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ORIGINAL ARTICLE

Jeffrey G. Supko · Robert L. Hickman
Michael R. Grever · Louis Malspeis

Preclinical pharmacologic evaluation of geldanamycin as an antitumor agent*

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Abstract The plasma pharmacokinetics of the antitumor antibiotic geldanamycin (GM; NSC 122750), a naturally occurring benzoquinoid ansamycin, was characterized in mice and a beagle dog. Concentrations of GM well above 0.1 μg/ml, which was typically effective against neoplastic cell lines responsive to the drug in vitro, were achieved in the plasma of the mice and the dog treated by i.v. injection. However, the systemic duration of the drug was relatively short. Plasma levels decayed below 0.1 μg/ml within 3–4 h after administration of the apparent maximum tolerated doses, which were approximately 20 mg/kg for the mice and 4 mg/kg for the dog.The drug exhibited linear pharmacokinetic behavior within the dose ranges studied. However, there were significant interspecies differences in its disposition. Whereas the mean biological half-life of GM was slightly longer in the mice (77.7 min) than in the dog (57.9 min), its mean residence time in the dog (46.6 min) was more than twofold greater than that observed in the mice (20.7 min). Nevertheless, the drug was cleared from plasma much faster by the dog (49.4 ml/min per kg) than by the mice (30.5 ml/min per kg). These apparent anomalies were principally associated with differences in the relative significance of the terminal phase upon overall drug disposition. The liver appeared to be the principal target organ of acute drug toxicity in the dog. Doses of 2.0 and 4.2 mg/kg both produced elevations in serum levels of the transaminases and other indicators of liver function characteristic of acute hepatic necrosis. Additional effects included symptoms of minor gastrointestinal toxicity and alterations in serum chemistry parameters consistent with less severe nephrotoxicity. Drug-related toxicity appeared to be reversible. In consideration of the potential for acute hepatotoxic reactions to GM, as well as to the other benzoquinoid ansamycins based upon structural analogy, additional pharmacological and therapeutic information is required to ascertain whether these compounds are viable candidates for clinical development.

Key words Ansamycins · Antineoplastic agents · Pharmacokinetics

Abbreviations *GM* Geldanamycin · *IS*, internal standard · GI_{50}, drug concentration for 50% inhibition of cell growth in vitro · LC_{50} drug concentration resulting in 50% lethality in cultured cells · *NCI*, National Cancer Institute · *AUC* area under plasma concentration-time curve between time zero and infinity · *CL*, total plasma clearance · *MRT*, mean residence time · $t_{1/2,z}$ biological half-life · V_1 central compartment apparent volume of distribution · V_{ss}, apparent volume of distribution at steady state · V_z total body apparent volume of distribution · *CPK* creatine phosphokinase · *LDH* lactic dehydrogenase · *SGOT* serum oxaloacetic transaminase · *SGPT* serum glutamic-pyruvic transaminase · *DMSO* dimethyl sulfoxide

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J.G. Supko (✉) · M.R. Grever · L. Malspeis
Laboratory of Pharmaceutical Chemistry, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Frederick, MD 21701, USA

R.L. Hickman
Pathology Associates, Inc., Frederick, MD, 21701, USA

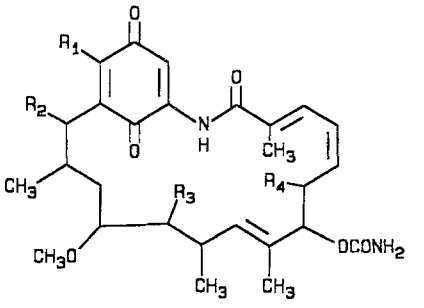
Introduction

Geldanamycin (GM) was isolated from the culture filtrates of a streptomycete and identified as the first known benzoquinoid ansamycin antibiotic [3, 31]. All previously characterized ansamycin antibiotics, which included the streptovaricins, rifamycins, naphthomycin and tolypomycins, were macrocyclic compounds comprised of an aliphatic ansa bridge

linked through a naphthalenic moiety [27]. Several other antibiotics that are structurally very similar to GM, the macbecins and herbimycins, have since been identified [17, 21]. The structures of these compounds are shown in Fig. 1. The pharmacological properties of the benzoquinoid subclass differ considerably from those of the naphthalenic ansamycins [16, 27, 32, 39].

The potent cytotoxic effects of GM against cultured murine L1210 leukemia and nasopharynx KB cells were reported at the same time as its discovery [3]. Shortly thereafter, evidence of antitumor activity against solid sarcoma 180 and Ehrlich ascites carcinoma in mice was demonstrated for two semisynthetic derivatives of GM [32]. The macbecins also show significant in vivo antitumor activity against P388 leukemia, B16 melanoma and Ehrlich carcinoma in mice [20, 24]. More recently, noncytotoxic concentrations of herbimycin A (0.5 μg/ml), macbecin I (1.0 μg/ml) and GM (0.1 μg/ml) have been found to cause Rous sarcoma virus-transformed cells to revert to normal morphology by inhibiting the function of *src* protein tyrosine kinase [5, 10, 34]. This effect is not exhibited by the naphthalenic ansamycins [33]. Evidence has been obtained that suggests that these compounds may prove useful in the treatment of human malignancies associated with elevated tyrosine kinase activity, such as chronic myelogenous leukemia and colon cancer [6, 11–13].

GM has been recently selected for preclinical development as an antitumor agent by the National Cancer Institute (NCI) based upon the demonstration of activity against cancer cells derived from human tumors[1]. At concentrations considerably lower than required to cause sensitive neoplastic cells to revert to normal phenotype, GM and herbimycin A are cytotoxic toward a select group of poorly differentiated neuronal and melanoma human tumor cell lines in vitro, without affecting the viability of several types of nontumorigenic cells or tumor cells with a higher degree of differentiation [36]. Values for the 50% inhibitory concentration against responsive cell lines in vitro range from 2.8 to 4.5 ng/ml for GM and from 18 to 36 ng/ml for herbimycin A. In addition, moderate growth inhibition of CHP-100 cells implanted subcutaneously into athymic nude mice is elicited upon daily i.p. treatment with GM. The mechanism of cytotoxicity appears unrelated to the effect of these compounds on the *src* oncogene or inhibition of its protein product [36]. Studies conducted by Whitesell and Myers at the NCI, which have subsequently been confirmed [26], have revealed that GM is also active against prostate cancer cells in vitro[1]. Furthermore, as described in the present report, GM elicited notable activity against the panel of human tumor cell lines that constitute the NCI's current in vitro screen for antitumor agents.



