

Safety Profile of Thalidomide after 53 Weeks of Oral Administration in Beagle Dogs

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Fifty-six adult beagle dogs (28 male, 28 female) were orally administered thalidomide at 43, 200, or 1000 mg/kg/day for 53 weeks. Sixteen (2/sex/dose group) and 32 (4/sex/dose group) dogs were euthanized and necropsied after 26 and 53 weeks of dosing, respectively. The remaining 8 animals (2/sex/group; high-dose and control groups) were dosed for 53 weeks, euthanized, and necropsied at 58 weeks after a 5-week recovery period. There were no deaths during the study. The only observed clinical signs attributable to thalidomide administration were green-colored urine, white-colored fecal residue presumed to be unchanged thalidomide, enlarged and/or blue coloration of female mammary tissue, and prolonged estrus. There were no thalidomide-related changes in body weights, food consumption, electrocardiography, ophthalmoscopy, neurological function, and endocrine function. The mostly slight and/or transient variations observed in some hematology and blood chemistry values of dosed dogs were considered to be toxicologically insignificant and were supported by the lack of histopathologic correlates. The only gross finding attributable to thalidomide was a yellow-green discoloration of the femur, rib, and/or calvarium that was observed at each euthanization interval including recovery. There was no microscopic correlate for this finding. No thalidomide-related microscopic changes were seen in any of the organs and tissues at 26 weeks. Mammary duct dilatation and/or glandular hyperplasia observed in females at 53 and 58 weeks and hepatic bile pigment exhibited by high-dose males at 53 weeks were microscopic changes considered to be thalidomide-related. There was no gross and histopathologic evidence of any tumors. In summary, thalidomide at up to 1000 mg/kg/day for 53 weeks did not induce any major systemic toxicity or tumors in dogs. The NOAEL was 200 mg/kg/day.

Key Words: thalidomide; beagle dogs; chronic toxicity, oral; 53 weeks.

Thalidomide [α (N-pthalimido)glutarimide] was widely used in Europe as a nonbarbiturate sedative-hypnotic. It was withdrawn from the market in 1961 when its teratogenic effect

first became evident in humans (McBride, 1961). Approximately 5000–12000 babies from 46 countries were born with various external and internal deformities including phocomelia, deafness, facial and oculomotor paralysis, and cardiac, uterine, and vaginal malformations (Calabrese, 1999; Mellin and Katzenstein, 1962; Gardner-Medwin, 1996). It is not known how many babies died as a result of thalidomide. Thalidomide is currently in use in Mexico and South America, where cases of its teratogenicity are still being reported (Castilla *et al.*, 1996). In 1965 thalidomide was found to be effective in treating erythema nodosum leprosum (ENL), an acute inflammatory reaction of lepromatous leprosy (Sheskin, 1965). An application for approval as a sedative in the United States was submitted by the Richardson-Merrell company in 1960. Frances Kelsey, the U.S. Food and Drug Administration (U.S. FDA) reviewer, was concerned about reports of peripheral neuritis and requested additional data on fetal safety and action of thalidomide in humans (Kelsey, 1988). The company was not forthcoming and the drug was never approved. The thalidomide tragedy gave rise to greater enforcement powers for the U.S. FDA through the 1962 Kefauver-Harris Drug Amendments to the Food, Drug, and Cosmetic Act of 1938. These amendments were the impetus for the current stricter regulatory process that requires greater safety and efficacy data for new drug entities.

