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ORIGINAL ARTICLE

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Preclinical toxicity of a geldanamycin analog, 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG), in rats and dogs: potential clinical relevance

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Abstract *Purpose*: 17-DMAG is a hydrophilic derivative of the molecular chaperone inhibitor 17-(allylamino)-17-demethoxygeldanamycin (17-AAG; NSC-330507), which is currently being evaluated for the treatment of cancer in clinical trials. 17-DMAG offers a potential advantage over 17-AAG because its aqueous solubility eliminates the need for complicated formulations that are currently used for administration of 17-AAG. In addition, 17-DMAG undergoes only limited metabolism compared to 17-AAG. The present results are from preclinical toxicity studies evaluating 17-DMAG in rats and dogs. *Methods*: Doses of 0, 2.4, 12 and 24 mg/m^2 per day were administered to rats, while dogs received doses of 0, 8 or 16 mg/m^2 per day. In both species, 17-DMAG was administered i.v. (slow bolus for rats; 1-h infusion for dogs) daily for 5 days. An additional cohort of dogs received 16 mg/m^2 per day orally for 5 days. Clinical observations were noted, and standard hematology and clinical chemistry parameters were monitored. Selected tissues were evaluated microscopically for drug-related lesions. Tissue and plasma 17-DMAG concentrations were measured by HPLC/MS at selected time-points on days 1 and 5. *Results*: Daily i.v. administration of 17-DMAG at doses of 24 mg/m^2 per day in rats or 16 mg/m^2 per day in dogs produced lethality on day 6, approximately 24 h following the last dose. Body weight loss was common in rats and dogs. Drug-related gastrointestinal, bone marrow and hepatic toxicities were also common in rats and dogs. Dogs also exhibited signs of renal and gallbladder toxicity. Plasma concentrations of 17-DMAG increased proportionately with dose in rats and disproportionately with dose in dogs. In rat tissues, however, only fourfold to sixfold increases in 17-DMAG concentrations were observed with a tenfold increase in dose. The highest concentrations of 17-DMAG were found in the liver of rats, with progressively lower concentrations in the spleen, lung, kidney and plasma. Regardless of the route of administration, higher drug concentrations were present in plasma (rat and dog) and tissue (rat) samples obtained on day 5 compared to those obtained on day 1. Although plasma concentrations decreased with time, 17-DMAG was still detected in dog plasma for at least 24 h after drug administration. *Conclusions*: With the recent approval of 17-DMAG for clinical use, the data generated from these preclinical studies will provide guidance to clinicians as they administer this drug to their patients. The

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MTD of 17-DMAG was 12 mg/m^2 per day in rats and 8 mg/m^2 per day in dogs; therefore, the recommended starting dose for phase I trial is 1.3 mg/m^2 per day for 5 days. Gastrointestinal and bone marrow toxicity were dose-limiting in rats, and gastrointestinal, renal, gallbladder and bone marrow toxicity were dose-limiting in dogs. All adverse effects were fully reversible in surviving animals after treatment was complete.

Keywords 17-DMAG · 17-AAG · Geldanamycin · Preclinical studies · Animal toxicity studies

Introduction

Heat shock protein 90 (HSP90) is a molecular chaperone responsible for maintaining correct folding and conformation of "client proteins", many of which are important in cell proliferation and signaling (e.g., raf 1, ras, src and p185^{erb2}) [26]. Benzoquinone ansamycin compounds, e.g., geldanamycin and 17-(allylamino)-17-demethoxygeldanamycin (17-AAG), bind to HSP90 and disrupt the interaction between HSP90 and its client proteins [2, 19, 24]. This disruption can result in increased turnover and rapid degradation of these proteins both in vitro and in vivo [2, 19, 24]. Indeed, several investigators have shown a correlation between exposure to geldanamycin or its derivatives and the depletion of various cell signaling proteins [2, 12, 19, 24]. Thus, in the case of oncogenic client proteins, this novel mechanistic pathway could theoretically shut down their uncontrolled proliferative pathways simultaneously, leading to tumor cell growth arrest, and ultimately, cell death [2, 19, 24]. However, the binding of geldanamycin derivatives to HSP90 may also disrupt the interaction between HSP90 and suppressor proteins (e.g., HSF1), thereby causing the activation of the heat shock or stress response pathways [3, 22]. How much of a protective effect this could confer on cancer cells is unknown, considering the "competing" cytotoxic pathway, but it is clear that antitumor effects can be achieved in several animal models with geldanamycin derivatives [5, 23]. Currently 17-AAG is undergoing clinical investigation [4, 10, 14, 25]. However, 17-AAG suffers from a number of shortcomings including its propensity to undergo extensive metabolism to form potentially toxic metabolites [7], and its limited aqueous solubility, which necessitates the use of dimethyl sulfoxide and egg phospholipid for solubilization. These issues have prompted continuing efforts by the NCI to search for improved derivatives of geldanamycin.

A water-soluble, stable geldanamycin derivative, 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG, Fig. 1), has recently emerged from preclinical evaluation and phase I clinical trials have been initiated. With an aqueous solubility of greater than 10 mg/ml, 17-DMAG can be formulated in saline or 5% dextrose in water for patient use, thus eliminating the formulation problems that are associated with 17-AAG. 17-DMAG (bulk drug product) is stable for at least 2 months at room temperature. In addition, 17-DMAG undergoes limited metabolism compared to 17-AAG [7, 9], and has higher oral bioavailability in mice (50% bioavailability) than 17-AAG (24%) [8, 9]. Finally, less 17-DMAG is bound to plasma proteins (30–45%) as compared to 17-AAG (>90%) [8, 9].

In vitro and in vivo efficacy studies performed with 17-DMAG have also shown a potential advantage over 17-AAG. In the NCI 60-cell-line human tumor screening assay [1], 17-DMAG demonstrated in vitro activity against a variety of tumor types including colon, melanoma and breast [23]. In many cases, these cell lines were more sensitive to 17-DMAG than to 17-AAG. In vivo efficacy was demonstrated with both agents in athymic mouse xenograft studies with s.c. implanted MEXF melanomas [5, 23], as shown by the significant partial regressions in two of four models with both 17-AAG and 17-DMAG. However, significant partial regressions were seen in two of two LXF lung models with 17-DMAG and only one of two models with 17-AAG after i.v. administration using a variety of dosing schedules. It is important to note, however, that maximum tolerated doses of 45 mg/m^2 per day (17-DMAG) and 180 mg/m^2 per day (17-AAG) were required to achieve efficacy in these mice (two times daily for 5 days schedule). Significant reduction of tumor burden in liver was also seen after oral administration of 17-DMAG to athymic mice in an orthotopic metastasis model using human pancreatic AsPC-1 tumor cells [5]. In contrast, 17-AAG had no effect in this model [5]. The aims of the present studies were to estimate a safe clinical starting dose, to define the spectrum of 17-DMAG toxicity in rats and dogs, and to determine the plasma concentrations that were associated with observed adverse events to support phase I studies in humans.

