Cancer Chemother Pharmacol (1996) 38 : 329–334

ORIGINAL ARTICLE

Joel M. Reid · Denise L. Walker · Matthew M. Ames

Preclinical pharmacology of ecteinascidin 729, a marine natural product with potent antitumor activity

Received: 23 September 1994/Accepted: 6 December 1995

Abstract Ecteinascidins are marine natural products with potent antiproliferative activity under evaluation as chemotherapeutic agents by the National Cancer Institute. Ecteinascidins bind the minor groove of DNA and may form covalent adducts with DNA by binding the N-2 of guanine in a fashion similar to saframycin antibiotics. The most potent ecteinascidin is ET-729 with antitumor activity observed following administration of 3.8 and 10 μg/kg to mice bearing P388 leukemia and B16 melanoma, respectively. A reverse-phase high-performance liquid chromatography (HPLC) assay and an L1210 cell bioassay were developed for ET-729 and utilized for stability and murine pharmacokinetic studies. HPLC analysis showed that ET-729 was stable in organic solvents, mobile phase and acidic buffer ($t_{1/2} > 100$ h). Stability was diminished under neutral and basic conditions ($t_{1/2} < 14$ h). Following a 48-h incubation with L1210 cells in growth medium in the absence and presence of 2.5% murine plasma, the 50% growth inhibitory concentrations (IC_{50}) of ET-729 were 37 and 72 p*M*, respectively. Following intravenous administration of ET-729 to male $CD2F_1$ mice, the disappearance of antiproliferative activity determined by the bioassay was described by a two-compartment open model. The mean values of the elimination half-life and plasma clearance were 28 min and 39.7 ml/min per kg, respectively. Following intraperitoneal administration, peak plasma concentrations of antiproliferative activity were observed 6–15 min after injection and antiproliferative concentrations remained above 1 n*M* for longer than 1 h. Intraperitoneal bioavailability varied over a wide range (20–91%). Antiproliferative activity was detected in every urine sample following intravenous and intraperitoneal administration, but the total 48-h urinary recovery was less than 0.1%.

Key words Ecteinascidin · Mice · Pharmacokinetics

Introduction

Ecteinascidins are marine natural products selected by the National Cancer Institute (NCI) for development based on potent in vivo and in vitro antiproliferative activity. Extracts of the marine tunicate *Ecteinascidia turbinata* were first discovered to have cytotoxic activity in the late 1960s, but purification and identification of active components was not completed until 1990 [1,2]. The most potent ecteinascidin is ecteinascidin-729 (ET-729, NSC638718, Fig. 1) with antitumor activity observed following administration of 3.8 and 10 μg/kg to mice bearing P388 and B16 melanoma, respectively.

Ecteinascidins are structurally related to the safracin class of antitumor antibiotics since they contain three tetrahydroisoquinoline rings and a carbinolamine moiety which is believed to be responsible for the antitumor activity of the latter compounds [3]. Saframycins bind the minor groove of DNA and form covalent adducts, presumably by reaction of the N-2 of guanine with the carbinolamine moiety [3]. By analogy, a model of ET-DNA adducts [4, 5] has been prepared based on data from the three-dimensional structure of adducts between daunorubicin and DNA in the presence of formaldehyde. This model predicts four or five hydrogen bonding interactions between ET and DNA with minimal disturbance of the minor groove. As a result of covalent binding to DNA in the minor groove, ET-729 may inhibit DNA replication and transcription [5].

Supported in part by National Cancer Institute Contract CM27709

J.M. Reid · D.L. Walker · M.M. Ames (✉)
Mayo Clinic and Foundation, Department of Oncology, Division of Developmental Oncology Research, 200 First Street Southwest, Rochester, MN 55905, USA
(Fax: 507–284–3906)

