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ORIGINAL ARTICLE

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Preclinical toxicity studies of kahalalide F, a new anticancer agent: single and multiple dosing regimens in the rat

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Abstract *Purpose:* Kahalalide F (KF) is a new anticancer agent currently in clinical trials for solid tumors, including prostate cancer. During the preclinical development of this drug, the studies reported here were conducted to determine the acute and multiple dose toxicities of KF when administered intravenously (i.v.) to rats. This dosing route is the intended route of clinical administration. *Methods:* KF was administered i.v. to male and female CD rats using single- and multiple-dose (daily for 5 days) schedules. Animals were observed for clinical signs, and body weight, hematology, and clinical chemistry parameters determined. Animals were necropsied, gross observations and organ weights recorded, and numerous tissues were collected and examined microscopically. *Results:* KF produced lethality at 375 and 450 μg/kg in males and females, respectively, and the maximum tolerated dose (MTD) was estimated to be 300 μg/kg (1800 μg/m^2). The nervous system appeared to be a potential site of action for the production of lethality. Single-dose administration of KF at 150 and 300 μg/kg produced organ toxicity in which the kidney was the primary target. Injury to distal convoluted tubules was the most toxicologically significant lesion, and was observed on day 4. However, by day 29, resolution of renal toxicity had occurred in the 150-μg/kg group, but only partial resolution was seen at 300 μg/kg. Renal injury correlated with increased serum creatinine, BUN, and kidney weights at 300 μg/kg, indicating impairment of renal function. Subacute, necrotizing inflammation of bone marrow and peritrabecular osteocyte hyperplasia of bone were seen at 300 μg/kg on day 4, with recovery thereafter. Injury to blood vessels and surrounding tissue at the injection site were produced by KF, likely due to local cytotoxicity. In general, reversibility of toxicity was seen at 150 μg/kg but not at 300 μg/kg. When KF was administered once daily for five consecutive days at a dose of 80 μg/kg per day (400 μg/kg total dose), slightly decreased body weight gain was the primary drug-related effect. Therefore, the no-adverse-effect dose was at or near 80 μg/kg per day (480 μg/m^2 per day). *Conclusions:* These findings demonstrate that fractionation of a lethal or MTD dose of KF by daily administration for 5 days reduces drug-induced toxicity, and appears to be a viable option for the clinical evaluation of KF for the treatment of cancer.

Keywords Cancer chemotherapeutic · Cytotoxic · Antitumor · Renal toxicity · Kidney toxicity · Nervous system

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Introduction

Prostate cancer is responsible for a huge medical, economic, and emotional burden on the population, and produces approximately 40,000 deaths per year in the United States alone [9]. Primary therapy for advanced prostate cancer consists of androgen ablation via pharmaceutical or surgical castration (orchiectomy), which controls metastatic disease in a majority of cases. Blockade of the androgenic signal, due to decreased levels of testosterone and its metabolite, dihydrotestosterone, results in decreased activation of the androgen receptor leading to apoptosis of carcinoma cells [11]. Unfortunately, virtually all men with metastatic prostate cancer develop androgen-independent disease. Once hormone-refractory prostate carcinoma develops, treatment is palliative and the median duration of survival is between 6 and 12 months [10].

Kahalalide F (KF) is a new anticancer agent that is cytotoxic to prostate tumor cells and other solid tumors.

KF is a cyclic peptide toxin isolated from the herbivorous marine mollusk, *Elysia rufescens*, found in Hawaii. *Elysia rufescens* is a sarcoglossan mollusk capable of sequestering functioning chloroplasts from an algal diet of *Bryopsis pennata* with subsequent biosynthesis of secondary metabolites including KF [3]. KF is comprised of a lateral peptide chain (Fig. 1) formed by residues of D-*allo*threonine-D-*allo*isoleucine-L-ornithine-D-proline-D-valine-L-valine-L-threonine-D-valine and 5-methylhexanoic acid, and a cyclic region containing L-valine-(*Z*)-didehydroaminobutyric acid-L-phenylalanine-D-valine-D-*allo*isoleucine [5].