Materials and methods

Drug

17-DMAG (NSC-707545) was supplied in crystalline solid form by the Developmental Therapeutics Program of the National Cancer Institute. In these studies, 17-DMAG was formulated in 5% dextrose in water. Final

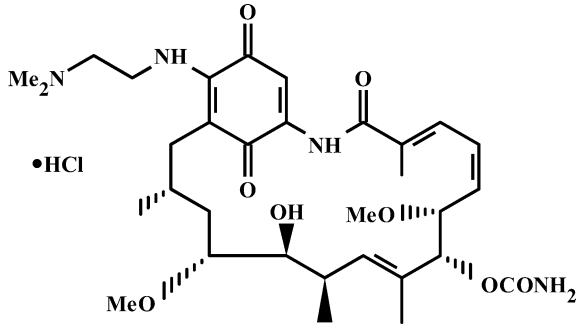

Fig. 1 Structure of 17-DMAG

drug concentrations ranged from 0.2 to 3 mg/ml for the rats and 0.2–0.75 mg/ml for the dogs.

Animals

All animals were housed and cared for in accordance with the guidelines of the US Department of Agriculture (Animal Welfare Act, Public Law 99-198) and those of the Guide for the Care and Use of Laboratory Animals, 7th edn (Institute of Laboratory Animal Resources, Commission of Life Sciences, National Research Council, National Academy Press, Washington, DC, 1996).

Rats

Fischer 344 rats were purchased from Taconic Farms (Germantown, N.Y.). Rats were approximately 7–8 weeks of age upon receipt and approximately 9 weeks old on the first day of dosing. They were housed individually in polycarbonate cages in animal rooms that were maintained in the temperature range 20–26°C with a relative humidity of 30–70%. Room lights were controlled automatically to provide 12 h of light and 12 h of dark. All rats had free access to tap water and Certified Purina Laboratory Chow no. 5002 (PMI Feeds, St. Louis, Mo.).

Dogs

Beagle dogs were obtained from Marshall Farms USA (North Rose, N.Y.). Dogs were approximately 7–8 months old upon initiation of treatment. These animals were singly housed in a temperature-controlled (18–29°C) and humidity-controlled (30–70%) room with a 12-h light/12-h dark cycle. Dogs were allowed access to approximately 350 g of Certified Canine Diet no. 5007 (PMI Feeds) for 1–2 h per day. Tap water was provided ad libitum.

Study design

Doses for the definitive (IND-directed) studies were derived from the results of initial range-finding studies, where doses of 15, 30, 60 and 90 mg/m^2 per day (2.5, 5, 10 and 15 mg/kg per day, rats), and 15 and 30 mg/m^2 per day (0.75 and 1.5 mg/kg per day, dogs) were administered i.v. (bolus for rats; 1-h i.v. infusion for dogs) daily for five consecutive days. In the present IND-directed rat study, i.v. (bolus, over 30 s) doses of 0, 2.4, 12 and 24 mg/m^2 per day (0, 0.4, 2 and 4 mg/kg per day) were administered at a constant volume of 2 ml/kg to male and female rats (ten per sex per dose group for the main study in rats; nine males per dose group for satellite rats) for five consecutive days. Vehicle-control rats received 2 ml/kg sterile 5% dextrose in water daily for five consecutive days. In the IND-directed dog study, doses of 0, 8 and 16 mg/m^2 per day (0, 0.4 and 0.8 mg/kg per day) were administered as a 1-h i.v. infusion to male and female beagle dogs (two per sex per dose group) at a rate of 2 ml/kg/h once a day for five consecutive days. Vehicle-control dogs received sterile 5% dextrose in water at a rate of 2 ml/kg/h over 1 h on five consecutive days. Additional male and female dogs (two per sex per dose group) received an oral (gavage) dose of 16 mg/m^2 per day at a volume of 2 ml/kg for five consecutive days.

Animals were monitored for morbidity and mortality twice daily during the dosing period and at least once daily thereafter until study termination (days 29 and 35 for the rat and dog studies, respectively). Rats and dogs were also monitored for clinical signs of toxicity daily until study termination. Body weights were measured before the dosing period, on dosing days, and weekly thereafter. Hematology and clinical chemistry measurements were performed on days 8, 15, 22 and 29 for rats and days-4, 2, 4, 6 (moribund dogs only), 8, 12, 15, 22, 29 and 35 for dogs. Blood was drawn for plasma 17-DMAG concentration measurements on days 1 and 5. Scheduled necropsies (including gross and microscopic pathologic evaluations) were performed on vehicle-control animals and animals in selected (main study) treated groups killed on days 8, 29 (rats only) and 35 (dogs only). In addition, necropsies were performed on all animals that died or were killed in a moribund condition.

Plasma and tissue drug concentrations

Blood samples were collected into heparinized tubes from three satellite rats per dose group at each of the following time-points: 2, 4, and 8 h after dosing on days 1 and 5. The blood samples were mixed by gentle inversion, placed on ice and then centrifuged to separate the plasma. At each time-point, selected tissues (liver, kidney, lung, spleen, brain, heart, bone marrow and sections of the gastrointestinal tract) were also collected at necropsy for determination of 17-DMAG concentrations. For the dog definitive study, blood was collected 2 min before the end of infusion and 2 h and 24 h after infusion on days 1 and 5 for plasma drug concentration determinations. For the orally dosed group, blood was collected at 30 min and 2 h and 24 h after dosing on days 1 and 5. Dog tissue samples were not assayed for 17-DMAG. Plasma and tissue samples were stored frozen until analysis.

Concentrations of 17-DMAG in plasma, tissues and red blood cells (RBC) were determined using a modification of a previously published method [9]. Briefly, 10 µl of 1 µg/ml 17-AAG internal standard, which was prepared in methanol/distilled water (50:50, v/v), was added to 0.2 ml plasma, RBC or tissue homogenate. After addition of internal standard, 1 ml ethyl acetate was added to each tube, and tubes were vortexed and then centrifuged at 14,000 *g* for 6 min. The resulting supernatants were transferred to 12×75 mm borosilicate glass tubes and evaporated to dryness under nitrogen.