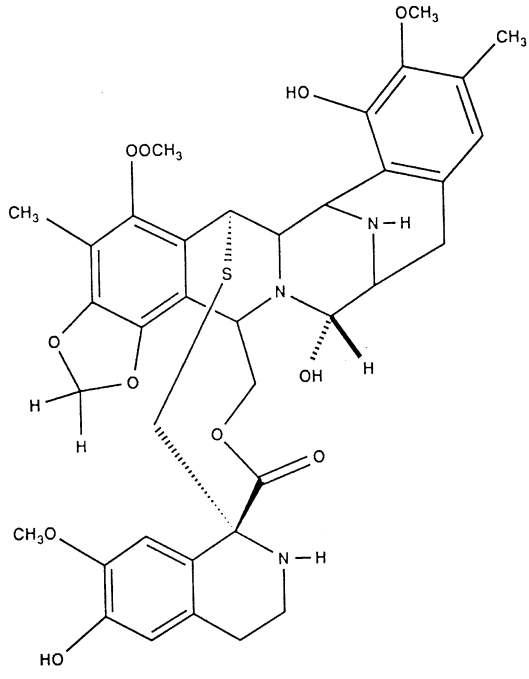

Fig. 1 Structure of ET-729

As part of NCI-sponsored investigations of ET-729, we developed analytical methodology to characterize the pharmacokinetics following intravenous (i.v.) and intraperitoneal (i.p.) administration to male $CD2F_1$ mice. Since it was believed that administered doses (40–80 μg/kg) would result in plasma concentrations of drug well below detection limits of standard analytical methods, we adapted an L1210 cell bioassay for detection of ET-729 in biological fluids.

Materials and methods

Chemicals

ET-729 was provided by the Pharmaceutical Resource Branch, Division of Cancer Treatment, NCI (Bethesda, Md.). Fetal calf serum and RPMI-1640 medium were obtained from Gibco (Gaithersburg, Md.). All chemicals were of analytical reagent grade and all solvents were of HPLC grade.

Sample preparation

Aqueous samples (buffer, plasma; 0.1 ml) were added to 1-ml Baker C_{18} SPE columns which had been rinsed sequentially with 1 ml each of methanol, distilled water and 50 m*M* ammonium acetate, pH 7.0. Following sample application, columns were washed with 1 ml each of 50 m*M* ammonium acetate, pH 7.0, and distilled water. ET-729 was eluted from the column with 2 ml methanol. Samples were evaporated to dryness under a nitrogen stream and reconstituted in HPLC mobile phase (0.1 ml) prior to chromatography.

HPLC analysis

Reverse-phase HPLC analyses were performed on a Hewlett-Packard 1090 M ternary gradient liquid chromatograph equipped with a diode array detector set at 254 nm. Separations were achieved on a Whatman Partisil 10 ODS-3 analytical column (250 × 4.6 mm i.d.), protected with a Brownlee Newguard RP18 column (15 mm × 3.2 mm i.d., 7 μm particles) and maintained at 40°C. The mobile phase consisted of methanol and 50 m*M* ammonium acetate, pH 7.0 (70:30, v/v) and was delivered at a flow rate of 1.0 ml/min.

Growth inhibition assay

Exponentially growing murine leukemia L1210 cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum. Cultures were maintained at 37°C in an incubator under a humidified atmosphere (100% relative humidity) of 5% CO_2:95% air. The day prior to each experiment, a portion of the medium was aspirated from the flasks and sufficient fresh medium was added to reduce cell numbers to $5–8 \times 10^5$ cells/ml. Standard samples were prepared by serial dilution of an ET-729 stock solution (54.9 μ*M* in ethanol) with ethanol (1:10) followed by cell culture medium (1:10 and 1:13.3) to obtain solutions containing 0.549 and 0.413 μ*M* drug, respectively. A set of working solutions with concentrations in the range 3.43 to 549 n*M* was prepared from each of these solutions by serial dilution with cell culture medium containing 10% ethanol. Aliquots of these solutions (20 μl) were then mixed with 380 μl medium containing the appropriate proportion (10–50%) of buffer, plasma or urine. Unknown samples were prepared for the bioassay by dilution (1:2–1:10) of buffers, plasma or urine with cell culture medium. Aliquots of standard or unknown samples (0.15 ml) were added to flasks containing 2.85 ml cell culture medium and 1.14×10^5 L1210 cells. The resulting incubations contained 0.5% ethanol, 0.5% to 2.5% mouse plasma and 10% fetal calf serum. Following an incubation period of 48 h, cell numbers in culture aliquots (1 ml) were determined by Coulter counter. All samples were assayed in duplicate. Growth inhibition was expressed as percent of cells in drug-treated flasks relative to the number of cells in control flasks.