KF has demonstrated anticancer activity against hormone-independent prostate tumors (IC_{50} values < 1 μ*M*), along with neu$^+$ (Her2-overexpressing) breast tumor cells, and neuroblastomas [3]. In in vivo animal models KF has shown cytotoxicity against hormone-independent prostate tumors [2, 3]. Assays in vitro have indicated that cytotoxicity is not schedule-dependent, as a minimum exposure of 1 h is as potent as an exposure of 48 h [3]. Cytotoxicity against human patient tumor specimens, upon incubation with 1 μ*M* KF, has been seen with breast, colon, non-small cell lung, and ovarian tumors [7]. KF is also cytotoxic against mesenchymal chondrosarcoma and osteosarcoma cells following in vitro incubations for as short a time as 10 min [13]. A primary mechanism of action of KF-induced cell killing appears to be disruption of lysosomal structure, possibly due to hydrophobic insertion in lysosomal membranes [4]. Prostate cells contain large numbers of lysosomes, which may explain, in part, KF's activity against this tumor type. These findings demonstrate that KF is a potent and rapid cytotoxic agent with promising activity against a variety of solid tumors.

The studies reported here were conducted to determine the toxicity of intravenous (i.v.) KF and the reversibility of KF toxicity in rats following both single and multiple (daily for 5 days) administrations. KF is currently being evaluated in clinical trials utilizing the i.v. route of administration [12]. These studies were conducted in accordance with the US FDA Good Laboratory Practice regulations to support the clinical trials program (21 Code of Federal Register, Part 58 – Good Laboratory Practice for Nonclinical Laboratory Studies).

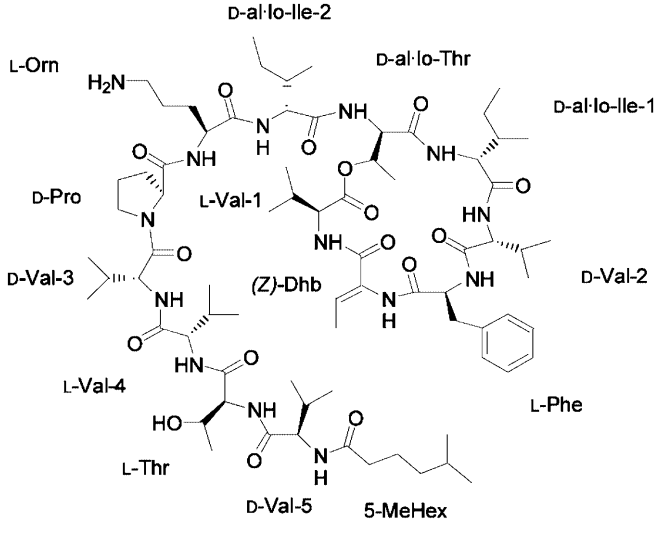

Fig. 1. Kahalalide F (KF)

Materials and methods

Test drug

KF was received from PharmaMar USA (Cambridge, Mass.) and stored at –70ºC to –80ºC, at ambient humidity, and protected from light. KF was prepared as a solution in 10% dimethylformamide/90% sterile saline (v/v) for the maximum tolerated dose (MTD) and single-dose studies, and as a solution in 5% Cremophor EL/5% ethanol/90% sterile saline (v/v/v) for the multiple-dose study. All dosage formulations were prepared on the day of use.

Animals

Male and female CD virus antibody-free rats were received from Charles River Laboratories (Portage, Mich., facility for MTD and single-dose studies; Raleigh, N.C., facility for the multiple-dose study). The animals were approximately 7 to 9 weeks old and weighed approximately 200–300 g (males) and 150–200 g (females) at dosing initiation. The animals were housed singly in polycarbonate cages with Anderson-bed-o-cob bedding (Heinhold, Kankakee, Ill.) in a temperature-controlled (18–26ºC) and humidity-controlled (30–70%) room with a 14-h light/10-h dark cycle. The animals were housed in an animal facility accredited by AAALAC International. Certified Rodent Chow No. 5002 (PMI Feeds, St. Louis, Mo.) and tap-water were provided ad libitum. All animals were quarantined for at least 1 week prior to dosing, and were approved by a clinical veterinarian for placement on study. General procedures for animal care and housing were in accordance with the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The protocols for these studies were approved by the University of Illinois at Chicago Animal Care Committee.

Maximum tolerated dose study

Using a table of random numbers, 20 males and 20 females were randomized by sex into dose groups ($n = 4$ per sex per group). KF was administered once by i.v. bolus injection into the lateral tail vein at doses of 150, 300, 375, 450, and 600 μg/kg (900, 1800, 2250, 2700, and 3600 μg/m^2, respectively) in a dose volume of 1.2 ml/kg. All animals were observed twice on the day of dosing, at approximately 1–2 and 4–6 h after dosing, and once daily thereafter for clinical signs of toxicity. In addition, animals were observed twice daily, in the morning and afternoon, for morbidity/mortality. All surviving animals were killed on day 15. No other measurements/procedures were conducted.