Even though thalidomide was banned in the United States, it has been available on an investigational basis for the treatment of various inflammatory conditions such as ENL, AIDS wasting, rheumatoid arthritis, graft-versus-host disease, Behçet's syndrome, cutaneous lupus, and refractory aphthous ulcerations in AIDS patients (Georghiou and Allworth, 1992; Gunzler, 1992; Vogelsang *et al.*, 1992; Youle *et al.*, 1989). The fever, weight loss, and debility associated with these diseases are thought to be due to the production of cytokines such as tumor necrosis factor α (TNF- α) by macrophages (Girardin *et al.*, 1988; Tracey *et al.*, 1988). Thalidomide's anti-inflammatory and immunomodulatory mechanism of action has been shown to be due to the degradation of mRNA encoding the TNF- α protein in monocytes (Moreira *et al.*, 1993). Thalido-

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mide has also been found to have potent antiangiogenic activity (D'Amato *et al.*, 1994). The combination anti-inflammatory and antiangiogenic actions make it an important therapeutic agent with significant potential in various debilitating diseases. In 1998 the U.S. FDA approved thalidomide (Thalomid®) for the treatment of ENL. Thalidomide is currently being evaluated in clinical trials for various cancers, Crohn's disease, and cancer cachexia (Glass *et al.*, 1999; Vasilias *et al.*, 1999; Watanabe *et al.*, 1999). It recently showed significant antitumor activity in refractory multiple myeloma and almost eliminated the gastrointestinal side effects from irinotecan (Campstar) chemotherapy in metastatic colorectal cancer (Govindarajan *et al.*, 2000; Singhal *et al.*, 1999).

Long-term use of thalidomide in humans has been associated with peripheral neuropathy (Ochonisky *et al.*, 1994). Some patients taking thalidomide have experienced peripheral neurological disturbances such as hypo- and hyperalgesia, impaired temperature sensitivity, impaired vegetative functions, and polyneuritis (Gunzler, 1992). There is evidence of significant species differences in the metabolism and teratogenicity of thalidomide. Different colored hydrolysis chromophores were detected in urine of rabbits, rats, mice, and guinea pigs. The formation of these products is thought to be due to spontaneous hydrolysis (Schumacher *et al.*, 1965a). Thalidomide produces variable teratogenic responses in rabbits. Certain primates produce live fetuses with defects similar to humans (Hendrickx and Peterson, 1997). Rodents are somewhat resistant, responding with increased resorptions (Schumacher *et al.*, 1968; Szabo and Steelman, 1967). If a nonrodent species such as rabbit had also been used in the initial testing, the teratogenicity of thalidomide would have been evident and it would not have been approved in Europe. Although studies on thalidomide analogs found that an intact phthaloyl or phthalimidine group is required for teratogenicity, the mechanism of action has not been determined, even though numerous mechanisms have been postulated (Helm *et al.*, 1981; Schumacher, 1975; Stephens, 1988). More recent work has suggested mechanisms of action involving inhibition of neovascularization in fetal limbs and free radical oxidation of embryonic macromolecules (D'Amato *et al.*, 1994; Parman *et al.*, 1999). It is not known if the terata are caused by thalidomide, its enantiomers, or hydrolysis products.

Despite the 40 years that thalidomide has been studied and in restricted human use, there is still a lack of chronic toxicity data (with the exception of reproductive toxicology) generated under modern protocols and performed under the U.S. FDA's Good Laboratory Practice regulations. Previous studies in various species are dated, lacked comprehensive measurements, or were replete with discrepancies (Kunz *et al.*, 1956; Somers, 1960; Locker *et al.*, 1971). Subchronic rodent toxicology studies were recently completed to fill this data gap (Teo *et al.*, 1999a). The current study was performed to fulfill regulatory requirements for chronic toxicity testing in a nonrodent species. The main aim of the study was to determine the systemic

toxicity in dogs after single daily oral doses for 53 weeks. Interim, terminal, and recovery sacrifices were performed at 26, 53, and 58 weeks, respectively. The potential for development of any tumors was also assessed.