Murine pharmacokinetics

Non-tumored male $CD2F_1$ mice (20–30 g), supplied by the National Cancer Institute, were housed five per cage on commercially obtained pure wood shaving bedding in an on-site facility with light provided from 6:00 a.m. to 8:00 p.m. Food (Purina Rodent Chow) and tap water were provided *ad libitum*. Stock solutions of ET-729 (1 mg/ml in ethanol) were diluted to concentrations of 5 or 10 μg/ml with sterile normal saline and administered to male $CD2F_1$ mice intravenously or intraperitoneally (40 or 80 μg/kg) using a 1 ml tuberculin syringe fitted with a 27 gauge needle. The intravenous dose was administered via the tail vein to mice restrained in standard Broome-type restraints.

In a single experiment, ET-729 was administered either intravenously or intraperitoneally to separate groups of 10 (40 μg/kg) or 13 (80 μg/kg) mice. Blood samples (one mouse per time point) were obtained by cardiac puncture from mice anesthetized under ether vapors during the 24-h period after drug administration. Specimens were drawn into citrated syringes (150 μl CPDA anticoagulant/ml whole blood) and transferred to silanized microcentrifuge tubes. Plasma was separated by centrifugation (10 000 rpm × 3 min), transferred to silanized microcentrifuge tubes and immediately frozen. Frozen samples were stored at −20°C until analysis, usually within 2 days. To determine urinary excretion of ET-729 following intravenous or intraperitoneal administration 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.

Plasma concentrations of ET-729 were determined with the L1210 bioassay described above, utilizing the linear portion of the growth inhibition versus ln(drug concentration) graphs. Plasma samples were routinely subjected to twofold dilution with cell culture medium prior to their assay. Urine samples were subjected to a tenfold dilution with cell culture medium prior to their assay.

Data analysis

Mean values of the percent growth inhibition were graphed as a function of the natural logarithm of the ET-729 concentration in the incubation flasks. The IC_{50} values were calculated from these data as the concentration of drug to produce 50% inhibition of cell growth in flasks incubated with drug during a 48-h period. The linear portion of the dose-response curve was fitted by linear least squares regression to the equation $y = m\ln(x) + b$, where x is the concentration of drug added to the culture flasks, y is the percent growth inhibition, m is the slope of the linear portion of the dose response curve and b is a constant. Concentrations of drug in unknown samples were calculated by fitting growth inhibition values to the standard curve regression equations and adjusted for the dilution required to incubate the sample with cells.

Interassay variability was assessed by comparing mean growth inhibition values of each standard concentration in the linear range and fitted slope values of the corresponding standard curves.

Plasma concentration–time data were fitted to a two-compartment open model by non-linear least squares regression using the program PCNONLIN version 3.0 (Statistical Consultants, Lexington, Ky.). Plasma concentration–time data were also analyzed by non-compartmental methods as described by Gibaldi and Perrier (6). AUC values were determined by trapezoidal approximation from zero time to the time of the last detectable plasma concentration (C_{last}). Residual area after C_{last} was calculated by $AUC_r = C_{last}/k_{el}$, where k_{el} is the elimination rate constant. The concentration at zero time (C_0) was estimated by $C_0 = D/V$, where D is the dose and V is the distribution volume.

Results

Reverse-phase HPLC of ET-729 with a C_{18} column proved satisfactory when the mobile phase was buffered with ammonium acetate, pH 7.0, and the column temperature was maintained at 40°C (Fig. 2). Standard curves of peak area versus ET-729 plasma concentration were linear over the range 5 to 100 μg/ml and the lower limit of detection was 0.12 μg on column. Due to the high potency of ET-729, in vivo concentrations were below the detection limits of HPLC. Therefore, the HPLC method was used primarily to monitor stability.

We and others have shown that a bioassay method, based on L1210 growth inhibition in tissue culture flasks, provides sensitive and reliable determination of potent cytotoxic agents in biological fluids. We have applied the bioassay to the characterization of pharmacokinetics of other potent agents following administration to mice [7]. However, such bioassays lack specificity, since inhibition of cell growth does not distinguish between parent drug and cytotoxic chemical degradation products and/or metabolites.