Compound	R_1	R_2	R_3	R_4
Geldanamycin	OCH_3	H	OH	OCH_3
Herbimycin A	H	OCH_3	OCH_3	OCH_3
Macbecin I	H	OCH_3	OCH_3	CH_3

Fig. 1 Chemical structures of the benzoquinoid ansamycin antibiotics.

Although nearly 25 years have elapsed since the antineoplastic effects of the benzoquinoid ansamycins were first established, information concerning the pharmacokinetics and toxicology of these compounds in preclinical animal models has not been previously reported. Preliminary investigations of in vitro stability and plasma pharmacokinetics in mice have suggested that the systemic duration of GM in vivo is significantly greater than either macbecin II or herbimycin A. Therefore, with the objective of evaluating the suitability of GM as a candidate for clinical development as an antineoplastic agent, the studies presently described were initiated to characterize the disposition of GM in the mouse and beagle dog. Efforts to identify the principal acute toxicities in the dog were concurrently undertaken.

Materials and methods

In vitro assessment of antitumor activity

NCI anticancer drug screen

The current NCI anticancer drug screen provides an in vitro evaluation of antitumor activity [2, 8]. Briefly, agents are tested over a broad concentration range against 60 human tumor cell lines. The tumor cells are inoculated into a standard 96-well microtiter plate and incubated for 24 h prior to addition of the test compound at five ten-fold dilutions starting with the highest soluble concentration in dimethyl sulfoxide (DMSO). Following 48 h of exposure to the test agent, the cells are fixed in situ with trichloroacetic acid, washed and dried. Cellular protein is then quantified spectrophotometrically upon addition of sulphorhodamine B using automated reading devices.

NCI prostate cancer subpanel assay

Compounds exhibiting demonstrable activity in the NCI in vitro anticancer drug screen are referred for evaluation against cancer cell strains derived from primary prostatic carcinoma [26]. The techniques employed to cultivate and characterize the prostate tumor

[1] Minutes of the Decision Network Committee, 20 April, 1992, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD 20892, pp 4–12.Copies of this public document can be obtained by written request to the Associate Director, DTP, DCT, NCI

cells have been described in detail [25]. In the subpanel assay, agents are tested against three different cell strains, selected to represent a range of Gleason grades, in a manner analogous to the primary NCI screen, except that the exposure period is extended to 6 days. Those compounds demonstrating 50% growth inhibition (GI_{50}) at concentrations less than 1 n*M* are considered of sufficient interest for in vivo evaluation against human prostate tumor models in mice.

Drug disposition studies

Drug formulation

GM (NSC 122750) was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, NCI (Bethesda, Md.). Purity was verified by reversed-phase gradient elution HPLC with diode array UV/VIS detection and high-resolution mass spectrometry. Although quite stable in the solid state, GM is subject to photolytic degradation in aqueous as well as in nonaqueous solutions. Amber glass and plastic did not provide adequate protection from prolonged exposure to normal laboratory lighting. Therefore, the flasks, vials and delivery devices used to contain solutions of GM were routinely wrapped in aluminum foil. In addition, procedures that involved handling drug solutions, including biological specimens, were performed in rooms illuminated with indirect, dim lighting.

Unfavorable solubility properties necessitated formulating GM as a solution in neat DMSO (Sigma, St. Louis, Mo.) such that the intended dose was delivered in volumes of 0.5 µl/g and 86–172 µl/kg body weight for the mouse and dog studies, respectively. These doses of DMSO have been shown to be well tolerated by both species when administered by rapid i.v. injection [28, 37].Solution densities were estimated by weighing three 10- µl aliquots, measured with a Hamilton syringe (Reno, Nev.), on a Cahn C-31 microbalance (Cahn Instruments, Cerritos, Calif.). The concentration of GM in each dosing solution was ascertained by HPLC analysis. The solutions were stored at − 20°C when not in use.

Mice

Unfasted male Harlan $CD2F_1$ mice, weighing 25.4 ± 2.4 g (mean ± SD, $n = 84$), were treated with 8.7 and 20.6 mg/kg of GM by 0.5 min tail-vein injection using a 26 gauge, 3/8 inch Precision Glide Needle (Becton Dickinson, Rutherford, N.J.) without anesthesia. The animals were given free access to food and water. At 15 time intervals, ranging from 3 min to 4 h postinjection, three mice were anesthetized with methoxyfluorane and bled by retroorbital puncture using heparinized Pasteur pipets. A 25 mg/kg dose of GM was also administered by rapid intragastric injection to a group of 12 mice (19.2 ± 1.5 g) using a 22 gauge, 1 inch Animal Intubation Needle with a 1.25 mm ball diameter (Popper & Sons, New Hyde Park, N.Y.). They were similarly bled at 15, 30, 60 and 120 min after dosing. Each blood specimen was centrifuged (12000, *g*, 2 min, 25°C) immediately after collection. Plasma was rapidly separated from the pelleted blood cells, placed in an amber polypropylene microcentrifuge tube, flash frozen and stored at -20°C until assayed.

Dogs

A single male beagle dog (Hazelton Research Products, Kalamazoo, Mich.) was treated with two doses of GM (2.0 and 4.2 mg/kg) separated by an 8-week recovery period at the Frederick Research Center/Southern Research Institute (Frederick, Md.). The dog was fully acclimated and given a complete physical examination prior to approval for use by an attending veterinarian. The age of the animal was 10 months during the first study. It was fasted the day prior to dosing and weighed 8.7 and 8.9 kg on the treatment day of the first and second studies, respectively.