Dried residues were reconstituted in 100 µl mobile phase, consisting of methanol/distilled water/formic acid (60:40:0.1, v/v/v), and 10 µl was injected onto the LC/MS. Along with unknown samples, duplicate plasma standard curves containing concentrations of 1, 3, 10, 30, 100, 300 and 1000 ng/ml 17-DMAG in the appropriate biologic matrix were run. Duplicate quality control samples containing concentrations of 3, 30 and 300 ng/ml were also included with each run.

The LC/MS instrumentation consisted of an Agilent 1100 autosampler and quaternary pump (Agilent, Wilmington, Del.) fitted with a SYNERGI Hydro-RP 80A column (4 µm, 2×100 mm; Phenomenex, Torrance, Calif.). The mobile phase was pumped at 0.2 ml/min, and the column eluate was monitored with a Thermofinnigan Surveyor MSQ single quadrupole mass spectrometer (Thermofinnigan, San Jose, Calif.) operating in ESI positive-ion mode and monitoring *m/z* 617 for 17-DMAG and 584 for 17-AAG internal standard. The lower limit of quantitation in rat and dog plasma was 1 ng/ml, and the assay was linear between 1 and 1000 ng/ml. Criteria for run acceptance were those recommended by Shah et al. [20, 21].

Pathology

Each surviving main study animal was killed by CO_2 asphyxiation (rats; days 8 and 29) or barbiturate overdose followed by exsanguination (dogs; days 8 and 35), and subjected to a complete necropsy with gross and microscopic examination. Moribund animals were killed out of sequence. Tissues from all organ systems of rats and dogs were fixed in 10% neutral buffered formalin, except for the eyes and optic nerves, which were fixed in Davidson's solution. Fixed tissues from selected groups were processed by routine histopathologic methods, stained with hematoxylin and eosin, and evaluated microscopically.

Results

Mortality, clinical signs, body weights and food consumption

In the rat study, three (of ten) female rats in the 24 mg/m^2 per day dose group died on day 6 after receiving all five doses of 17-DMAG, but all ten male rats survived until study termination. Diarrhea was observed in all drug-treated groups, but the frequency and the number of rats affected increased with the increase in dose. Sores/ulcers of the tail at the site of injection were observed in males in the 24 mg/m^2 per day dose group. Consistent with the gastrointestinal-related effects, statistically ($P < 0.05$) significant decreases in group mean body weights ranging from 7% to 14% were seen in male rats receiving doses of 24 mg/m^2 per day and were noted as early as day 4; however, body weight changes were not observed in rats in any other dose group. No statistically significant changes in food consumption were noted in the rats.

In the dog study, the 16 mg/m^2 per day i.v. dose proved lethal, as three of four dogs (two males and one female) were killed in a moribund condition on day 6 after receiving all five doses. However, dogs that received oral doses of 16 mg/m^2 per day or i.v. doses of 8 mg/m^2 per day survived until study termination. The most common clinical signs of toxicity were emesis and diarrhea (which sometimes contained blood), and were seen in all drug-treated groups, including the 16 mg/m^2 per day oral dose group. Moribund dogs were also noticeably dehydrated on day 6 prior to being killed, as evidenced by the loss of elasticity of the skin at the back of the neck. Jaundice was observed on the skin and gums of one of the three dogs (female) in the 16 mg/m^2 per day i.v. dose group that was killed in a moribund condition. Modest decreases in body weight, ranging from 11% to 14%, were only seen in the 16 mg/m^2 per day i.v. dose group (four of four dogs) between day 1 and the day of death (days 6–8). More strikingly, dose-dependent decreases in food consumption were noted in all treated dogs. Dogs in the 16 mg/m^2 oral and i.v. groups had decreased their food consumption by 64–69% by days 5 and 4, respectively, and dogs in the 8 mg/m^2 i.v. group had decreased their food consumption by up to 30% on day 7. Surviving animals had resumed normal food consumption by day 14.

Hematology

In rats, 5 days of dosing with 17-DMAG produced dose-dependent thrombocytopenia in all treated groups on day 8 (Fig. 2). Reticulocytopenia was also observed in rats in the 12 and 24 mg/m^2 per day dose groups on day 8. In dogs, 17-DMAG produced neutropenia and lymphopenia on days 4 and 2, respectively (Fig. 3). While all treated animals were affected, leukopenia was more severe in dogs that received i.v. doses than in those that received oral doses. In dogs treated with i.v. doses of 16 mg/m^2 per day, moderate to marked increases in fibrinogen were observed on day 6, while dogs in the 8 mg/m^2 per day i.v. group and in the 16 mg/m^2 per day oral group exhibited mild to moderate increases in fibrinogen on day 8 (Fig. 3). No other drug-related changes in hematologic parameters occurred. By day 22, all hematologic parameters in surviving rats or dogs were within or trending towards normal ranges, suggesting reversibility of the hematologic effects. Coagulation changes were also reversible in dogs, as fibrinogen values were comparable to baseline values by day 22.

Clinical chemistry

In rats, drug-related, dose-dependent changes in clinical chemistry parameters that were indicative of hepatocellular toxicity were observed with 17-DMAG doses of 12

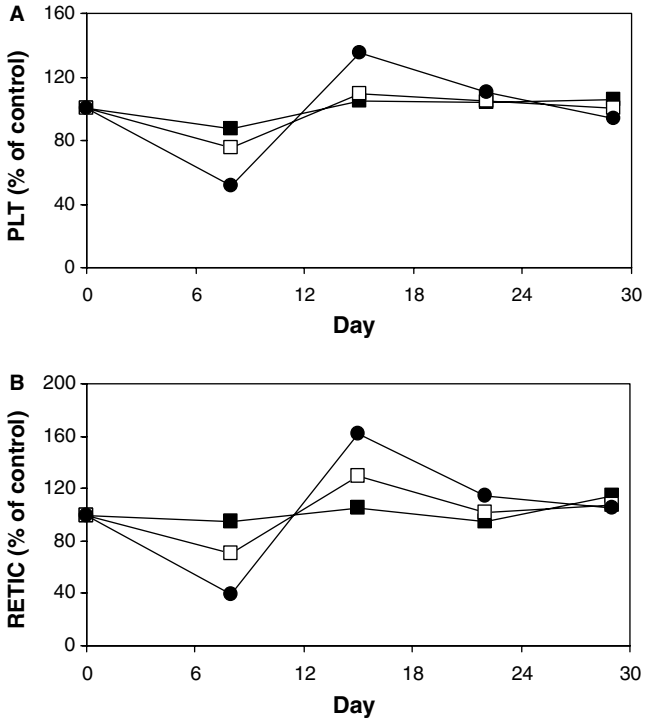

Fig. 2 Hematologic toxicity in Fischer 344 rats treated with 2.4 mg/m^2 (*filled squares*), 12 mg/m^2 (*open squares*) and 24 mg/m^2 (*filled circles*) per day 17-DMAG (**a** platelet counts, **b** reticulocyte counts). Blood was drawn for hematology determinations on days 8, 15, 22 and 29. Data are represented as percentages of control. No sex differences were seen in platelet or reticulocyte counts; therefore each data point represents an average for all animals in a given dose group

and 24 mg/m^2 per day. These included modest increases (up to threefold) in group mean transaminase activity (ALT, AST) and total bile acids. Other clinical chemistry changes included modest increases in alkaline phosphatase activity and cholesterol, and modest decreases in group mean triglyceride values (40–67%) in rats in the 12 and 24 mg/m^2 per day dose groups on day 8. Group mean triglyceride values were also lower (37% decrease) in female rats in the 2.4 mg/m^2 per day dose group. All changes in clinical chemistry parameters were reversible since all clinical chemistry values were within the normal range by day 15