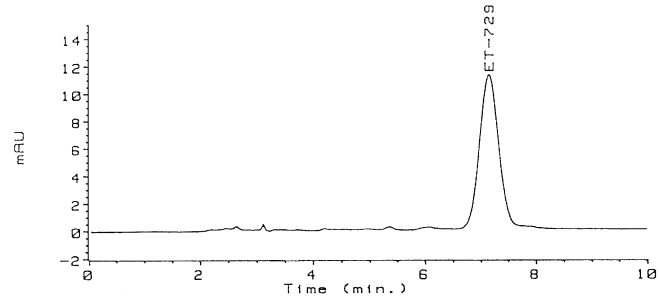

Fig. 2 Reverse-phase HPLC chromatogram of ET-729 (27 μ*M*, prepared in mobile phase) following elution with a mobile phase consisting of methanol and 50 m*M* ammonium acetate, pH 7.0 (70:30 v/v), at a flow rate of 1.0 ml/min. UV absorbance was monitored at 254 nm

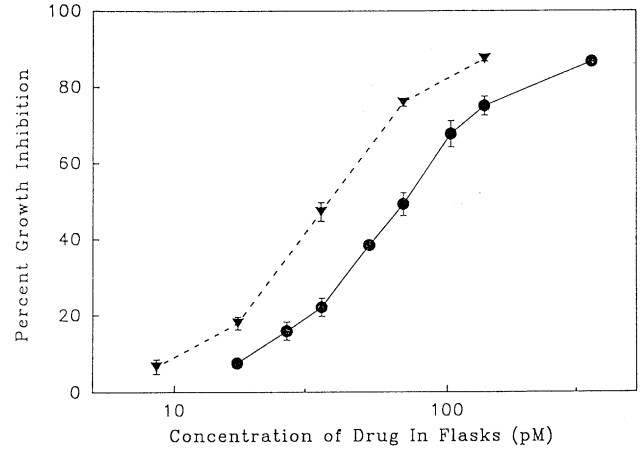

Fig. 3 Growth inhibitory activity of ET-729 prepared in cell culture medium (▲) and a 1:1 dilution of murine plasma (●) following 48 h of incubation with L1210 cells. Symbols represent the means ± standard deviation of five experiments in cell culture medium and of six experiments in cell culture medium containing 2.5% murine plasma

L1210 cell growth was assessed following a 48-h period of incubation in RPMI-1640 medium supplemented with 10% fetal calf serum. The graph of L1210 cell growth inhibition exhibited a linear response over the ET-729 concentration range of 20–80 p*M* with an IC_{50} value of 37 ± 2 p*M* (Fig. 3), confirming the potency of this agent. L1210 cell growth and assay sensitivity were reduced when the cell culture medium contained dilute (final concentration, 0.5% to 2.5%) mouse plasma. The linear range of growth inhibitory activity was 34–103 p*M* ET-729 and the IC_{50} value was 72 ± 8 p*M* when the cell culture medium contained 2.5% plasma (Fig. 3). The reproducibility of the low (34 p*M*) and high (103 p*M*) concentration standards in the linear range of the standard curve were 28% and 10%, respectively. The interassay variability determined from the coefficient of variation of the slope of the standard curves was 9%. Determination of ET-729 in mouse urine samples required 1:10 dilution of urine prior to addition to cell culture flasks (data not shown). The limits of quantitation of ET-729 (adjusted for

sample dilution) in plasma and urine were 0.7 n*M* and 5.0 n*M*, respectively.

The stability of ET-729 in aqueous solutions, organic solvents, culture medium and plasma was first studied by HPLC under conditions which allowed detection of potential degradation products formed during the incubation. Excellent stability was observed ($t_{1/2} >$ 100 h) in acidic (pH 4) buffer, mobile phase and organic solvents. However, stability was reduced under neutral (pH 7.4) and basic (pH 10) conditions with half-life values of 13.1 h and 4.3 h, respectively. ET-729 was relatively stable when incubated in plasma and medium, with half-life values of 39 h and 55 h, respectively. New peaks, corresponding to potential degradation products, were not observed in HPLC chromatograms during the incubation period.