The dog was lightly sedated by i.v. injection with 2.5% thiamylal (Bio-Ceutic, St. Joseph, Mo.) 1–2 h prior to the administration of GM. A percutaneous 17 gauge IV Intrafusor with an 18 gauge 11.4 cm catheter (Sorenson Research, Salt Lake City, Utah) was placed into the left saphenous vein for introduction of the dosing solution. A 17 gauge CVP Intrafusor with an 18 gauge 53.3 cm catheter (Sorenson Research) was inserted percutaneously via the right saphenous vein for blood sampling. Sterile 0.9% sodium chloride (Abbott Laboratories, Chicago, Ill.), used throughout as a catheter flushing solution, was delivered at ~10 ml/h using a Cormed ML6-8 infusion pump (Dakmed, Buffalo, N.Y.) to maintain patency of the venous catheters. After inserting a Buster 8F urinary catheter, 2 × 500 mm (A.J. Buck & Sons, Owings Mills, Md.), the dog was placed in a sling (Alice King Chatham Medical Arts, Los Angeles, Calif.) and permitted to recover from the anesthetic.

Shortly before dosing, two 10–ml aliquots of blood were collected and centrifuged to provide pretreatment plasma. In addition, the urinary bladder was flushed with saline and the contents drained via the catheter. The dosing solution was then manually delivered from a weighed syringe into the venous catheter using a three-way stopcock over an intended period of 60 s. The syringe was removed, capped and weighed to determine the amount injected. Residual dosing solution in the stopcock and catheter was passed into the vein by injecting 4–5 ml of pretreatment plasma. Pharmacokinetic blood specimens (1.5 ml) were drawn into heparin-treated syringes at 23 time intervals during 8 h postinjection and processed as described above. The urinary bladder was drained every 2 h, or more frequently as required, and flushed with saline (3 ml) until colorless. The volume and pH of the urine and combined volume of the recovered bladder flushes were measured. The specimens were placed in polypropylene tubes, flash frozen and maintained at − 20°C until assayed.

Blood samples for clinical pathology evaluations were acquired on two or three different days before drug administration, which included a specimen drawn just prior to dosing, and at 4, 8, 12, 24, 48 and 96 h postinjection. Additional samples were obtained as necessary until all parameters approached pretreatment values. Tests performed included a standard hematology profile with differentials, reticulocyte and platelet counts, prothrombin time, and a standard serum chemistry profile plus creatine phosphokinase (Veterinary Diagnostic Services, Maryland Medical Laboratory, Baltimore, Md.).

Data analysis

Actual doses were calculated from the known volume of solution delivered and the assayed drug concentration.The beginning and ending times of the drug input and sample collection intervals were monitored using a digital timer and recorded to the nearest second. Time points were calculated as the difference between the midpoint of the blood collection interval and starting time of dose administration. For the disposition studies in mice, the geometric mean plasma concentration was calculated from the observed concentrations in three animals at each time point. Plasma concentration-time profiles were pharmacokinetically analyzed according to model-independent methods [7]. Conventions recommended by Rowland and Tucker for symbols of pharmacokinetic terms were adopted [29] (see Abbreviations). Thus, the appropriate equation having the general form [1]

$$C = \sum_{i=1}^{n} C_i (\lambda_i \tau)^{-1} (e^{-\lambda_i t'} - e^{-\lambda_i t})$$

was fitted to the observed time courses of the GM plasma concentration by nonlinear least squares regression using PCNONLIN (Statistical Consultants, Lexington, Ken.). The value of t' is zero until

the infusion of duration τ has terminated, upon which it becomes defined as

$$t' = t - \tau$$

where t denotes the time from the initiation of treatment. The coefficients C_i are intercept values, corresponding to i.v. bolus administration of the dose, of each log-linear phase with slope $-\lambda_i$, such that $\lambda_1 > \lambda_2 > \ldots > \lambda_z$. Parameters corresponding to the terminal decay phase are designated with a subscript z by convention. Each plasma profile was subjected to repeated regression analyses whereby the number of exponential terms in the fitted equation and the influence of the weighting factor, $y_{obs}^{-n} (0 \leq n \leq 2)$, were both evaluated to identify the simplest equation that best described the data. Parameter values of the best-fit equation were used to calculate all pharmacokinetic terms.

HPLC analysis

Chemicals

All reagents and chemicals were obtained from commercial sources in grades appropriate for direct use. Distilled water was deionized and stripped of dissolved organics by passage through mixed-bed resins and activated carbon (Hydro Water Systems, Rockville, Md.).

Analytical solutions

Glassware was deactivated by treatment with a 2% (v/v) solution of SurfaSil (Pierce Chemical, Rockford, Ill.) in hexane and oven dried. Stock solutions of the drug and internal standard (IS), *N*-phenyl-1-naphthylamine, were prepared in DMSO at concentrations of 1.0 mg/ml. A series of ten working solutions of GM, ranging in concentration from 5 to 500 μg/ml, were made by diluting various volumes of the stock solution to 1.0 ml with DMSO. These solutions were stored at 5°C. Standard solutions were prepared in amber polypropylene microcentrifuge tubes by thoroughly mixing each working solution of GM (3 μl) with drug-free plasma or urine (300 μl) to provide concentrations of 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, 2.0, 3.0, 4.0 and 5.0 μg/ml. They were stored at −20°C and used for no longer than 1 week. The IS solution used in the assay (0.70 μg/ml) was made daily by adding 14 μl of the IS stock solution to acetonitrile (20 ml).

Sample preparation

Frozen specimens were thawed in a refrigerator and thoroughly mixed prior to analysis. Plasma or urine samples (50 μl) were pipetted into an amber polypropylene microcentrifuge tube, thoroughly mixed with the IS solution (150 μl) by vortexing for 15 s, and then centrifuged for 2 min at 12 000 *g*. The clear supernatant (165 μl) was separated from the pellet and diluted with an equivalent volume of ammonium acetate buffer (0.05 *M*, pH 4.7) before injecting a 250-μl aliquot into the chromatograph.