Single-dose study

Using a computer-generated randomization procedure, 30 males and 30 females were randomized by sex into dose groups ($n = 10$ per sex per group) on the basis of body weight. KF was administered once by i.v. bolus injection into the lateral tail vein on day 1 at 0 (vehicle control), 150, and 300 μg/kg (900 and 1800 μg/m^2, respectively) in a dose volume of 1.2 ml/kg. In addition to regular observations described above, animals underwent a physical examination once weekly. Body weights were determined at randomization, on day 1, and twice weekly thereafter (including termination). Blood was collected and clinical pathology parameters

measured in five rats per sex per group on days 4, 15, and 29. Hematology measurements included red blood cell parameters, leukocyte count and differential, and platelet and reticulocyte counts. Clinical chemistry parameters included alanine aminotransferase (ALT), albumin, alkaline phosphatase (ALKP), total bile acids, globulin, albumin/globulin ratio (A/G), glucose, phosphate, potassium, sodium, sorbital dehydrogenase (SDH), calcium, chloride, cholesterol, creatinine, total protein, triglycerides, and urea nitrogen (BUN). Five rats per sex per group were necropsied on days 4 and 29. The necropsy procedure was a thorough examination of the viscera and carcass, and the collection and fixation of approximately 40 tissues. In addition, the adrenals, brain, heart, kidneys, liver, lungs, ovaries, spleen, and testes/epididymides were weighed. All collected tissues were examined microscopically by a veterinary pathologist.

Multiple-dose study

Using a computer-generated randomization procedure, 40 males and 40 females were randomized by sex into dose groups ($n = 10$ per sex per group) on the basis of body weight. KF was administered once daily for five consecutive days (starting on day 1) by i.v. bolus injection into the lateral tail vein at 0 (vehicle control), 10, 30, and 80 μg/kg per day (60, 180, and 480 μg/m^2 per day, respectively). The dose volume was 1.0 ml/kg per day. Body weights were determined at randomization, on days 1–5, twice weekly thereafter, and at termination. Animals were observed for clinical signs and physical examinations were conducted as in the single-dose study. Blood was collected on days 8, 18, and 32 from five rats per sex per group, and hematology and clinical chemistry parameters measured as in the single-dose study. Five rats per sex per group were necropsied on days 8 and 32, tissues collected and fixed, and organ weights determined as previously described. All tissues collected from animals in the vehicle control and high-dose groups, along with the injection sites (tail vein) from low- and medium-dose animals necropsied on day 32, were examined microscopically by a veterinary pathologist.

Statistical analyses

Linear regression analysis of mortality data in the MTD study (dose and log dose plotted) was performed to estimate the LD_{10} and LD_5. The LD_{50} was based upon the incidence of 50% mortality in each sex and the MTD was estimated based upon the LD_5 to LD_{10} range. For animals in the single- and multiple-dose studies, analysis of variance tests were conducted on body weight, hematology, clinical chemistry, and organ weight data. Organ weight analysis considered weights relative to brain weight (percent of brain weight). If a significant F ratio was obtained ($P \leq 0.05$), Dunnett's t-test was used for pair-wise comparisons with the concurrent control group.

Results

Maximum tolerated dose study

KF administered i.v. produced mortality in two of four, three of four, and four of four males at 375, 450, and 600 μg/kg, respectively, and in one of four and two of four females at 450 and 600 μg/kg, respectively. Deaths occurred on the day of dosing with the exception of one male (on day 2) in the 375-μg/kg group. Clinical signs of toxicity were seen on day 1, and to a lesser extent on day 2, and included ataxia, tremors, labored breathing, increased respiratory rate, decreased activity, and piloerection. These signs suggest that KF elicited nervous system effects, which were the likely cause of death. The LD_{50} in males was 375 μg/kg and the LD_{50} in females was 600 μg/kg, suggesting a sex difference. Linear regression analysis of the mortality data determined that the LD_5 to LD_{10} range in males was 270–300 μg/kg and in females was 380–410 μg/kg. Based upon these results, the single-dose MTD was estimated to be 300 μg/kg.

Single-dose study

Based upon the results of the MTD study, doses of 150 and 300 μg/kg were selected for the single-dose study. No deaths occurred and clinical signs of toxicity were seen on day 1 only at 300 μg/kg. Clinical signs included decreased activity in all males and one female, agitation (observed as excessive head shaking and vocalization) in all females, and piloerection in one female. Animals in the 300-μg/kg group had generally lost a few grams of body weight by day 4 (approximate weight loss of 1% from day 1), whereas weight gain occurred in the control and 150-μg/kg groups (data not shown). Recovery from the weight changes occurred thereafter.