All clinical chemistry parameters in dogs were within the normal historical range prior to dosing (day-4). On day 6, changes indicative of severe hepatotoxicity were observed in moribund animals in the 16 mg/m^2 i.v. dose group that were killed in a moribund condition. These animals (both males and one female) exhibited moderate-to-marked increases in serum liver transaminase (ALT, AST), alkaline phosphatase and gamma-glutamyl transpeptidase (GGT) activities, as well as total bilirubin, prior to being killed on day 6 (Fig. 4). Both direct and indirect bilirubin were increased in these dogs. Cholesterol and triglycerides were also increased, threefold to fourfold and threefold to tenfold, respectively, in moribund animals prior to being killed (data not shown). Slight hepatotoxicity may also have been present in one or more of the surviving dogs, as modest (about twofold) increases in AST, alkaline phosphatase and/or GGT were observed (Fig. 4).

Evidence of renal toxicity was also present in moribund animals prior to being killed on day 6 (Fig. 5). In these dogs, BUN, phosphorous and potassium were elevated. Creatinine was also increased, but only in the male dogs (two of two) of this dose group (16 mg/m^2 per day i.v.). Evidence of renal toxicity was also present in one male dog in the 8 mg/m^2 per day dose group; This dog had marked treatment-related changes in BUN, creatinine, phosphorous and potassium on day 12.

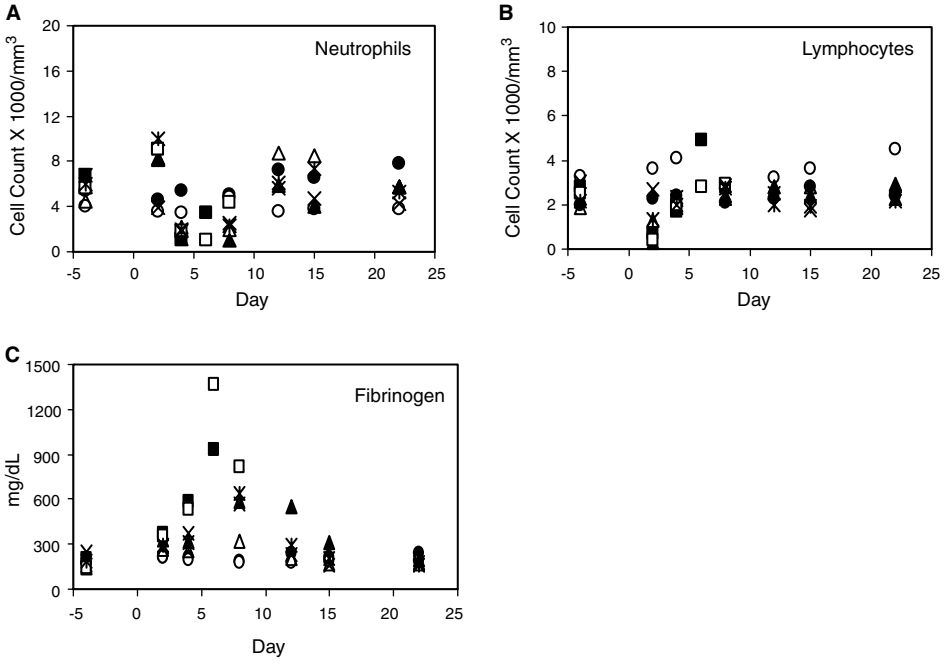

Fig. 3 Hematologic toxicity in beagle dogs treated with 17-DMAG (**a** neutrophil counts, **b** lymphocyte counts, **c** fibrinogen levels; *filled circles* control males, *open circles* control females, *filled triangles* 8 mg/m^2 per day i.v. males, *open triangles* 8 mg/m^2 per day i.v. females, *filled squares* 16 mg/m^2 per day i.v. males, *open squares* 16 mg/m^2 per day i.v. females, *asterisks* 16 mg/m^2 per day oral males, *crosses* 16 mg/m^2 per day oral females). Doses shown are daily doses that were administered. All data points are the average values for two dogs except for days 6 and 8 for the 16 mg/m^2 per day i.v. females for which only one dog is represented per day