MATERIALS AND METHODS

Animals. This study was conducted in compliance with the Good Laboratory Practice Regulations (21 CFR 58) and the Animal Welfare Act of 1970 for the care of animals. Twenty-eight male and 28 female beagle dogs approximately 8–10 months of age were obtained from White Eagle Laboratories, Inc. (Doylestown, PA). They were housed individually in stainless steel pens and maintained according to currently acceptable practices of good animal husbandry (NIH, 1985). They were acclimated for approximately 4 weeks prior to the initiation of dosing. Dogs were fed approximately 300 g Certified Purina Laboratory Canine Diet #5007 (Ralston Purina Co., St. Louis, MO) once daily and municipal water was supplied *ad libitum*. Residual food from the previous day was removed and food consumption was recorded approximately 1 h prior to dosing. All dogs were fed each day approximately 1 h after oral dosing. Dogs were ranked by body weight according to sex and assigned to dose groups and length of time (26, 52, or 58 weeks) on study using a computer-generated table of random numbers.

Environmental conditions. Temperature and relative humidity in animal rooms were regularly measured and ranged between 16–26°C and 41–100%, respectively. The high humidity was due to room cleanings. A 12-h light/12-h dark cycle was used throughout the study.

Thalidomide. Thalidomide is a white powder stable at room temperature and insoluble in water. Four batches of thalidomide powder with an average purity of 100% (99.3, 100, 100, and 100%) were used. Purity was determined by HPLC. The HPLC system (LCM-1, Waters, Milford, MA) was equipped with a 33 × 4.6 mm MICRA NPS C18 (1.5 µm) column (MICRA Scientific, Northbrook, IL). Each run was performed isocratically using a 1% acetic acid mobile phase at a flow rate of 1.0 ml/min. Peaks were monitored using a Waters 486 UV detector set to monitor at 230 nm. Phenacetin was used as the internal standard. Thalidomide eluted at approximately 3.30 min. Thalidomide powder was stored at room temperature in closed airtight containers and protected from light. It was weighed and packed into size 11 gelatin capsules (Torpac, Inc., East Hanover, NJ) at least once weekly.

Twenty-eight-day dose selection study. Doses for the 53-week study were selected after reviewing toxicity and toxicokinetic data from a 28-day oral range-finding toxicity study. Five male and five female dogs were divided into 5 groups of 2 dogs per group. Dogs (1/sex/group) were dosed once daily with capsules containing 12, 100, 1000, or 2000 mg/kg thalidomide. Capsules were prepared based on most recent body weights. Control dogs were given empty capsules. Parameters monitored were clinical observations, body weights, food consumption, clinical pathology (hematology, serum chemistry, urinalysis), gross and histopathology, and absolute and relative organs weights. Clinical observations were performed daily, body weights and food consumption were performed at week –1 and weekly thereafter and clinical pathology at weeks –1 and 4. Discolored (green and brown) urine was observed in all thalidomide-dosed dogs, with the duration increasing with dose. At the 12 mg/kg dose, 1 dog had discolored urine 2 out of 29 days, the other, 6 out of 29; at 100 mg/kg, both dogs were affected 24 out of 29 days; at 2000 mg/kg, the incidence was greater than 28 days. White residue, presumably unabsorbed thalidomide, was also observed in the feces of all dogs dosed with 1000 and 2000 mg/kg, with the duration increasing with dose. At 1000 mg/kg, white residue was observed in the feces 14 and 19 days out of 29 and at 2000 mg/kg, 25 and 28 days. There were no changes in the body weight, food consumption, and hematologic parameters in any of the dogs. The only significant clinical pathology finding was a 14–30% decrease in serum glucose levels in thalidomide-treated dogs at 29 days. There were no changes in organ weights, gross pathology, and histopathology. Toxicokinetic data indicated that oral doses beyond 1000

mg/kg/day did not increase systemic exposure to thalidomide in dogs. Based on these results, 1000 mg/kg was chosen as the high dose. Assuming a 10-kg dog, 43 mg/kg was chosen as the low dose to reflect the high end of the human dose (200–400 mg/day) for ENL. The mid dose was a multiple of this at 200 mg/kg.