Clinical chemistry changes occurred in the 300-μg/kg group only (significant changes indicated by Dunnett's t-test). On day 4, BUN was increased by 282% and 458% in males and females, respectively, and serum creatinine was increased by 56% in males and 141% in females (Table 1). Serum bile acids were increased by 115% in males and 94% in females on day 4. Also on day 4, decreases in serum albumin (9–14%) were seen in both males and females, and serum globulin was increased in females resulting in a decreased A/G ratio. Females on day 4 also had increased serum cholesterol (48%) and slightly decreased serum chloride levels. On day 15, serum albumin was nonsignificantly decreased in females in the 300-μg/kg group, resulting in a significant decrease in A/G ratio. However, the other changes seen on day 4 had resolved by day 15. No other changes in clinical chemistry parameters were seen and complete recovery had occurred by day 29. Decreases in lymphocyte counts were seen on day 15 in females only at 300 μg/kg (Table 1). This change was transient and no other changes in hematology parameters were seen.

Changes in organ weights were seen in the 300-μg/kg group only (Table 2). On day 4, weights of the kidneys were increased by 33% and 45% in males and females, respectively. Weights of the heart and liver were decreased by 9% and 17%, respectively, in males on day 4, most likely due to body weight loss. Weights of the adrenal gland were increased by 16% in the females on day 4, possibly due to a stress response. On day 29, kidney weights were still increased in the females (18%), but not in the males.

Drug-related gross lesions were seen on day 4 in the 300-μg/kg group only, which included pale pigmentation of kidneys in three of five males and one of five females. These observations were related to the renal lesions

Table 1. Select clinical chemistry and hematology parameters in the single-dose study. Values are means ± SD (*A*/*G* albumin/globulin)

Parameter	Males			Females		
	Dose (μg/kg)			Dose (μg/kg)		
	0	150	300	0	150	300
BUN (mg/dl)						
Day 4	17 ± 2	19 ± 2	65 ± 37*	19 ± 2	21 ± 3	106 ± 87*
Day 15	17 ± 2	17 ± 2	17 ± 1	19 ± 2	16 ± 1	18 ± 2
Creatinine (mg/dl)						
Day 4	0.34 ± 0.05	0.33 ± 0.04	0.53 ± 0.16*	0.41 ± 0.04	0.39 ± 0.05	0.99 ± 0.64
Day 15	0.32 ± 0.05	0.36 ± 0.04	0.40 ± 0.05	0.41 ± 0.04	0.37 ± 0.04	0.38 ± 0.03
Bile acids (mg/dl)						
Day 4	46 ± 27	38 ± 27	99 ± 51	34 ± 18	32 ± 15	66 ± 24*
Day 15	50 ± 38	52 ± 26	43 ± 13	49 ± 38	22 ± 15	59 ± 58
Cholesterol (mg/dl)						
Day 4	82 ± 5	81 ± 12	99 ± 18	71 ± 6	85 ± 12	105 ± 18*
Day 15	63 ± 9	63 ± 10	67 ± 8	73 ± 11	72 ± 11	72 ± 7
Chloride (mmol/l)						
Day 4	106 ± 1	105 ± 2	104 ± 0.2	107 ± 2	105 ± 1	102 ± 4*
Day 15	106 ± 2	105 ± 3	105 ± 1	107 ± 2	107 ± 2	108 ± 2
Albumin (g/dl)						
Day 4	4.6 ± 0.3	4.7 ± 0.04	4.2 ± 0.2*	5.2 ± 0.2	4.9 ± 0.3	4.5 ± 0.3*
Day 15	4.5 ± 0.2	4.5 ± 0.4	4.5 ± 0.1	5.3 ± 0.2	5.1 ± 0.2	4.9 ± 0.4
Globulin (g/dl)						
Day 4	1.8 ± 0.1	1.8 ± 0.2	1.9 ± 0.2	1.6 ± 0.1	1.7 ± 0.2	2.0 ± 0.2*
Day 15	1.9 ± 0.1	1.8 ± 0.2	1.9 ± 0.1	1.6 ± 0.2	1.8 ± 0.2	1.7 ± 0.1
A/G ratio						
Day 4	2.58 ± 0.29	2.67 ± 0.22	2.24 ± 0.22	3.20 ± 0.17	2.83 ± 0.28	2.23 ± 0.29*
Day 15	2.41 ± 0.25	2.54 ± 0.12	2.45 ± 0.16	3.36 ± 0.28	2.91 ± 0.39	2.84 ± 0.14*
Lymphocytes (10^3/μl)						
Day 15	9.5 ± 2.0	9.3 ± 2.6	8.3 ± 2.3	8.3 ± 2.9	5.7 ± 4.0	4.7 ± 3.8