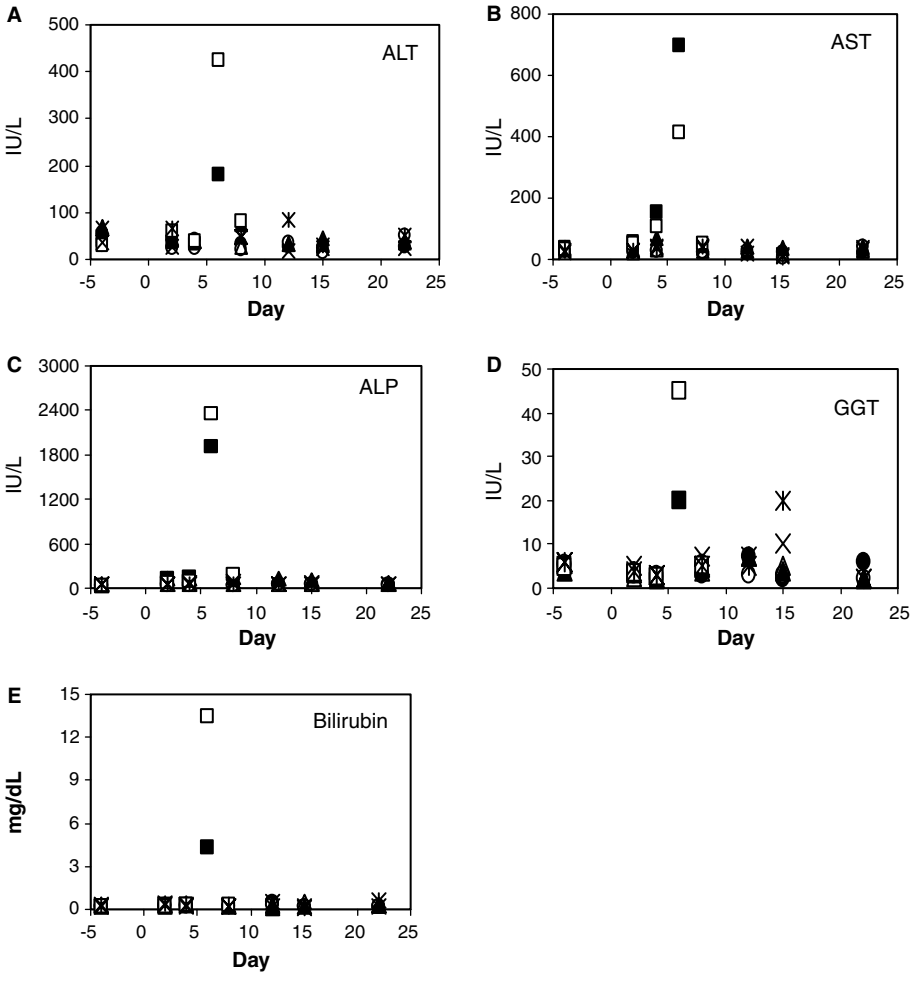

Fig. 4 Hepatic toxicity in beagle dogs treated with 17-DMAG (**a** ALT activities, **b** AST activities, **c** ALP activities, **d** GGT activities, **e** total bilirubin levels; *filled circles* control males, *open circles* control females, *filled triangles* 8 mg/m^2 per day i.v. males, *open triangles* 8 mg/m^2 per day i.v. females, *filled squares* 16 mg/m^2 per day i.v. males, *open squares* 16 mg/m^2 per day i.v. females, *asterisks* 16 mg/m^2 per day oral males, *crosses* 16 mg/m^2 per day oral females). Doses shown are daily doses that were administered. All data points are the average values for two dogs except for days 6 and 8 for the 16 mg/m^2 per day i.v. females for which only one dog is represented per day

Moderate-to-marked increases in creatine kinase (3-fold to 17-fold), lactate dehydrogenase (twofold to eightfold) and globulin (about threefold), and decreases (about 20%) in glucose, albumin and chloride, were also observed, but only in moribund animals prior to being killed on day 6. By day 22, all clinical chemistry parameters were normal in surviving animals (8 mg/m^2 per day i.v. group and 16 mg/m^2 per day oral group).

Plasma and tissue drug concentrations

Following i.v. administration to rats, plasma 17-DMAG concentrations were proportional to dose. In tissues, however, only fourfold to sixfold increases in 17-DMAG concentrations were observed with the tenfold increase in dose (Fig. 6). 17-DMAG was widely distributed to tissues, with the highest concentrations found in the liver (up to 100-fold greater than plasma concentrations), and progressively lower concentrations in the spleen, intestines, lung, kidney and heart. 17-DMAG concentrations in red blood cells were low, but detectable at all time-points. For all dose groups, concentrations of 40–80 ng/g were achieved 2–4 h after dosing on day 1, and concentrations of 30–160 ng/g were achieved 2–4 h after dosing on day 5 (data not shown). Very little drug crossed the blood-brain barrier and permeated the brain. Overall, these findings are consistent with previously reported pharmacokinetic data for 17-DMAG in CD_2F_1 mice and Fischer 344 rats [9]. 17-DMAG could be detected in rat plasma and tissues for at least 8 h after drug administration. Higher drug concentrations of 17-DMAG were detected in the plasma and tissues on day 5 (compared to day 1), suggesting drug accumulation on the daily five times schedule.

In the dog study, plasma concentrations of 17-DMAG increased with the i.v. dose, but the extent of the increase was greater than the twofold increase in dose (Table 1). Although plasma concentrations decreased with time, 17-DMAG was still detected in dog plasma for at least 24 h after i.v. administration, which resulted in higher plasma concentrations being achieved on day 5 than on day 1. Measurable concentrations of 17-DMAG were detected after oral administration on days 1 and 5 at all time-points (0.5, 2 and 24 h). Although area-under-the-curve (AUC) values were not calculated for this limited data set, the plasma values were consistent with a previous pharmacokinetic/bioavailability data study in dogs in which a 30 mg/m^2 oral dose produced a bioavailability of 35–39% (data not shown).

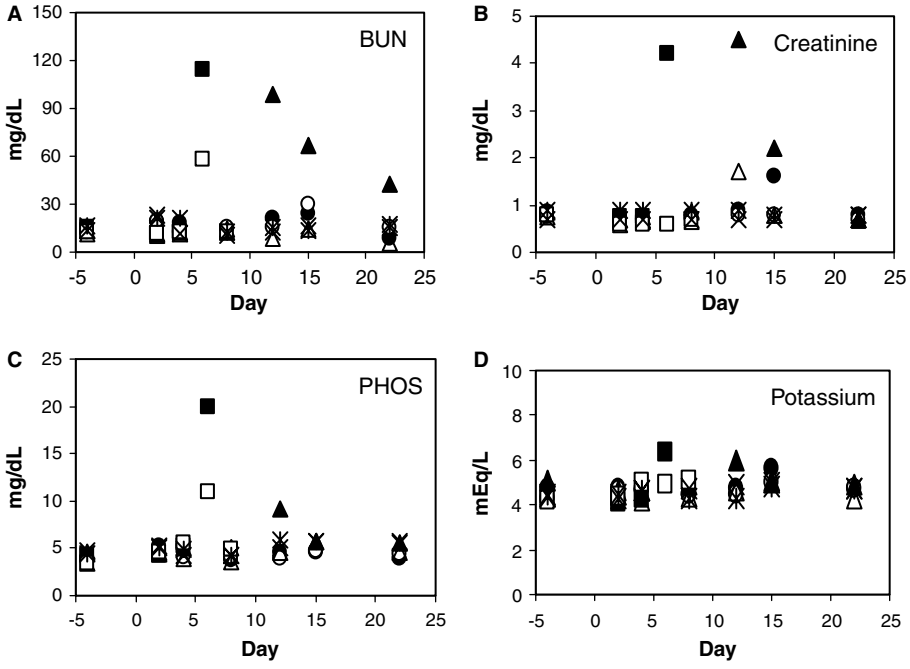

Fig. 5 Renal toxicity in beagle dogs treated with 17-DMAG (**a** BUN levels, **b** creatinine levels, **c** phosphorus levels, **d** potassium levels; *filled circles* control males, *open circles* control females, *filled triangles* 8 mg/m^2 per day i.v. males, *open triangles* 8 mg/m^2 per day i.v. females, *filled squares* 16 mg/m^2 per day i.v. males, *open squares* 16 mg/m^2 per day i.v. females, *asterisks* 16 mg/m^2 per day oral males, *crosses* 16 mg/m^2 per day oral females). Doses shown are daily doses that were administered. All data points are the average values for two dogs except for days 6 and 8 for the 16 mg/m^2 per day i.v. females for which only one dog is represented per day