Chromatography

HPLC was performed using an HP 1050 Series isocratic pump (Hewlett-Packard, Palo Alto, Calif.) and a Bio-Rad AS-100T autosampler equipped with a 500-μl sample loop. Light was excluded from the sample compartment of the autosampler with aluminum foil. Separations were conducted at ambient temperature under isocratic, reversed-phase conditions on a 3.9 mm × 15 cm stainless steel column packed with 4 μm Nova-Pak C_{18} (Millipore, Milford, Mass.). The analytical column was protected by a 0.5 μm postinjector filter and a 1.5 cm Brownlee RP-18 precolumn (Rainin Instrument Co., Woburn, Mass.). The precolumn cartridge was routinely replaced after approximately 60 specimens had been analyzed. A mobile phase composed of methanol/0.05 *M* ammonium acetate buffer, pH 4.7 (67:33, v/v) was employed at a flow rate of 1.0 ml/min. The solution was degassed in an ultrasonic bath for 15 min before use. UV absorbance of the column effluent was monitored at 304 nm (8 nm bandwidth) using an HP 1050 Series Variable Wavelength Detector fitted with an 8 μl flow cell (10 mm pathlength). The 1 V output of the detector was provided as the signal to an HP 3396 Series II integrator, configured to report peak area using a 0.2 min peak width, a threshold setting of 1, and baseline construction through each detected valley point.

Quantitation

Standard curves were constructed by plotting the peak area ratio of GM to the IS against the concentration of drug. Linear least squares regression was performed using a weighting factor of y_{obs}^{-2}, without inclusion of the origin, to determine the slope, y-intercept and correlation coefficient of the best-fit line. The analyte concentrations in unknown samples were calculated using the results of the corresponding regression analysis. Specimens with an analyte concentration exceeding the standard curve were reassayed upon appropriate dilution with drug-free plasma or urine. All samples were initially assayed in duplicate. Additional analyses were performed if the two determinations deviated from their average by more than 10%.

Assay validation

Absolute recovery of the drug from plasma was determined by comparing its peak area in chromatograms of plasma standards prepared for analysis to plasma-free solutions that contained the drug at concentrations corresponding to quantitative extraction. Precision, accuracy and reproducibility of the analytical method were evaluated by analyzing the predicted concentrations and regression parameters of ten independently prepared and assayed standard curves of GM in plasma. The coefficients of variation of the mean predicted concentration for the plasma standards provided a measure of precision. Relative recoveries of GM were calculated by comparing the mean predicted concentration to the known concentration in the plasma standards.

Results

Activity against human tumor cells in vitro

The in vitro activity of GM in the NCI anticancer drug screen was evaluated at concentrations ranging from 0.01 to 100 μ*M* in ten independent tests during a 20-month period. As illustrated in Fig. 2, following 48 h of exposure to drug concentrations near the mean GI_{50}, 0.18 μ*M*, there was a differential pattern of growth inhibition against the panel of 60 human tumor cell lines. In contrast, GM produced substantial cytotoxicity in most of the cell lines when present at a concentration of 10 μ*M* (mean LC_{50}, 21 μ*M*).

While the cell lines the prostate cancer panel (i.e. PC3 and DU-145) of the in vitro screen were no more