*$P < 0.05$ vs respective control

Table 2. Select organ weights in the single-dose study. Weights are expressed as percent of brain weight. Values are means ± SD

Parameter	Males			Females		
	Dose (μg/kg)			Dose (μg/kg)		
	0	150	300	0	150	300
Kidneys						
Day 4	132 ± 16	135 ± 5	175 ± 28*	95 ± 5	95 ± 9	138 ± 23*
Day 29	165 ± 34	169 ± 24	169 ± 21	101 ± 8	106 ± 6	119 ± 10*
Adrenals						
Day 4	2.16 ± 0.15	2.47 ± 0.35	2.63 ± 0.66	2.90 ± 0.29	3.16 ± 0.13	3.36 ± 0.21*
Day 29	3.17 ± 0.56	3.26 ± 0.36	3.15 ± 0.29	4.28 ± 0.22	4.23 ± 0.63	3.98 ± 0.85
Heart						
Day 4	53 ± 3	54 ± 2	48 ± 4*	42 ± 2	42 ± 4	41 ± 1
Day 29	67 ± 8	75 ± 14	67 ± 4	49 ± 5	50 ± 2	l47 ± 3
Liver						
Day 4	696 ± 69	665 ± 59	575 ± 72*	446 ± 23	469 ± 33	480 ± 19
Day 29	806 ± 141	796 ± 95	771 ± 86	508 ± 44	523 ± 41	475 ± 48

*$P < 0.05$ vs respective control

subsequently described. The kidneys were the primary target organ for toxicity in the study. On day 4, injury to distal convoluted tubules and cytomegaly in the outer medulla were seen, in a dose-dependent fashion, in males in the 300-μg/kg group, and females in the 150-μg/kg and 300-μg/kg groups (Table 3). Injury to distal convoluted tubules (minimal to marked in severity) was the most significant lesion and was characterized by a uniform presence of rays of basophilic staining of distal convoluted tubules and collecting ducts in the outer medulla and inner cortex. Features of the altered tubules included regions of thin, attenuated epithelium, basophilic granular material (mineralization) in the tubule lumen, and an increased incidence of mitotic figures in

Table 3. Drug-related lesions on day 4 in the single-dose study showing the incidence and (in parentheses) the mean group severity scores (1 minimal, 2 mild, 3 moderate, 4 marked). Five animals per sex per group were necropsied on day 4 (– lesion not observed)

Lesion	Males			Females		
	Dose (μg/kg)			Dose (μg/kg)		
	0	150	300	0	150	300
Kidney						
Injury, distal convoluted tubule	–	–	4/5 (2.4)	–	3/5 (0.6)	5/5 (3.4)
Cytomegaly, outer medulla	–	–	4/5 (2.2)	–	2/5 (0.4)	5/5 (3.4)
Mineralization, tubule epithelium	–	–	2/5 (1.0)	–	–	5/5 (2.2)
Inflammation, focal, subacute, necrotizing	–	–	–	–	–	2/5 (0.6)
Thyroid gland						
Colloid depletion	–	–	4/5 (2.2)	–	1/5 (0.2)	5/5 (2.4)
Bone (femur)						
Peritrabecular osteocyte hyperplasia	–	–	2/5 (0.8)	–	–	2/5 (0.8)
Bone marrow (femur)						
Inflammation, subacute, necrotizing	–	–	2/5 (0.8)	–	–	3/5 (1.0)
Tail vein (injection site)						
Basophilic granular material	–	1/5 (0.2)	2/5 (0.8)	–	3/5 (0.8)	4/5 (1.0)
-perivascular necrosis	1/5 (0.2)	1/5 (0.2)	2/5 (0.6)	–	2/5 (0.4)	4/5 (1.2)

the basophilic tubule epithelium. Cytomegaly was defined as enlarged basophilic cells in the outer medulla. Mineralization of the distal tubule epithelium was observed at 300 μg/kg only.