Histopathology

In the rat study, drug-related lesions were found in the bone marrow (atrophy), thymus (atrophy), cecum (congestion, edema, hemorrhage and inflammation) and liver (necrosis and infiltration of mixed cells) in male and female rats in the 24 mg/m^2 per day dose group (Table 2). Lesions were also observed at the site of

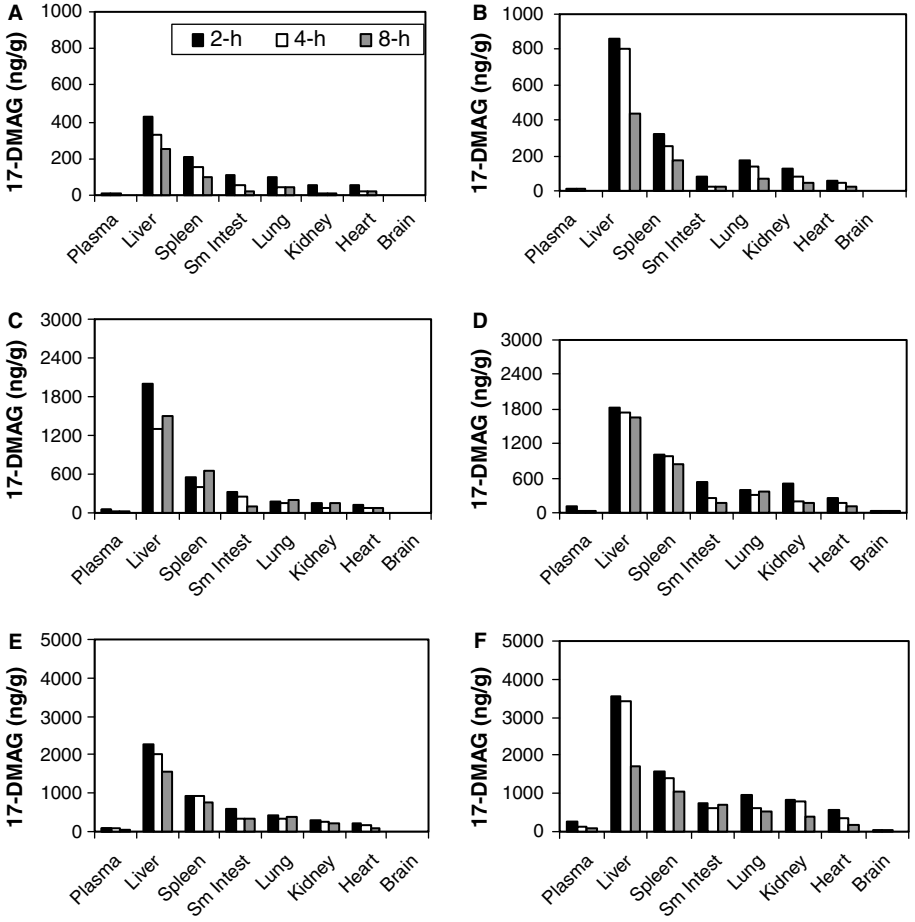

Fig. 6 Concentrations of 17-DMAG in plasma, and tissues of rats injected i.v. daily for 5 days with 2.4 mg/m^2 (**a**, **b**), 12 mg/m^2 (**c**, **d**) or 24 mg/m^2 (**e**, **f**) per day 17-DMAG (**a**, **c**, **e** day 1; **b**, **d**, **f** day 5). Plot scales vary with dose. Three rats were killed at each time-point. Values shown are the averages of 17-DMAG concentrations in plasma or tissue from three rats

Table 1 Plasma concentrations of 17-DMAG in dogs (no sex differences were noted, therefore values shown are averages for four dogs, two male and two female)

Day	Time (h)[a]	Plasma concentration (n *M*)		
		8 mg/m^2 i.v.	16 mg/m^2 i.v.	16 mg/m^2 oral
1	0/0.5	138	442	82.9
	2	37.2	107	42.5
	24	8.21	13.1	5.28
5	0/0.5	297	1231	99.4
	2	91.3	436	99.1
	24	22.8	221	19.9

[a]Plasma concentrations were determined 2 min before the end of the i.v. infusion (about 0 h) and at 2 and 24 h after i.v. dosing, and at 0.5, 2 and 24 h after oral dosing

injection (hemorrhage, inflammation, necrosis and ulceration) in rats in the 24 mg/m^2 per day dose group as well as in control animals, although the severity in control animals was considerably lower than in treated animals. This suggests that these lesions were at least partially due to mechanical injury resulting from the injection itself and not to the drug. All target tissues (except liver, which showed lesions of minimal-to-mild severity in animals in the 24 mg/m^2 per day dose group) were subsequently evaluated in the lower dose groups and in recovery animals. On day 8, male and female rats that were given i.v. doses of 12 mg/m^2 per day also had lesions in the bone marrow and thymus and at the injection site. However, these lesions were less severe and found in fewer animals than with the 24 mg/m^2 per day dose. No drug-related lesions were noted in the 2.4 mg/m^2 per day dose group (day 8). On day 29, one male rat in the 24 mg/m^2 per day dose group had a drug-related lesion (mild inflammation of the cecum; data not shown).

In the dog study, drug-related lesions were found in all animals treated i.v. with 16 mg/m^2 per day (Table 3). In general, lesions found in the moribund animals were considerably more severe than in the surviving female in the 16 mg/m^2 per day i.v. dose group. Affected organs included the stomach (congestion, inflammation and necrosis), small and large intestine (congestion and hemorrhage), gallbladder (edema, hemorrhage, inflammation and necrosis), liver (hemorrhage, hyperplasia and infiltration of mixed cells), lymphoid tissues (atrophy of lymphoid cells in the spleen, thymus and tonsil), bone marrow (atrophy), adrenal gland (congestion), and/or the skin at the injection site (hemorrhage, inflammation and ulceration). A proteinaceous exudate was also observed within the lumen of the renal tubules, but visible histologic damage to the glomeruli or renal tubules was not noted.