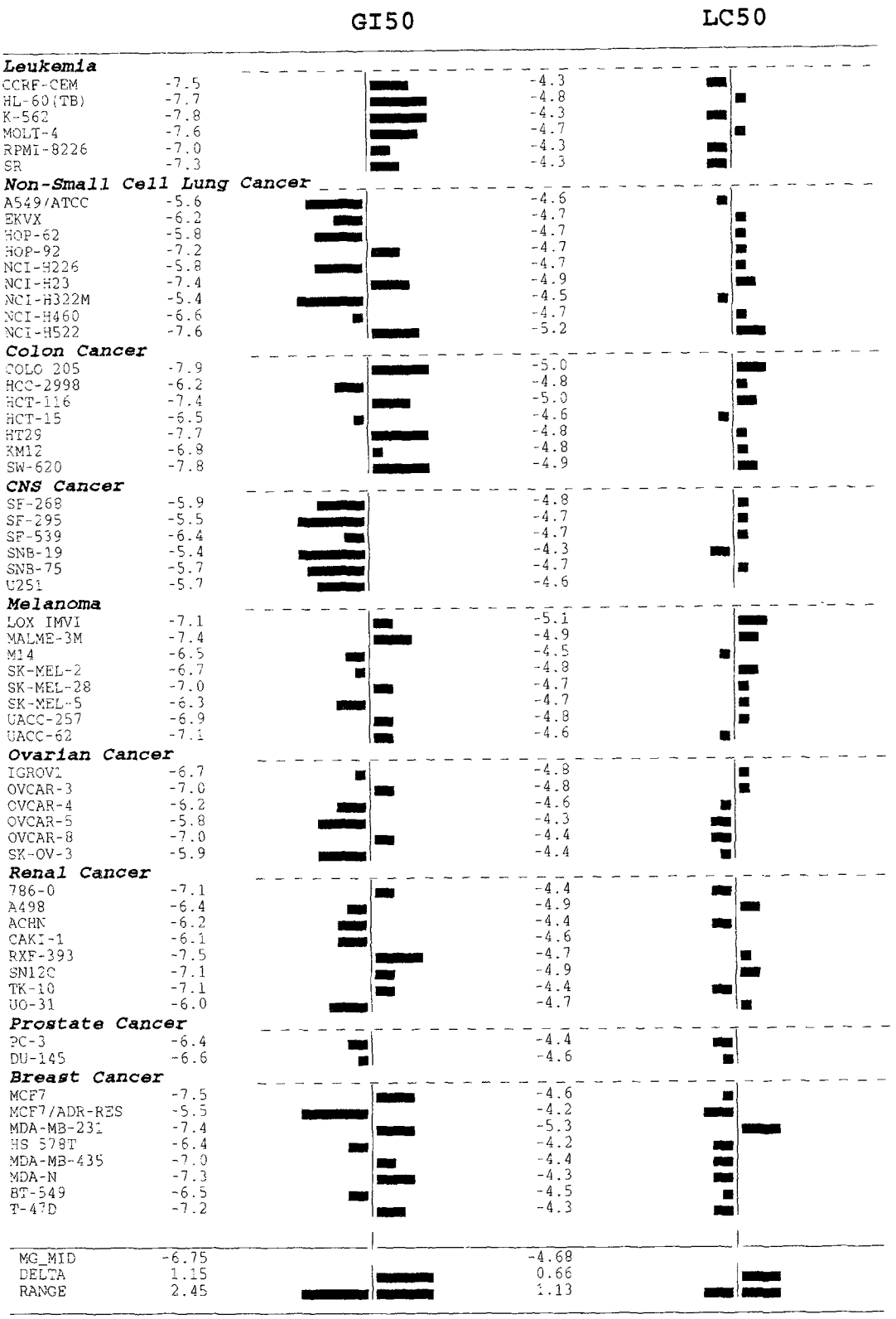


Fig. 2 Graphic representation of GM activity against the human tumor cell lines which comprise the NCI in vitro anticancer screen observed during multiple experiments. The vertical lines (MG_MID) represent the mean values of the logarithmic drug concentrations effecting 50% growth inhibition (mean GI_{50}, 0.18 μM) and 50% cytotoxicity (mean LC_{50}, 21 μM) for all 60 cell lines. The individual response of each cell line to GM is shown as a bar extending either to the right or to the left, depending on whether the cell line was more or less sensitive, respectively, than the average response. The length of each bar is proportional to relative sensitivity as compared to the mean value. It is clear that, relative to the average response of all cell lines, individual cell lines exhibit a greater difference in sensitivity to GM at concentrations near the mean GI_{50} than the mean LC_{50}

sensitive to the growth inhibitory effects of GM than the other cell lines, interest in the potential of this compound for chemotherapy of prostate cancer had been generated by data presented to the Decision Network Committee of the NCI's Division of Cancer Treatment[1]. Therefore, the drug was further evaluated against the NCI prostate subpanel assay, in which primary tumor cells obtained from patients are exposed to the test agent for 6 days. The GI_{50} of GM in this assay was 0.1 n*M*, thus confirming its in vitro potency against human prostate cancer.

Analytical method

No methods to quantitate any of the benzoquinoid ansamycins, suitable for use in pharmacokinetic studies, have previously been reported. GM is a relatively large (M_r 560.65) lipophilic molecule with a UV spectrum featuring a prominent absorption band at 304 nm ($\varepsilon = 19\,300\ M^{-1}cm^{-1}$) due to the benzoquinone chromophore [3, 31]. These physical characteristics were employed in the development of an assay for GM in biological fluids utilizing reversed-phase HPLC with UV detection. A sample size of 50 µl was selected to permit replicate determinations of drug concentration in plasma specimens acquired from individual mice. The removal of endogenous macromolecules from the sample matrix by precipitation induced upon mixing with three volumes of acetonitrile proved to be a satisfactory and convenient method for sample preparation. The drug was quantitatively recovered in the supernatant resulting from centrifugation of the mixture.

HPLC chromatograms of plasma specimens obtained from mice before and at selected times after 8.7 mg/kg of GM was administered by rapid (0.5 min) i.v. injection are shown in Fig. 3. Under the chromatographic conditions employed, GM and the IS typically eluted within 6.6–7.9 and 9.8–11.8 min, respectively. There were no endogenous components that interfered with the detection of GM or the IS. Aside from several minor components eluting prior to GM, chromatographic peaks consistent with drug metabolites were not observed. Considerations for selecting *N*-phenyl-1-naphthylamine as the IS included its UV absorption, chromatographic retention, recovery and chemical stability. It served primarily to monitor the reproducibility of pipetting and the performance of the automatic sampler.

Samples prepared for analysis showed no evidence of GM or IS degradation during 18 h at ambient temperature, facilitating overnight analysis with an autosampler. The lower limit of quantitation of the method, defined as the minimum concentration of GM in a 50-µl plasma sample amenable to analysis with a CV not exceeding 10%, was found to be 50 ng/ml. The chromatographic peak area ratio GM:IS was directly proportional to drug concentration for plasma standards ranging from 0.05 to 5.0 µg/ml. For ten standard curves of GM in plasma run during an 11 month-period, the mean correlation coefficient was 0.998 ± 0.002 (SD), the mean intercept value (0.012 ± 0.013) did not differ significantly from the origin and the CV of the mean slope (0.647) was 9.3%. The relative recovery of GM, compiled from the back-calculated concentrations of these standard curves, was independent of the drug concentration, exhibiting mean values that ranged from 97.9% to 105.4% (CV = 4.0–7.9%). In addition, GM was efficiently isolated from plasma throughout the concentration range of the standard curve. Its absolute recovery ranged from 98.4 ± 4.7% in a 0.1 µg/ml sample to 102.8 ± 10.0% at 5.0 µg/ml (mean ± SD, $n = 4$ or 5). The analytical method was therefore considered to be accurate and reproducible.

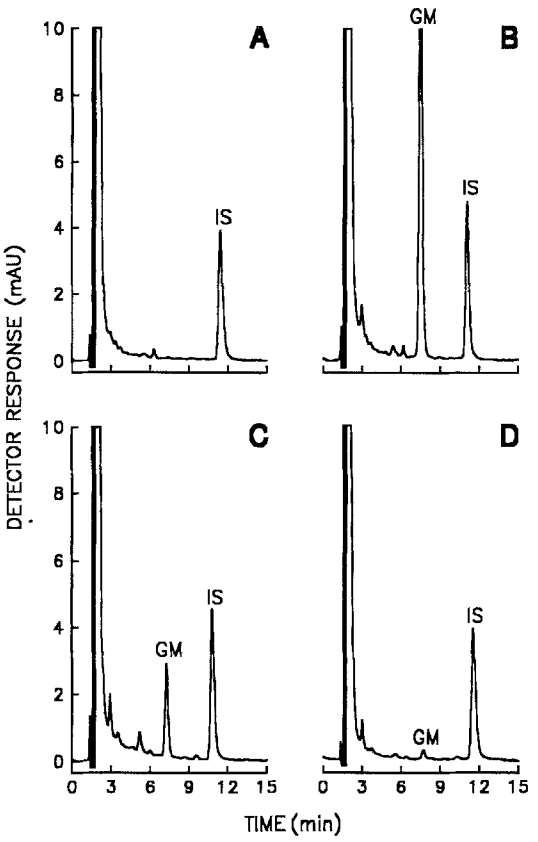


Fig. 3A-D Liquid chromatograms of plasma specimens obtained from mice before (**A**) and at 4.8 min (**B**), 45.1 min (**C**) and 2.0 h (**D**) after 8.7 mg/kg of geldanamycin was administered by rapid i.v. injection. The 5-min sample was assayed upon five fold dilution with drug-free plasma. The plasma concentrations of geldanamycin were 25.0 µg/ml at 5 min, 0.79 µg/ml at 45 min and 0.14 µg/ml at 2 h. Chromatographic peaks: *GM* geldanamycin; *IS* internal standard (*N*-phenyl-1-naphthylamine)

Plasma pharmacokinetics in mice

Acute toxicity resulting in rapid death (< 1 min) was evident in all mice ($n = 4$) treated by i.v. injection with

50 mg/kg of GM. While 25 mg/kg also proved to be toxic, as indicated by the occurrence of minor convulsions for several minutes after dosing, animals given doses of 20 mg/kg and lower showed no evidence of toxicity. Accordingly, pharmacokinetic studies were conducted at dose levels of 20.6 and 8.7 mg/kg administered by rapid i.v. injection. The sensitivity of the analytical method did not permit adequate definition of the plasma profile at lower doses.