Also on day 4, focal subacute necrotizing inflammation in the kidney was observed in two of five females at 300 μg/kg. This lesion was characterized by focal loss of normal architecture in the outer medulla with neutrophils and macrophages in the foci. On day 29, renal nephropathy, characterized by the presence of foci of basophilic staining tubule epithelium in the cortex and an occasional eosinophilic cast in the lumen of affected nephrons, was observed in all groups, including the vehicle control (Table 4). However, mild nephropathy was only present in the 300-μg/kg group, whereas minimal nephropathy, considered to be an incidental finding, was present in the control and 150-μg/kg groups. Therefore, nephropathy was considered to be drug-related at 300 μg/kg. The incidence and severity of chronic inflammation, characterized by infiltration of lymphocytes in the interstitial tissue around basophilic renal tubules, were slightly increased in females at 300 μg/kg, and considered to be secondary to the mild nephropathy present in these animals.

Subacute necrotizing inflammation of bone marrow (femoral) and peritrabecular osteocyte hyperplasia of bone (femur) were seen on day 4 in males and females at 300 μg/kg (Table 4). Subacute necrotizing inflammation was characterized by the replacement of normal bone marrow with mature neutrophils, lymphocytes, fibroblasts and macrophages. This lesion occurred at the metaphysis and resulted in a notably lighter staining region of bone marrow. Peritrabecular osteocyte hyperplasia was characterized by the presence of a layer of hypertrophied osteocytes one to three cells thick at the interface between trabeculae of metaphysis and bone marrow. The osteocyte hyperplasia was associated with subacute necrotizing inflammation of the adjacent bone marrow. It is not known if the slight lymphopenia observed in females at 300 μg/kg was related to these lesions. Recovery from the bone and bone marrow changes was seen by day 29.

Colloid depletion in the thyroid gland was seen on day 4 in males at 300 μg/kg, and females at ≥150 μg/kg (Table 3). This lesion was characterized by a reduced size of most thyroid follicles, increased basophilia and thickness of follicular epithelial cells, and the occasional presence of degenerated epithelial cells in the follicular lumen. This change can occur with increased secretion of

Table 4. Drug-related lesions on day 29 in the single-dose study showing the incidence and (in parentheses) the mean group severity scores (1 minimal, 2 mild, 3 moderate, 4 marked). Five animals per sex per group were necropsied on day 29 (– lesion not observed)

Lesion	Males			Females		
	Dose (μg/kg)			Dose (μg/kg)		
	0	150	300	0	150	300
Kidney						
Nephropathy	1/5 (0.2)	3/5 (0.6)	5/5 (1.6)	3/5 (0.6)	1/5 (0.2)	5/5 (1.6)
Chronic inflammation	1/5 (0.2)	1/5 (0.2)	2/5 (0.4)	–	1/5 (0.2)	3/5 (0.6)
Tail vein (injection site)						
Fibrosis	–	–	2/5 (0.6)	–	–	1/5 (0.2)

thyroid hormones, but the biological significance of this morphological change was equivocal and not considered to be toxicologically significant. This change was not observed on day 29.

Perivascular necrosis and basophilic granular material of the tail vein were observed in a dose-dependent fashion on day 4 in KF-treated males and females (Table 3). Perivascular necrosis was characterized by a lack of differential staining, light eosinophilic staining, and occasional fragments of cell debris. The presence of this lesion in one control male was likely due to local tissue trauma resulting from i.v. administration of the vehicle. The presence of basophilic granular material could represent mineralization of lipid released from damaged lipocytes, possibly following leakage of drug during or after dose administration. On day 29, fibrosis of the tail vein, characterized by the presence of eosinophilic staining of collagen in the affected perivascular tissue, was seen in two of five males and one of five females in the 300-μg/kg group (Table 4). Fibrosis was likely a response to the tissue injury observed on day 4 and is considered a lesion of minimal biological significance.

Multiple-dose study

A summary of drug-related effects is presented in Table 5. No deaths occurred and drug-related clinical signs of toxicity were not seen. Body weight changes were not seen during the dosing period. At the end of the study (day 32), total weight gain was decreased by 20%, resulting in a mean body weight that was significantly decreased by 10% in males at 80 μg/kg per day, compared to the control group. Body weight changes were not seen in females. Serum globulin levels were slightly, but significantly, increased on day 8 in males at 80 μg/kg per day, resulting in a decreased A/G ratio. Serum glucose was increased on day 8 in females at 80 μg/kg per day. These clinical chemistry changes are not considered toxicologically significant and resolution had occurred by day 18. Liver weight (as a percent of brain) was decreased by 17% in males at 80 μg/kg per day on day 32, but this change was not observed on day 8 or in females, and likely reflects the decreased body weight of these animals (Table 5). Drug-related gross and histological lesions were not seen. Minimal to mild perivascular fibrosis at the injection site (tail vein), characterized by an increased proportion of collagen connective tissue in the perivascular stroma, was seen on day 32 in several KF-treated animals. This change was not seen in a dose-dependent fashion and was not supported by other histological changes (such as inflammation or necrosis) at the injection site (including on day 8). Therefore, fibrosis was interpreted as an indication of irritant drug leakage, likely from the needle tip, during or after dose administration.