The stability of ET-729 (55 μ*M*) in plasma and culture medium was also evaluated with the bioassay. The decline in growth inhibitory activity paralleled the decreased peak area detected by HPLC during the 48-h incubation period. The half-life values for the decline of growth inhibitory activity in plasma and culture medium were 45 h and 34 h, respectively. The stability of ET-729 was also evaluated in PBS, plasma and culture medium at the much lower concentration of 11 n*M*, which was in the range of concentrations expected in vivo. The drug concentration in PBS fell rapidly during the first hour of incubation, presumably due to adsorption to the walls of the incubation vessel. During the next 47-h period the growth inhibitory activity declined with an apparent half-life value of 163 h. The growth inhibitory activity of ET-729 in plasma and culture medium remained unchanged during a 10-h period (data not shown).

Growth inhibitory activity in plasma of male $CD2F_1$ mice was determined following intravenous and intraperitoneal doses of 40 and 80 μg/kg ET-729. These doses were chosen on the basis of toxicity and activity data available from NCI. Both doses were below the MTD in $CD2F_1$ mice [8] and the lower dose (40 μg/kg) was within the range of doses with observed antitumor activity in mice. The 80 μg/kg dose was chosen to examine linearity of the pharmacokinetics. We did not examine higher doses due to limited drug availability.

Plasma profiles of ET-729 growth inhibitory activity displayed a biphasic pattern (Fig. 4) with mean values of the distribution and elimination half-lives of 1.0 min and 28 min, respectively (Table 1). Following a 40 μg/kg intravenous dose, ET-729-related growth inhibitory activity was detected in mouse plasma for 60–120 min. When the dose was increased to 80 μg/kg ET-729-related growth inhibitory activity was detected for as long as 180 min (data not shown). Compartmental analysis of the plasma concentration-time data from individual experiments yielded estimates of Cl_{TB} and V_{ss} that varied over a wide range (Table 1). This was due primarily to error in the estimation of values for the intercept (A) and the distribution rate constant (α) as a result of a single value of growth inhibitory activity observed in the distribution phase of the plasma profile. Noncompartmental analysis of the plasma concentration-time data was performed with an estimate of V based on the assumption of distribution of ET-729 into whole blood immediately after injection (Table 1). There were modest differences in the mean parameter

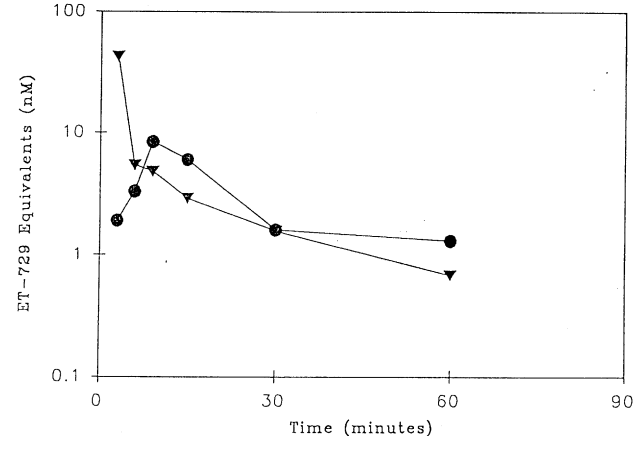

Fig. 4 Representative plasma profiles of ET-729-related growth inhibition activity following intravenous administration (▲) and intraperitoneal administration (●) of 40 μg/kg to male $CD2F_1$ mice

Table 1 Summary of ET-729 pharmacokinetic parameters following intravenous administration to male $CD2F_1$ mice

Experiment	Dose (μg/kg)	Compartmental analysis						Noncompartmental analysis		
		A (n*M*)	B (n*M*)	$t_{1/2\alpha}$ (min)	$t_{1/2\beta}$ (min)	V_{ss} (l/kg)	Cl_{TB} (ml/min per kg)	MRT (min)	Cl_{TB} (ml/min per kg)	V_{ss} ml/kg
1	40	1095	5.2	0.6	20	224	48.6	4.1	42.6	175
2	40	121	5.2	1.2	31	3027	125	9.7	49.8	485
3	40	555	10.7	0.9	37	1071	43.8	18.6	32.9	611
Mean		590	7.0	0.9	29	1441	72.5	10.8	41.8	424
4	80	1257	19.8	1.2	25	624	49.9	19.9	33.9	676
5	80	166	18.9	1.7	27	2563	96.2	12.1	37.3	452
Mean		712	19.4	1.4	26	1594	73	16	35.6	564