Similar initial plasma concentrations of the drug (82 μg/ml) were observed after treatment with either dose and the levels remained above 0.1 μg/ml for 3–4 h postinjection. Notwithstanding, both of the plasma concentration–time profiles were distinctly triexponential (Fig. 4A, B), with comparable values of the concentration-independent pharmacokinetic parameters (Table 1), indicative of linear drug disposition. Although the two initial disposition phases were very rapid, with harmonic mean half-lives of 1.6 and 10.8 min, respectively, their combined contribution to the AUC was 89.4%. Thus, the MRT of GM (20.7 min) was much shorter than its $t_{1/2,z}$ (77.7 min) and the CL was correspondingly rapid (30.5 ml/min per kg). The low values of V_1 (0.16 l/kg) and V_{ss} (0.68 l/kg) exhibited by this lipophilic compound suggest that it has a much greater affinity for binding to plasma protein than for interaction with peripheral tissue. Quantifiable plasma levels of GM (> 0.05 μg/ml) were not achieved in mice 0.5–2 h after they had received an oral dose of 25 mg/kg.

Drug disposition and toxicity in the beagle dog

In the male beagle dog treated with 2.0 mg/kg of GM, i.v. given over 1.2 min, plasma levels of the drug decreased in a distinctly biexponential manner from 2.1 μg/ml to less than 0.1 μg/ml within 90 min (Fig. 4C). A 4.2 mg/kg dose given as a 1.8 min i.v. injection to the same dog 8 weeks later also provided a biexponential plasma profile. The concentration of drug achieved at the end of the injection was 4.6 μg/ml and plasma levels exceeded 0.1 μg/ml for ~3 h. Values of the pharmacokinetic parameters determined by nonlinear regression analysis of the GM plasma profiles for the two doses were very similar (Table 1). The harmonic mean half-lives for the initial and terminal disposition phases, which contributed equally to the AUC on average, were 6.3 and 57.9 min, respectively. The MRT of GM in the dog was 46.6 min, with a mean CL of 49.4 ml/min per kg, and values of 0.86 l/kg for V_1 and 2.31 l/kg for V_{ss}. The cumulative amount of unchanged drug eliminated by urinary excretion during 8 h after treatment accounted for 6.4% of the 2.0 mg/kg dose and 10.7% of the 4.2 mg/kg dose.

The dog presented only a few adverse clinical signs of relatively minor severity. During the first several hours after receiving each dose, the animal exhibited periodic

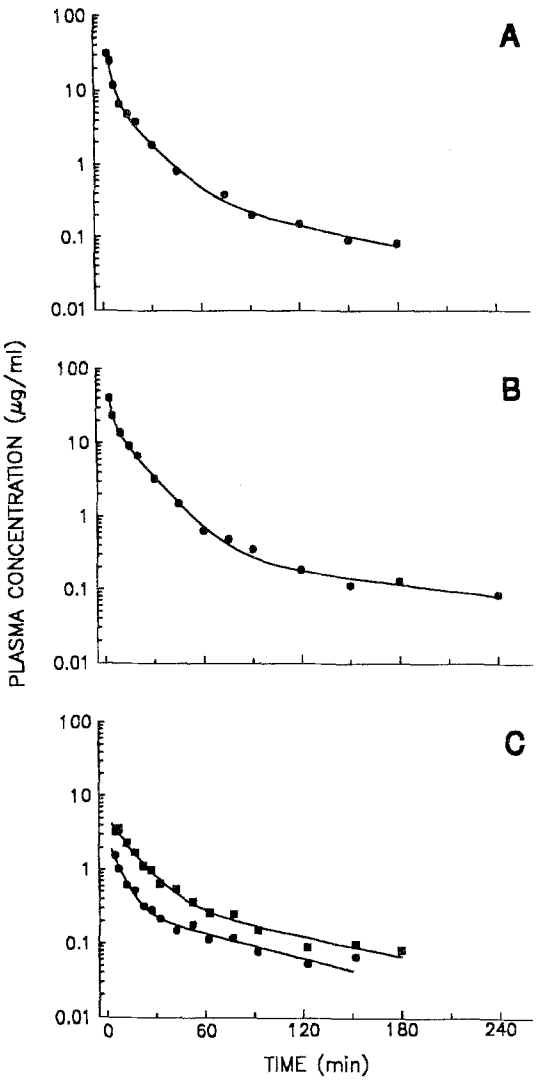


Fig. 4A-C Plasma concentration–time profiles of geldanamycin in mice and dogs. Mice were treated by 0.5 min i.v. injection with doses of (**A**) 8.7 and (**B**) 20.6 mg/kg. In these profiles, the data points are geometric means of the observed plasma levels in at least three mice per time point. (**C**) The drug was administered to a single beagle dog by 1.2–1.8 min i.v. injection at doses of 2.0 mg/kg (●) and 4.2 mg/kg (■). The curves are the best-fit of the experimental data as determined by nonlinear regression

restless behavior and its mucous membranes (gingivae) became pale. The animal vomited the day after treatment with the 4.2 mg/kg dose of GM and experienced some loss of appetite for several days. In both studies, recurrent emesis, together with diarrhea and loose stools, were noted 4–6 days after dosing. Clinical pathology results showed significant changes in several hematological and serum chemistry parameters (Table 2). The WBC count became elevated with corresponding increases in banded neutrophils and reticulocytes 1–7 days postinjection. Beginning the day after each treatment, hepatic enzyme levels in serum increased profoundly and remained above normal for 2 weeks or longer. Peak enzyme levels occurred 4–7 days postinjection coincident with the delayed gastrointestinal

Table 1. Pharmacokinetic and derived parameters of geldanamycin in the mouse and dog. $C(\tau)$, plasma concentration of drug at the end of infusion, C_i y-intercept of the ith disposition phase; $t_{1/2,i}$ half-life of the ith disposition phase, AUC_i contribution of the ith disposition phase to AUC, $f_{ex,ur}$(0–8 h) fraction of the dose excreted in the urine between 0 and 8 h after dosing, *ND* not determined.