Discussion

KF is a new cytotoxic anticancer agent that has demonstrated activity against various solid tumors in preclinical investigations. The current studies were conducted to characterize the toxicity associated with

Table 5. Drug-related effects in the multiple-dose study. KF was administered once a day for 5 days. Values are means ± SD (*A*/*G* albumin/globulin)

Parameter	Males				Females			
	Dose (μg/kg/day)				Dose (μg/kg/day)			
	0	10	30	80	0	10	30	80
Body weight (g)								
Day 1	275 ± 10	276 ± 9	274 ± 14	271 ± 11	196 ± 9	193 ± 8	194 ± 12	193 ± 10
Day 32	463 ± 11	453 ± 19	434 ± 24	418 ± 32*	267 ± 19	256 ± 18	269 ± 24	261 ± 22
Albumin (g/dl)								
Day 8	4.4 ± 0.2	4.3 ± 0.2	4.2 ± 0.1	4.4 ± 0.2	4.7 ± 0.3	5.0 ± 0.2	4.9 ± 0.2	4.8 ± 0.2
Day 18	4.3 ± 0.2	4.2 ± 0.1	4.4 ± 0.1	4.3 ± 0.3	4.7 ± 0.2	4.8 ± 0.2	4.7 ± 0.3	4.7 ± 0.1
Globulin (g/dl)								
Day 8	1.7 ± 0.2	1.8 ± 0.1	1.8 ± 0.2	2.0 ± 0.1*	1.8 ± 0.1	1.9 ± 0.0	1.9 ± 0.2	1.8 ± 0.1
Day 18	2.1 ± 0.2	1.9 ± 0.1	2.1 ± 0.2	2.1 ± 0.3	2.0 ± 0.2	1.8 ± 0.2	2.0 ± 0.1	2.0 ± 0.2
A/G ratio								
Day 8	2.66 ± 0.30	2.36 ± 0.09	2.37 ± 0.22	2.21 ± 0.13*	2.53 ± 0.10	2.67 ± 0.12	2.64 ± 0.27	2.62 ± 0.12
Day 18	2.08 ± 0.23	2.18 ± 0.13	2.07 ± 0.14	2.12 ± 0.29	2.42 ± 0.15	2.71 ± 0.12*	2.36 ± 0.19	2.36 ± 0.18
Glucose (mg/dl)								
Day 8	162 ± 22	142 ± 5	138 ± 15	136 ± 12	124 ± 8	138 ± 10	132 ± 8	178 ± 43*
Day 18	139 ± 24	143 ± 22	128 ± 8	134 ± 19	137 ± 17	139 ± 30	137 ± 19	127 ± 10
Liver weight[a]								
Day 32	948 ± 41	918 ± 41	888 ± 50	788 ± 116*	561 ± 47	540 ± 87	570 ± 95	1574 ± 52

*$P < 0.05$ vs respective control
[a]Expressed as percent of brain weight

i.v. administration of KF and to support initiation of clinical trials.

Initially, male and female rats received a single i.v. dose of KF to determine the MTD. Lethality was produced at ≥375 μg/kg, generally occurred on the day of dosing, and was accompanied by ataxia, tremors, labored breathing, increased respiratory rate, and decreased activity. These results suggest that KF elicits nervous system effects, possibly central, as a mechanism for lethality. Nervous system effects were further indicated by the observation of transient, excessive head shaking (likely tremors) and vocalization in females at 300 μg/kg. Although histological evidence for neurotoxicity was not seen, KF may elicit a pharmacological effect on the nervous system, or produce microscopic lesions not visible using light microscopy. Additional studies will be needed to further clarify KF-nervous system interactions. Amino acid analysis has demonstrated sequence similarity between KF and a precursor of Botulinum neurotoxin type F, an endopeptidase that cleaves the synaptic vesicle membrane protein VAMP/synaptobrevin at a glutamine-lysine bond [4]. Botulinum neurotoxin is produced by *Clostridium botulinum* and induces paralysis by inhibiting the release of acetylcholine at the neuromuscular junction [1, 8]. Recently, in vitro cell culture studies have indicated that 10 μ*M* KF can produce cytotoxicity to central nervous system neurons but not to astrocytes or sensory and motor neurons [6].