Study design. The 56 dogs were divided into 3 groups based on the phase of the study. The first group represented the interim phase consisting of 16 dogs (8 males and 8 females). They were divided into 4 subgroups of 2 males and 2 females for the control, low (43 mg/kg), mid (200 mg/kg), and high (1000 mg/kg) doses and were euthanized at 26 weeks. The second group was the terminal phase consisting of 32 dogs (16 males and 16 females). They were similarly divided into 4 subgroups of 8 dogs (4 males and 4 females) each and were euthanized at 53 weeks. The third group consisted of 8 dogs and was the 4-week recovery phase with euthanization at 58 weeks. The dogs were divided into 2 subgroups of 4 dogs (2 males and 2 females) for the control and high dose. All dogs were dosed once daily by capsule. Control dogs were given empty capsules.

Antemortem observations and measurements. A series of pre necropsy observations and measurements were performed in the study. These included the following:

- Clinical observation: each animal was examined twice daily for moribundity and changes in general appearance and behavior.
- Body weight: dogs were weighed on day –12, day –2, and once weekly thereafter and just prior to necropsy.
- Food consumption: the amount of food consumed was qualitatively determined by estimating the fraction remaining. Consumption was recorded daily throughout the pretest week –1, dosing, and recovery periods.
- Ocular examination: this was done on pretest week –4 and during weeks 25 and 51 by a board-certified veterinary ophthalmologist. Pupillary reflexes were tested in subdued light, followed by inspection of the anterior regions of the eye. The pupils were then dilated (1% USP tropicamide ophthalmic solution, Sight Pharmaceuticals, Inc., Tampa, FL) and focally illuminated, and the refractive elements of the eyes and the fundus were examined by indirect ophthalmoscopy.
- Physical examination: the rectal temperature, respiration rate, and electrocardiogram (EKG) were taken during pretest week –1 and weeks 25 and 52 on the 53-week dogs. EKGs were performed by a board-certified veterinary cardiologist. The electrocardiographic leads consisted of limb leads I, II, III, aVR, aVL, and aVF, and chest leads rV₂, V₂, V₄, and V₁₀. All EKGs were recorded using Cambridge MC 6400 analog (Cambridge, UK) and Schiller CS 612 digitized (Schiller-Reomed AG, Switzerland) instruments. Heart rates of the animals were obtained from the EKGs.
- Neurological examination: to assess the incidence of hypo- and hyperalgesia, impaired temperature sensitivity, impaired vegetative functions and polyneuritis, neurological and nerve conduction studies were performed by a board-certified veterinary neurologist. The examination was performed at pretest week –2 and weeks 13, 26, 38, and 51 on 53-week dogs. Recovery dogs were also evaluated at week –2. The neurologist was blinded to the dogs' assignments. Tests were done on cerebral, cerebellar, cranial nerves, fore and hindlimb proprioception (long tracts), posture and gait, reflexes (myotatic, flexor withdrawal, panniculus, perineal), and sensory functions (Kimura, 1989).

• Clinical pathology: hematology, blood chemistry, and urinalysis were performed at pretest week –2 and weeks 13, 26, 38/39, 52, and 58 on all animals. Blood samples were obtained by jugular venipuncture into EDTA-coated tubes following an overnight fast. Urine samples were collected by free catch during the 16-hour period preceding blood collection. Hematological measurements performed consisted of total and differential leukocyte, erythrocyte, reticulocyte counts, hematocrit, platelet count, mean corpuscular hemoglobin count (MCHC), mean corpuscular volume (MCV), prothrombin time (PT), and activated partial thromboplastin time (APTT). Blood plasma was analyzed for glucose, urea nitrogen (BUN), creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP), total

bilirubin, cholesterol, triglycerides, protein, albumin, globulin, albumin/globulin ratio, sodium, potassium, calcium, chloride, and inorganic phosphate. Urine was examined for color, volume, pH, total protein, specific gravity, glucose, ketones, bilirubin, urobilinogen, occult blood, sodium, potassium, and chloride. The sediment was microscopically examined.

• Thyroid function: previous studies in rats have purportedly shown that thalidomide can affect thyroid (Esser and Heinzler, 1956; Murdoch and Campbell, 1958) function. To assess its effect in dog thyroid function, blood samples were collected from 53-week dogs in the morning prior to dose administration during week –1 and weeks 13, 26, 39, and 52. Samples were taken in the morning to eliminate any diurnal cycle variation. Serum samples were separated, frozen, and analyzed for thyroid-stimulating hormone (TSH), T₃, and T₄ levels.