Table 2 Intraperitoneal bioavailability of ET-729 (based on trapezoidal approximation)

Experiment	Dose (μg/kg)	AUC (n*M* · min)		
		i.p.	i.v.	Ratio i.p./ i.v.x 100%
1	40	360	1674	22
2	40	332	1681	20
3	40	1819	2161	84
4	80	1214	4124	29
5	80	3493	3826	91

estimates obtained for each dose level that may be due to the low parameter values obtained for the first experiment. The mean values of Cl_{TB} and V_{ss} obtained by noncompartmental analysis were 39.7 ml/min per kg and 484 ml/kg, respectively.

Peak plasma concentrations of ET-729 were observed 6–15 min after intraperitoneal administration (Fig. 4). Following administration of 40 μg/kg, plasma concentrations remained above 1 n*M* for approximately 1 h in two of three experiments and concentrations remained above 1 n*M* for longer than 4 h in one experiment. Following administration of an 80 μg/kg dose, plasma concentrations remained above 1 nM for longer than 4 h. The mean value of the elimination half-life was 231 min (range, 100–462 min). The intraperitoneal bioavailability was determined as the ratio of intraperitoneal and intravenous AUC values (Table 2). In three experiments, the apparent intraperitoneal bioavailability fell in a narrow range of 20–29%. In the two remaining experiments, the apparent intraperitoneal bioavailability was greater than 80%.

Growth inhibitory activity was detected in all urine samples, but the total 48-h urinary recovery was less than 0.1%.

Discussion

Ecteinascidins are potent cytotoxic marine natural products that are potentially useful as antitumor agents. Evaluation of the pharmacokinetics of these molecules, especially the most potent analog ET-729, requires the availability of a sensitive method of detection in biological fluids. Due to the potency of ET-729, standard detection methods such as UV absorbance or fluorescence detection do not provide sufficient sensitivity to allow detection of the minute plasma concentrations of drug expected following administration of doses in the low microgram range. Development of methods with greater sensitivity which include chemical derivatization or immunoassay was not possible due to limited availability of ET-729.

As an alternative, we chose to adapt an L1210 bioassay which had been found to be useful in the characterization of the preclinical pharmacology of the CC-1065 analogs adozelesin [9] and bizelesin [7]. This assay was based on inhibition of L1210 cell growth in culture flasks following a 48-h exposure to medium containing drug. ET-729 was extremely potent with an IC_{50} value of 37 p*M* and growth inhibitory activity was linear over the concentration range of 20–80 p*M*. Sensitivity was reduced when a portion of the medium was replaced with plasma. A linear response was observed over the range 34–103 p*M* when the culture medium contained 2.5% plasma as a result of twofold dilution of plasma prior to addition to culture flasks. The assay was reproducible and as little as 0.7 n*M* ET-729 was detectable in mouse plasma samples. It should be emphasized that the assay is not specific for the parent drug since all species with growth inhibitory activity will contribute to the total growth inhibitory activity of samples and therefore concentrations determined with this assay may include metabolites and/or chemical decomposition products which may form in vivo.

An HPLC method for ET-729 was developed concurrently with the bioassay to determine the stability profile in aqueous buffers, cell culture medium and biological fluids. While the HPLC method was less sensitive, it provided an opportunity to determine the effect of drug decomposition on its antiproliferative activity. Parallel profiles of loss of drug and growth inhibitory activity when ET-729 was incubated in mouse plasma or cell culture medium indicate that loss of drug was not accompanied by the formation of cytotoxic byproducts.