The kidneys were the primary target organ for KF toxicity following single administration at nonlethal doses. On day 4, injury to distal convoluted tubules and cytomegaly in the outer medulla were seen in males at 300 μg/kg and females at ≥150 μg/kg. Injury to distal convoluted tubules was the most toxicologically significant lesion seen in the study. Other renal lesions observed on day 4 included mineralization of the distal tubule epithelium and focal subacute necrotizing inflammation at 300 μg/kg. The incidence and severity of the renal lesions were greatest in females. These renal lesions correlated with biologically significant increases in BUN and serum creatinine, and increased kidney weights, on day 4 at 300 μg/kg. Decreased serum chloride on day 4 in females at 300 μg/kg may represent renal loss of this ion. These results indicate impairment of renal function in the 300-μg/kg group. Although renal lesions were observed in some females at 150 μg/kg, those lesions were of minimal severity and did not result in clinical chemistry or kidney weight changes. Changes in clinical chemistry parameters indicative of renal dysfunction were not seen on day 15, indicating resolution of renal injury. This was confirmed on day 29, as mild nephropathy and chronic inflammation were the only lesions seen in the 300-μg/kg group. Although kidney weights were still increased in females at 300 μg/kg on day 29, the increases were less than on day 4. Complete resolution of the renal changes occurred in the 150-μg/kg group. These results indicate that i.v. administration of KF results in acute but reversible renal injury. The mechanism for KF-induced renal toxicity is unknown and is under further investigation.

Single-dose i.v. administration of KF resulted in local injury to the tail vein. Perivascular necrosis and basophilic granular material were observed on day 4 at ≥150 μg/kg, and indicate that KF produced direct cytotoxicity to the vasculature and surrounding tissue at the injection site. By day 29, these lesions had resolved in the 150-μg/kg group but progressed to fibrosis in the 300-μg/kg group. Although fibrosis is considered to be of minimal biological significance, its presence indicates that greater tissue injury occurred at 300 μg/kg. Although perivascular fibrosis at the injection site was seen in a few animals following multiple administrations of KF, the relationship to the drug was uncertain because it did not occur in a dose-dependent manner and was not supported by other histological changes at the injection site. These results suggest that vascular injury produced by KF is dependent upon the local drug concentration administered i.v. rather than upon the total dose given. In the single-dose study, the lowest drug concentration administered was 125 μg/ml, whereas the highest drug concentration administered in the multiple-dose study was 80 μg/ml.

In the single-dose study, serum total bile acids, cholesterol, and globulin were increased, and albumin was decreased at 300 μg/kg, primarily on day 4. Although these changes suggest altered hepatic function (possibly a cholestatic event), serum levels of liver enzymes were not elevated and histological evidence of liver injury was not seen. These changes were transient and not observed on day 29.

In the multiple-dose study, the slight decrease in body weight gain in males at 80 μg/kg per day was the primary treatment-related effect. Although minimal clinical chemistry changes were seen at this dose, the toxicological significance was equivocal as tissue injury and other indices of toxicity were not seen. KF administered at 80 μg/kg per day for 5 days, which is equivalent to a total dose of 400 μg/kg, is greater than the single lethal dose (375 μg/kg) and the single MTD dose (300 μg/kg). These results demonstrate that fractionation of a lethal or MTD dose of KF by daily administration for 5 days reduces drug toxicity. The mechanism for this may be due to the pharmacokinetics of KF. Preliminary pharmacokinetic data in mice demonstrate that KF administered i.v. is rapidly cleared from plasma, has limited binding to extravascular tissues, and does not accumulate upon repeated dosing [2]. Furthermore, in a phase I clinical trial in cancer patients, the terminal half-life of KF administered i.v. was 0.46 h [12]. These results suggest that daily i.v. administration of KF can be performed due to the rapid clearance of the drug and apparent absence of cumulative toxicity.

In conclusion, the minimal lethal dose following a single i.v. administration was 375 μg/kg (2250 μg/m^2). The nervous system appeared to be a potential site of action for the mechanism of lethality. In general, reversibility of toxicity was seen at 150 μg/kg, but not at

300 μg/kg (900 μg/m^2 and 1800 μg/m^2, respectively). When KF was administered once daily for 5 days, the no-adverse-effect level was at or near 80 μg/kg per day (480 μg/m^2 per day). These results indicate that KF can be safely administered in multiple daily doses and that toxicity does not appear to be cumulative. KF has recently entered clinical trials in patients with androgen-refractory prostate cancer utilizing a schedule consisting of a 1-h i.v. infusion once daily for five consecutive days every 3 weeks [12].

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