The histopathologic effects of 17-DMAG at doses of 8 mg/m^2 per day i.v. and 16 mg/m^2 per day oral were judged to be minimal to mild on day 8. These included mild lesions in the liver (infiltration of mixed cells and/or bile duct hyperplasia), thymus (atrophy) and/or skin at the injection site (8 mg/m^2 per day, hemorrhage and inflammation). Lesions of the skin at the injection site (hemorrhage and/or inflammation) were also noted in male and female dogs in the control group, but were

Table 2 Treatment-related lesions in rats treated with 17-DMAG. Tissues from all major organ systems from rats (five per sex per dose group) in the control group and 24 mg/m^2 per day group that were killed at scheduled necropsy on day 8 were evaluated microscopically. Also included are histologic results from the three female rats that died on day 6 (24 mg/m^2 per day). Target tissues only were evaluated in lower dose groups and in recovery animals (day 29); day 29 results are not shown. The incidence of target tissue lesions is presented with mean group severity scores (*1* minimal, *2* mild, *3* moderate, *4* marked) in parentheses (– lesion not observed, *n.d.* not determined)

	Males				Females			
	Dose (mg/m^2 per day)				Dose (mg/m^2 per day)			
	0	2.4	12	24	0	2.4	12	24
Large intestine (cecum)								
Congestion	–	–	–	–	–	–	–	1/8 (3)
Edema	–	–	–	1/5 (3)	–	–	–	4/8 (2.5)
Hemorrhage[a]	–	–	–	1/5 (3)	–	–	–	2/8 (4)
Inflammation	–	–	–	2/5 (2.5)	–	–	–	5/8 (2.4)
Bone marrow								
Atrophy	–	–	5/5 (1.4)	5/5 (3)	–	–	1/5 (1)	8/8 (2.2)
Injection site								
Hemorrhage	1/5 (1)	–	–	4/5 (2)	–	–	2/5 (2)	5/8 (2.6)
Inflammation	4/5 (1)	1/5 (1)	4/5 (1)	5/5 (2.2)	1/5 (2)	1/5 (1)	5/5 (1.4)	5/8 (2)
Necrosis	–	–	–	–	–	–	–	1/8 (3)
Ulcer	–	–	–	–	–	–	–	1/8 (3)
Liver								
Necrosis[a]	–	–	–	–	–	–	–	3/8 (1.7)
Infiltration, mixed cell	1/5 (1)	n.d.	n.d.	3/5 (1.3)	–	n.d.	n.d.	–
Lymphoid system								
Atrophy, thymus	–	–	2/5 (1)	5/5 (2.6)	–	–	2/5 (1)	6/8 (2.7)

[a]Lesions found only in rats that died on day 6

Table 3 Treatment-related lesions in dogs treated with 17-DMAG. Tissues from all major organ systems from all dogs were evaluated microscopically. Dogs (one per sex per group per day) that received doses of 8 (i.v.) or 16 mg/m^2 per day (oral) were necropsied on day 8 and day 35. Two male dogs and one female dog dosed at 16 mg/m^2 per day i.v. were killed in a moribund condition on day 6; the surviving female in this group was killed on day 8. Shown are the histologic results from dogs that were killed on days 6 or 8; day 29 results are not shown. The incidence of target tissue lesions is presented with mean group severity scores (*1* minimal, *2* mild, *3* moderate, *4* marked) in parentheses (– lesion not observed, *n/a* not applicable)

Lesion	Males				Females			
	Dose (mg/m^2 per day)				Dose (mg/m^2 per day)			
	0	8 (i.v.)	16 (i.v.)	16 (oral)	0	8 (i.v.)	16 (i.v.)	16 (oral)
Kidney								
Exudate, luminal, tubules	–	–	2/2 (3.5)	–	–	–	1/1 (1)	–
Gallbladder								
Edema	–	–	1/2 (3)	–	–	–	–	–
Hemorrhage	–	–	1/2 (3)	–	–	–	1/2 (4)	–
Inflammation	–	–	–	–	–	–	1/2 (2)	–
Necrosis, artery or mucosa	–	–	1/2 (1)	–	–	–	1/2 (4)	–
Stomach								
Congestion	–	–	1/2 (2)	–	–	–	–	–
Inflammation	–	–	–	–	–	–	1/2 (3)	–
Necrosis, blood vessel	–	–	1/2 (2)	–	–	–	–	–
Small intestine[a]								
Congestion	–	–	1/2 (2.5)	–	–	–	2/2 (2.2)	–
Hemorrhage	–	–	1/2 (3)	–	–	–	1/2 (3)	–
Large intestine								
Congestion	–	–	–	–	–	–	2/2 (2.3)	–
Hemorrhage	–	–	1/2 (3)	–	–	–	–	–
Bone marrow								
Atrophy	–	–	2/2 (2)	–	–	–	2/2 (2)	–
Injection site								
Hemorrhage	–	1/2 (2)	2/2 (3)	n/a	1/2 (2)	–	2/2 (2.5)	n/a
Inflammation	1/1 (1)	1/2 (2)	1/2 (3)	n/a	1/2 (2)	–	1/2 (3)	n/a
Ulcer	–	–	–	n/a	–	–	1/2 (2)	n/a
Liver								
Hemorrhage, blood vessel	–	–	2/2 (1.5)	–	–	–	1/2 (1)	–
Hyperplasia, bile duct	–	–	2/2 (1.5)	1/2 (1)	–	–	2/2 (2.5)	–
Infiltration, mixed cell	–	1/2 (2)	2/2 (2.5)	1/2 (3)	–	–	2/2 (2.5)	1/2 (2)
Lymphoid system								
Atrophy, spleen, thymus, tonsil, lymph nodes	–	–	2/2 (3)				2/2 (2.8)	1/2 (2)
Adrenal gland								
Congestion	–	–	1/2 (3)	–	–	–	1/2 (3)	–

[a]Both male dogs dosed at 16 mg/m^2 per day had lesions of the gastrointestinal tract on day 6; one exhibited evidence of congestion of the small intestine and the other exhibited signs of hemorrhaging in the small and large intestine

considerably less severe than in 17-DMAG-treated groups. Drug-related histopathologic lesions were absent in surviving animals on day 35 (data not shown), showing the reversibility of drug-related toxicity at non-lethal doses. Due to the early deaths, histopathologic analysis could not be performed on animals in the 16 mg/m^2 per day i.v. dose group on day 35.