	Mouse			Dog			
Parameter	8.7 mg/kg	20.6 mg/kg	Mean[a]	2.0 mg/kg	4.2 mg/kg	Mean[a]	Units
$C(\tau)$	81.7	82.2		2.1	4.6		μg/ml
C_1	79.3	68.0		2.1	4.4		μg/ml
C_2	10.6	21.3					μg/ml
C_z	0.5	0.4		0.3	0.4		μg/ml
$t_{1/2,1}$	1.6	1.5	1.6	4.7	9.3	6.3	min
$t_{1/2,1}$	10.7	10.8	10.8				min
$t_{1/2,1}$	63.7	99.7	77.7	52.3	64.8	57.9	min
MRT	17.8	24.7	20.7	48.2	45.2	46.6	min
CL	22.1	38.8	30.5	55.5	43.2	49.4	$ml/min\,kg^{-1}$
V_1	0.096	0.23	0.16	0.84	0.87	0.86	$l \cdot kg^{-1}$
V_{ss}	0.39	0.96	0.68	2.68	1.95	2.31	$l \cdot kg^{-1}$
V_z	2.03	5.58	3.81	4.19	4.04	4.11	$l \cdot kg^{-1}$
AUC	392.2	530.6		35.9	97.1		$\mu g \cdot min \cdot ml^{-1}$
AUC_1	47.2	27.6	37.4	39.7	60.2	50.0	%
AUC_2	41.6	62.4	52.0				
AUC_z	11.2	10.0	10.6	60.3	39.8	50.0	%
$f_{ex,ur}$(0–8 h)	ND	ND		6.4	10.7	8.6	% dose

[a] Harmonic mean half-lives and mean residence time

Table 2. Changes in hematological and serum chemistry parameters following the administration of geldanamycin to a beagle dog by rapid i.v. injection. Baseline values are the mean ± SD in specimens acquired on 2 (4.2 mg/kg) or 3 (2.0 mg/kg) separate days before dosing

	Dose = 2.0 mg/kg		Dose = 4.2 mg/kg		
Parameter	Baseline	Peak (days)[a]	Baseline	Peak (days)[a]	Units
White blood cells	10.3 ± 2.1	22.4 (2)	7.9 ± 2.4	24.8 (2)	$10^3/\mu l$
Banded neutrophils	0.3 ± 0.6	8 (4)	0 ± 0	7 (6)	%
Reticulocytes	0.7 ± 0.4	2.4 (4)	0.4 ± 0.1	5.2 (6)	%
SGPT	42 ± 3	1498 (4)	53 ± 8	2440 (6)	U/l
SGOT	39 ± 5	1239 (7)	34 ± 0	1660 (4)	U/l
Alkaline phosphatase	69 ± 7	1442 (7)	110 ± 30	1724 (6)	U/l
LDH	94 ± 40	204 (4)	53 ± 13	272 (4)	U/l
CPK	215 ± 22	3557 (0.5)	197 ± 94	1085 (0.5)	U/l
Blood urea nitrogen	15 ± 1	22 (4)	17 ± 2	27 (0.5)	mg/dl
BUN/creatinine	18.8 ± 1.3	28.3 (1)	19.6 ± 4.1	30.0 (0.5)	

[a] Time following drug administration

indications described above. The maximum serum glutamic-oxaloacetic transaminase (SGOT) and glutamic-pyruvic transaminase (SGPT) activities ranged from 32–49 times pretreatment values and the peak alkaline phosphatase level was 16–21 times normal. In addition, serum creatine phosphokinase (CPK) activity became rapidly elevated, with a peak value 6–17 times greater than baseline at 12 h, but returned to normal within 4–6 days. Elevations in serum lactic dehydrogenase (LDH) activity and blood urea nitrogen, although less dramatic, were also evident.

Discussion

The antiproliferative effects of the benzoquinoid ansamycins against human neoplasms in vitro are exerted through several distinct mechanisms of action characterized by cytostasis or cytotoxicity. Cytostatic growth inhibitory effects appear to be exclusively directed against malignancies associated with elevated tyrosine kinase activity by promoting differentiation, as in the case of K562 chronic myelogenous leukemia cells, or by inducing cytostasis without morphological change, as observed with human colon tumor cells [5, 6, 11]. Constant drug exposure is required to maintain cytostasis. Its removal abrogates growth inhibition and cells revert to neoplastic morphology [6, 11, 13, 33]. The ansamycins are also cytotoxic toward poorly differentiated human neuronal and melanoma tumor cell lines in vitro [36]. The mechanism of cytotoxicity is apparently unrelated to modulation of tyrosine kinase activity and requires only a relatively brief period of exposure to the agent. Thus, depending on the type of malignancy, dosing regimens that provide either

constant systemic levels of the drug or intermittent exposure may be therapeutically effective.

The in vitro evaluation of antitumor activity clearly shows that GM is a potent agent inducing both growth inhibition and cytotoxicity. While there may be a differential effect of this agent on various human tumor cell lines at lower concentrations (i.e. about 0.1 μM), GM produced substantial cytotoxicity at higher concentrations in the NCI's anticancer screen, in which cells are exposed to the test agent for 48 h. Cell strains cultivated from primary human prostatic tumors were markedly sensitive to GM (GI_{50}, 0.1 nM) following 6 days of exposure to the agent in vitro. Despite the in vitro evidence for tumor cell selectivity reported by others[1], our studies demonstrated that GM is a potent, broad spectrum inhibitor of both tumor cell growth and viability. As such, the necessity to evaluate the therapeutic index of GM in vivo was indicated, thereby providing the basis for the pharmacologic and limited toxicological studies presented in this report.