Following intravenous and intraperitoneal administration of 40–80 μg/kg doses, ET-729 was well tolerated and peak plasma concentrations were > 270-fold higher than the IC_{50} value against L1210 tumor cells. Plasma elimination of growth inhibitory activity was best fitted with a two-compartment open model with an elimination half-life of 28 min. ET-729 plasma clearance (39.3 ml/min per kg) was greater than the liver blood flow rate (21 ml/min per kg) for mice, while urinary excretion appeared to play a minor role in the clearance of growth inhibitory activity. Therefore, rapid clearance of cytotoxic activity may be due to extensive tissue distribution and binding to nucleophilic molecules including DNA.

The broad range for the Cl_{TB} and V_{ss} estimates calculated by compartmental analysis was associated with variability in estimates of A and α. Due to the rapid decline in the growth inhibitory activity immediately after injection, a single specimen was obtained during the distribution phase of the plasma profile. Small differences in the timing of specimen collection may lead to large differences in growth inhibitory activity observed for early time-points and may introduce large errors in parameter estimates based on these data. Due to technical limitations of the experimental procedures, it is not possible to obtain more specimens during this short time period or, alternatively, to improve the

precision in the timing of collection of the specimen. Noncompartmental analysis provided an alternative method to calculated the pharmacokinetic parameters and substantially reduced the variability in the estimates of Cl_{TB} and V_{ss}.

The variability in ET-729 intraperitoneal bioavailability may also be due to parameter estimation based on limited samples in the distribution phase. Low, bioavailability (20–29%) was found in three experiments, while high bioavailability ($> 80\%$) was found in two experiments. While every effort was made to obtain blood samples from the heart following intraperitoneal administration, the elevated bioavailability values may have been due to contamination from ET-729 remaining in the peritoneal cavity.

In summary, these studies indicate that growth inhibitory concentrations attributable to ET-729 are achieved in plasma following intravenous and intraperitoneal administration. These data are important to the development of ET-729 or other ecteinascidin analogs as anticancer agents.

Acknowledgements The authors would like to thank Ms. Wanda Rhodes for preparation of the manuscript.

References

1. Rinehart KL, Holt TG, Fregeau NL, Stroh JG, Keifer PA, Sun F, Li LH, Martin DG (1990) Ecteinascidins 729, 743, 745, 759A, 759B, and 770: potent antitumor agents from the Caribbean tunicate *Ecteinascidia turbinata*. J Org Chem 55:4512–4515
2. Wright AE, Forleo DA, Gunawardana GP, Gunasekera SP, Koehn FE, McConnell OJ (1990) Antitumor tetrahydroisoquinoline alkaloids from the colonial ascidian *Ecteinascidia turbinata*. J Org Chem 55:4508–4512
3. Rao KE, Lown JW (1990) Mode of action of saframycin antitumor antibiotics: sequence selectivities in the covalent binding of saframycins A and S to deoxyribonucleic acid. Chem Res Toxicol 3: 262–267
4. Sakai R, Rinehart KL, Guan Y, Wang AH-J (1992) Additional antitumor ecteinascidins from a Caribbean tunicate: crystal structures and activities in vivo. Proc Natl Acad Sci USA 89:11456–11460
5. Guan Y, Sakai R, Rinehart KL, Wang AH-J (1993) Molecular and crystal structures of ecteinascidins: potent antitumor compounds from the Caribbean tunicate *Ecteinascidia turbinata*. J Biomol Struct Dyn 10:793–818
6. Gibaldi M, Perrier D (1982) In: Pharmacokinetics, Chapter 11 (2nd ed.) Marcel Dekker, New York, pp 409–417
7. Walker DL, Reid, JM, Ames MM (1994) Preclinical pharmacology of bizelesin: a potent bifunctional analog of the DNA-binding antibiotic CC-1065. Cancer Chemother Pharmacol 34:317–322
8. Hill JR, Schindler-Horvat JE, Baldwin RC, MacGregor JT, Osborn BL, Donohue SJ, Tyson, CA (1994) Toxicity of ecteinascidin 729 (NSC-638718) in CD2F1 mice and beagle dogs. Proc. Amer Assoc Cancer Res, 35:467
9. Li LH, Kelly RC, Warpehoski MA, McGovren JP, Gebhard I, DeKoning TF (1991) Adozelesin, a selected lead among cyclopropylpyrroloindole analogs of the DNA-binding antibiotic, CC-1065. Invest New Drugs 9:137