• Endocrine function: prolactin, estradiol, cortisol, corticosterone, aldosterone, and ACTH levels were determined only after enlarged mammary tissue, production of milklike discharge, and prolonged estrus were observed in the study. These tests were performed only on the 16 interim euthanization animals at week 24. No measurements of progesterone levels were performed, as including them at this juncture would have involved determining the stage of the estrus cycle along with vaginal cytology and blood samplings. On the day of sampling, the interim animals were dosed such that blood was collected in the morning. The serum/plasma was separated and stored frozen at approximately –65°C and sent (Anilytics, Rockville, MD) for determination of endocrine function using a validated in-house antibody assay for prolactin and RIA kits for estradiol, cortisol, corticosterone, aldosterone, and ACTH.

Postmortem observations and measurements. Dogs for the 26-week interim and 53-week/recovery necropsies were anesthetized with pentothal and phenobarbital (Butler Co., Columbus, OH) before euthanization by exsanguination.

• Gross pathology and organ weights: 2 dogs/sex/group were necropsied after 26 weeks of dosing. Four dogs/sex/group were necropsied after 53 weeks of dosing. Following a 5-week recovery period, 2 dogs/sex from both the control and high-dose groups were necropsied. The brain, heart, liver, thymus, kidneys, adrenals, testes, ovaries, thyroid, epididymides, spleen, and uterus were weighed and organ-to-body weight ratios calculated for all animals. Paired organs were weighed together. The following tissues were collected from all animals and evaluated histopathologically from control and high-dose animals (see histopathology section): adrenals, bone and marrow (sternum, femur), brain (cerebellum, cerebrum and brain stem), cecum, colon, dorsal root ganglion (lumbar 2–6), duodenum, epididymides, esophagus, eyes, gall bladder, gross lesions, heart and aorta, ileum, jejunum, kidneys, lacrimal glands, liver, lungs and bronchi, lymph nodes (mesenteric), mammary gland, muscle (biceps femoris), nerve (sciatic, sural), ovaries, pancreas, pituitary, prostate, rectum, salivary glands (submandibular), skin (thigh, lateral), spinal cord (cervical, thoracic lumbar), spleen, stomach, testes, thymus or remnant, thyroid/parathyroid glands, trachea, tongue, urinary bladder, uterus, and vagina.

• Histopathology: organs/tissues collected from the control and high-dose animals, as well as all gross lesions, were fixed with 10% neutral buffered formalin, stained with hematoxylin and eosin, processed to slides, and examined microscopically. In addition, liver and mammary tissues from the 53-week low- and mid-dose dogs were similarly processed to slides and microscopically examined.

Data analysis. Group means and standard deviations were calculated for numerical data where appropriate and compared by one-way (dose) analysis of variance (ANOVA). If significant differences ($p < 0.05$) among the means were indicated, the Dunnett's test was used to determine which means were different from the control. A 2-way (dose, sex) repeated measures ANOVA was used to analyze body weight, blood and urine chemistry, hematology, endocrine function, body temperature, heart rate, and respiration over time. The ANOVA model consisted of the main effects of dose, sex, and time; the interactions time \times dose, time \times sex, and dose \times sex; and the nesting term subject [dose, sex]{random}. When preliminary analysis revealed a nonsignificant ($p > 0.05$) interaction term, it was removed from the model and the

ANOVA re-run. Changes in the hematology and blood chemistry parameters were expressed as least squares means to compensate for different covariate values and other factors in the model. All statistical analyses were performed using the JMP statistical software package (v 3.2.2, SAS Institute, Cary, NC).