In the present investigation we showed that concentrations of GM well above 0.1 μg/ml, which was effective against most of the responsive neoplastic cell lines in vitro [33, 36], were achieved in the plasma of mice and a dog following administration of the drug by rapid i.v. injection. However, the systemic duration of the drug was relatively short. Plasma levels decayed below 0.1 μg/ml within 3–4 h after treatment with the apparent maximum tolerated dose, which was approximately 20 mg/kg for mice and 4 mg/kg for the dog. Pharmacokinetic analysis of the GM plasma concentration–time profiles revealed that drug disposition, although apparently linear within the dose ranges studied, was nevertheless quite different in the two species. Whereas the mean biological half-life of GM was slightly longer in mice (77.7 min) than in the dog (57.9 min), its MRT in the dog (46.6 min) was more than twofold greater than that observed in the mice (20.7 min). This apparent anomaly arose from differences in the relative contribution of the terminal disposition phase to overall drug disposition. While the terminal phase comprised 50% of the AUC for the dog, the two initial disposition phases of GM in the mice, which had mean half-lives of 1.6 and 10.8 min, accounted for 89.4% of the AUC. Furthermore, despite having a significantly longer MRT in the dog, the drug was cleared from plasma much faster by the dog (49.4 ml/min per kg) than by the mice (30.5 ml/min per kg). This disparity may also be attributed to the influence of the quantitatively minor terminal disposition phase of GM in mice on the magnitude of its CL. Considering that CL is the product of V_z and λ_z, the longer terminal disposition phase of GM in mice results in a lower apparent CL, since the values of V_z were similar in the mice (3.81 ml/min per kg) and dog (4.11 ml/min per kg). Interspecies differences in plasma protein binding relative to tissue distribution may also contribute to the observed differences in the pharmacokinetic behavior of GM. This may be inferred from the values of V_1 and V_{ss}, which were both considerably larger in the dog, suggesting that the extent of drug distribution into peripheral tissue regions may have been greater in the dog than in the mice.

GM clearly produced dose-related toxic effects in the dog, as indicated by profound elevations in serum enzyme levels, altered hematological parameters and clinical observations. The liver appeared to be the principal target organ of acute drug toxicity. Elevations in SGPT, SGOT and serum alkaline phosphatase activities characteristic of acute hepatic necrosis were observed 4–7 days after both doses of the drug were given [38]. Histological verification of hepatotoxicity was not ascertained because the condition of the animal did not warrant sacrifice or biopsy. It is recognized that alternate pathological effects cannot be definitively excluded on the basis of serial measurements of serum enzyme levels alone. Varying amounts of the transaminases, alkaline phosphatase and LDH are released into the bloodstream during damage to tissues other than the liver, principally the myocardium, kidney, intestine, bone and skeletal muscle. Nevertheless, it is very unlikely that peak SGPT levels exceeding 1000 U/l could result from tissue damage to any organ other than the liver. However, the transient elevation of serum CPK activity shortly after the injection of GM cannot be attributed to hepatotoxicity, since the liver does not contain significant concentrations of the enzyme. Although cellular injuries to the heart and brain release CPK into the bloodstream, muscular trauma associated with the catheterization procedures performed shortly before administration of the drug is considered to be a more probable cause. Additional drug-related effects were limited to symptoms of minor gastrointestinal aggravation and alterations in serum chemistry parameters consistent with less severe nephrotoxicity. Changes in hematological parameters, comprised primarily of increases in total leukocytes, banded neutrophils and reticulocytes, could be a consequence of either drug-induced tissue damage or stress-induced leukemoid reactions. All clinical parameters returned to baselines values within 1 month indicating that the pathological effects resulting from acute exposure to GM are probably reversible. These observations suggest that future toxicological evaluations of GM should include analysis of LDH isoenzymes, serum 5′-nucleotidase activity, liver biopsy and tests for cardiovascular toxicity.

Previous studies of benzoquinoid ansamycin toxicity have been limited to determinations of median lethal doses in rodents [3, 15, 20, 22]. However, the toxicological effects of rifampin, a semisynthetic ansamycin with a naphthalenic hydroquinone function used clinically in the treatment of tuberculosis, has been thoroughly investigated [9]. While rifampin causes little direct

hepatotoxicity in patients with normal liver function, at least when given alone, it induces more serious and frequent side effects in dogs than seen in humans or other species [4]. Elevated hepatic enzyme activities in serum are the predominant manifestations of toxicity. Severe reactions are indicated by vomiting, diarrhea and even death.

The symptoms and clinical indications of GM toxicity in the dog are notably similar to those associated with acute acetaminophen poisoning [14]. Consideration of the mechanism of acetaminophen hepatotoxicity and chemical reactivity of the benzoquinone moiety of GM strongly suggests that these similarities are more than coincidental. Oxidative metabolism of acetaminophen yields an electrophilic quinoneimine which, at therapeutic doses, is probably detoxified by reacting with hepatic glutathione. Sufficiently large doses of acetaminophen diminish glutathione levels to the extent that essential hepatic macromolecules become subject to arylation by the metabolite which leads to the formation of lesions and necrosis [18, 19]. The reactivity of the quinone moiety of herbimycin A with nucleophiles [23, 30, 35], including sulfhydryl groups present in protein [5], implies that an analogous mechanism may be operative in the apparent hepatotoxicity of GM.

The potential for acute hepatotoxic reactions to GM, as well as to the other three benzoquinoid ansamycins based upon structural analogy, indicates that additional pharmacological and therapeutic information is required to ascertain whether any of these compounds are viable candidates for clinical development. Presently, we are attempting to examine the influence of the pattern and duration of systemic drug exposure on toxicity in the dog. Through these studies, we hope to determine whether the relatively brief initial period of high plasma levels of GM associated with administration by rapid i.v. injection is primarily responsible for its toxicity. If this is indeed the case, toxicity could be minimized by employing drug delivery techniques that avoid peak plasma concentrations that may not be necessary for therapeutic effects. The results of these investigations, and analogous studies of this agent in other animal species, will be reported in the future communications.

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