Discussion

17-DMAG has recently been approved for clinical use and phase I trials were recently initiated. The daily for 5 days schedule used in the preclinical toxicology studies reported here was chosen to support the various clinical schedules proposed for 17-DMAG, including: twice weekly, once a week for 3 weeks, daily for 3 days, daily for 5 days and daily for 5 days once every 3 weeks. The data generated from these preclinical studies will provide guidance to clinicians as they administer this agent to their patients. In particular, the maximally tolerated dose (MTD), defined as the highest dose tested that does not cause severe, irreversible toxicity [11, 13], was determined for both rats and dogs and allowed for the estimation of a "safe" starting dose in humans. The MTD of 17-DMAG was 12 mg/m^2 per day in rats and 8 mg/m^2 per day in dogs. Therefore, the recommended starting dose for phase I trials is 1.3 mg/m^2 per day for 5 days, calculated as one-sixth of the MTD in milligrams per meter squared in the non-rodent species [6, 11, 13].

The target organs and the potential for reversibility of drug-induced toxicity have also been determined and will provide further guidance to clinicians during the phase I trials. In general, target organs of 17-DMAG-induced toxicity were classified as drug-related target organs if one or both of the following criteria were met: (1) the toxicity was observed in more than one dose group, and (2) histopathologic evidence of toxicity was

present. Based on these criteria, the cecum was confirmed as a target in rats based on the presence of lesions in the cecum, and the associated clinical observations of diarrhea and body weight loss. Similarly, the lesions in the stomach and sections of the small and large intestines, along with the emesis and diarrhea observed, confirmed the gastrointestinal tract as a target in dogs. Of interest is the fact that the lesions of the gastrointestinal tract were observed with the administration of 16 mg/m^2 per day i.v., and not with oral administration at the same dose. This suggests that the lesions of the gastrointestinal tract in dogs were the result of a systemic effect, and not the result of local irritation.

Based on the minimal-to-mild lesions in the livers of surviving rats and dogs on day 8 and the modest increases in AST, alkaline phosphatase and GGT (dogs only), the liver was considered a drug-related target in rats and dogs. While it is likely that the effect on the liver was exacerbated in moribund animals due to their poor general health, the possible loss of gastrointestinal epithelial integrity due to lesions of the gastrointestinal tract and/or dehydration, the moderate-to-severe lesions in the gallbladder, minimal-to-moderate lesions in the liver and the presence of jaundice were all consistent with a direct drug-related effect on the liver and gallbladder.

The histopathologic evidence of renal tubular proteinaceous material (16 mg/m^2 per day i.v. dose group only) and the elevated BUN and creatinine levels observed in two dose groups (8 and 16 mg/m^2 per day i.v.) indicate that the kidney was also a drug-related target organ in dogs, despite any secondary effects on the kidney due to morbidity, dehydration and/or lesions of the gastrointestinal tract. However, this toxicity appeared to be species-specific, as renal changes were not observed in rats at doses higher than those which produced significant changes in dogs.

The bone marrow was confirmed as a target in rats and dogs based on the evidence of bone marrow atrophy in both species and the hematologic findings of reticulocytopenia and thrombocytopenia in rats, and leukopenia in dogs. Dogs appeared to be more sensitive to the hematologic effects of 17-DMAG, as the changes were more extensive for dogs at a lower dose (8 mg/m^2 per day) than for rats at the highest dose tested (24 mg/m^2 per day). While the presence of lesions in the lymphoid system (rats and dogs) and adrenal glands (dogs) might suggest a drug-related effect, typically these changes are responses secondary to stress.

In the present studies, dose-limiting toxicity was defined as an adverse event with sufficient change in one or more clinical chemistry parameter(s), and/or with sufficient target tissue damage, such that the event is considered severe or life-threatening. Based on this definition, only the toxicity to the gastrointestinal tract and bone marrow appeared to be dose-limiting in rats, while gastrointestinal, renal, gallbladder and bone marrow toxicity were considered dose-limiting in dogs. The liver toxicity observed in both species, which was judged as minimal-to-mild in severity at lethal doses, was not regarded as dose-limiting.

The 17-DMAG plasma concentrations exceeded the median IC_{50} value (53 n *M*) for at least 2 h on days 1 and 5 after the administration of 12 mg/m^2 per day i.v. to rats or 8 mg/m^2 per day i.v. to dogs. These doses produced minimal to mild toxicity in rats and moderate but reversible toxicity in dogs. Plasma concentrations approximated or exceeded the IC_{50} for at least 1.5 h (between 30 min and 2 h) after oral dosing at 16 mg/m^2 per day to dogs. High concentrations of 17-DMAG were observed in well-perfused organs relative to plasma concentrations, even though drug-related tissue damage was minimal with 12 mg/m^2 per day and lower doses in rats. It is noteworthy that the NCI's in vitro concentration versus time activity assay showed 50% growth inhibition in HL60 and LOX IMVI cell lines within 45 min of exposure to 50 and 25 n *M* 17-DMAG, respectively (NCI unpublished data).

The preclinical toxicity profile of 17-DMAG shares many similarities with that of 17-AAG. Gastrointestinal, hepatic, gallbladder and lymphoid tissue toxicity occur in dogs, and gastrointestinal, urinary bladder, bone marrow and lymphoid tissue toxicity occur in rats after treatment with 17-AAG [15, 16]. The adverse effects of 17-AAG are also reversible, as clinical signs, clinical pathology parameters and histopathology findings are all normal or tending toward normalcy by day 22 in surviving animals. However, while evidence of renal toxicity is seen in rats treated with 17-AAG, renal toxicity is not observed in dogs [15, 16]. With 17-DMAG the reverse is true, renal toxicity is observed in dogs but not in rats. Although gastrointestinal and hepatic adverse effects, and occasionally thrombocytopenia, appear to be dose-limiting in humans treated with 17-AAG, clinically significant renal toxicity has not emerged [18].

17-AAG and 17-DMAG do, however, differ in their effects on the hematopoietic system. While 17-DMAG depletes circulating red cells (rats), white cells (dogs) and bone marrow cells, 17-AAG produces bone marrow cellular depletion in rats, but circulating white and red cells are not affected [17]. For dogs, only mild increases in leukocytes are observed after dosing with 17-AAG [15]. In terms of a dose-relationship, the marked effects of 17-DMAG on the hematopoietic system appear to precede all other clinically relevant adverse events, as the reticulocytopenia and thrombocytopenia were the only evidence of toxicity in rats treated with 12 mg/m^2 per day. The first sign of drug-related toxicity in dogs also involved the hematopoietic system, as leukopenia was the only observed toxicity for the two females in the 8 mg/m^2 per day dose group.

17-DMAG and 17-AAG were both active at their MTD in xenograft studies with mice bearing MEXF 276 tumors [5, 23], However, 17-DMAG does have the advantages of higher oral bioavailability and lower plasma protein binding than 17-AAG [8, 9], which resulted in activity in a murine orthotopic liver metastasis

model with orally administered 17-DMAG, in which orally administered 17-AAG had no activity [5].

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