plasma concentration-time profiles are illustrated in Fig. 6 for three experiments in which bizelesin was injected i.v. via the tail vein. The plasma elimination of species with growth-inhibitory activity was best described by a two-compartment open model. A summary of the pharmacokinetic parameters obtained from those three experiments is presented in Table 1. Following i.p. administration, peak plasma concentrations were observed 30–60 min after administration, and elimination occurred over the following several hours. Drug exposure was at least 10 times lower following i.p. administration as compared with i.v. administration. Following i.v. or i.p. administration, recovery of material with growth-inhibitory activity in urine was $<0.1\%$ of the delivered dose based on the bioassay.

Discussion

The structurally related analogs CC-1065, adozelesin, and bizelesin are notable for their potency in both in vitro and in vivo screening systems. There is additional interest in bizelesin because it is the only bifunctional alkylating agent in this series of molecules. The remarkable potency of bizelesin required the development of analytical methods capable of quantitating plasma drug concentrations of <0.1 n*M*, well below the sensitivity of the HPLC method (0.5 μ*M*). The L1210 assay developed by Li et al. [5, 6] was readily applicable to studies with bizelesin and related molecules. However, an inherent limitation of this method is the potential presence of cytotoxic degradation products and/or metabolites of bizelesin in biological samples, which may contribute to the growth-inhibitory activity observed in the bioassay.

The antitumor activity of bizelesin is almost certainly related to conversion of the two chloromethyl moieties to cyclopropyl intermediates that, analogous to adozelesin and CC-1065, interact covalently with DNA following intercalation of the molecule [1, 4]. On the basis of HPLC analysis, we determined that when bizelesin was incubated in aqueous solutions (including plasma) at physiological pH, there was rapid conversion to a product with a retention time and an ultraviolet absorbance spectrum identical to those of the dicyclopropyl intermediate U-77809. In marked contrast to the observed HPLC degradation of bizelesin, there was little loss of growth-inhibitory activity over 24 h in the L1210 assay, consistent with a role for the dicyclopropyl product in the cytotoxicity of the parent compound. These findings are relevant to the murine pharmacokinetic data, which are based on the detection of growth-inhibitory species rather than the parent drug in plasma. This pharmacokinetic characerization is useful in that it represents the behavior of the potentially active and

Table 1. Summary of pharmacokinetic parameters obtained following i.v. administration of bizelesin at 15 μg/kg to male $CD2F_1$ mice

Experiment	$t_{1/2\alpha}$ (min)	$t_{1/2\beta}$ (h)	V_{SS} (ml/kg)	Cl_{TB} (ml min^{-1} kg^{-1})	AUC (ng ml^{-1} min^{-1})
1	0.9	2.7	4,772	23.2	647
2	4.3	11.0	8,146	8.92	1,681
3	5.4	8.2	10,004	16.9	886
Mean ± SEM	3.5±1.4	7.3±2.4	7,641±1,531	16.3±4.1	1,071±313

toxic species present after administration of the parent drug.

Plasma elimination of bizelesin-related growth-inhibitory activity was best characterized by a two-compartment open model following i. v. administration to mice. Systemic exposure was much lower following i. p. administration of bizelesin. The low urinary recovery of species active in the L1210 bioassay suggest an extensive interaction of the parent drug and other reactive species with macromolecules and/or further metabolism or degradation to inactive species.

Bizelesin is an extraordinarily potent DNA-binding and -alkylating agent that will be of interest to follow in the developmental process. The studies reported herein provide data on the preclinical pharmacology of this agent that will be of value as clinical trials of this interesting compound are initiated.

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