RESULTS

Clinical observations. There were no deaths during the 53 weeks of thalidomide administration or the recovery period. No gross and histopathologic evidence of tumors was seen. Green-colored urine, white particulate matter in the feces, enlarged female mammary tissue, and a prolonged duration of estrus evidenced by gross changes in the vulva and presence of discharge were the only observed clinical signs attributable to thalidomide administration. Generally, the time of onset of discolored urine was governed by dose, with the high-dose animals first producing green urine at an earlier point during the study (as early as day 1) than the low- and mid-dose animals (as late as week 13). During the first 6 months of thalidomide administration, green urine was intermittently observed for 11 of the 12 low-dose animals and all of the mid- and high-dose dogs. Following the 6-month interim euthanization, green urine was similarly observed for all surviving dogs receiving thalidomide. In addition, green urine was intermittently observed for all high-dose recovery dogs for 11–24 days after the last thalidomide dose.

What appeared to be unabsorbed thalidomide was periodically observed as a white particulate residue in the feces of 9 of the low-dose dogs and all the mid- and high-dose animals during the first 6 months of treatment. Chemical analysis of the residue was not performed. The presence of residue in the feces was observed with greater frequency with increasing dose. Following the 6-month interim euthanization, white residue was observed in the feces of 3 of the 8 remaining low-dose animals and in all the surviving mid- and high-dose dogs. White residue in the feces was not observed for any animal during the recovery phase of this study.

Slight to moderate enlargement of the mammary tissue was visually observed in 2 out of 6 females in each of the low- and mid-dose groups and in 5 out of 8 high-dose females. The duration of this enlargement varied from as few as 5 days to as many as 144 days and generally was noted to begin just prior to estrus, during estrus, or within the 2 months following estrus. Three of the affected females (1 low-dose and 2 high-dose dogs) experienced 2 separate episodes of mammary enlargement during the study. A light blue coloration of the area surrounding the nipples was also observed concomitantly in 5 of the 9 females with mammary enlargement. This blue coloration was also seen in 1 control and 1 high-dose dog in which mammary enlargement was not observed at any point during the study. This occurrence in a single control dog suggests that the increased incidence of blue coloration seen in the dosed females may be an exaggeration of a normal physiological phenomenon. In addition, a white watery discharge, with an

appearance consistent with colostrum, was expressed from the nipples of 1 mid-dose and 1 high-dose female, both of which also exhibited mammary enlargement. Mammary enlargement or blue discoloration was not observed during the recovery period. The high-dose dog returned to normal prior to the interim euthanization, and her mammary tissue was grossly and histologically normal.

Twenty-three of the 28 female dogs were observed to be in estrus at least once during the study. All of the remaining 5 females that did not come into estrus received thalidomide, however, 3 of them were on study for only 26 weeks and the remaining 2 non-estrus females were members of the low-dose group. All 8 control female dogs went into estrus. Of the 6 female dogs in each of the low- and mid-dose groups, 4 and 5 went into estrus, respectively. Of the 8 female dogs in the high-dose group, 6 went into estrus and none were from the 26 weeks dogs. The qualitative aspects of the observed estrus cycles such as external signs and behavior were normal for all dogs; however, the duration of the estrus or heat periods tended to lengthen with increasing thalidomide dose. A statistically significant ($p < 0.05$) difference in mean (\pm SD) estrus duration was observed for the high-dose females (21 ± 9 days) as compared with control (13 ± 4 days). In addition, 2 females (low and mid dose) exhibited what was thought to be a false heat, as they showed external signs of estrus 2 times, 16 and 35 days apart, respectively. This is a relatively common occurrence in breeding-age female dogs and is therefore not considered to be treatment or dose related.

Body weights, food consumption, ophthalmoscopy, physical examination, and electrocardiography. There was no effect of thalidomide dose on body weight. There was an expected significant ($p < 0.05$) sex effect on body weight, with the males approximately 2.0 kg heavier than females. Daily administration of thalidomide had no effect on food consumption at any dose level. The occasional statistically significant different food consumption of the mid-dose group as compared to controls was due to normal variation in dog appetites. Indirect ophthalmoscopy performed during weeks 25 and 51 revealed no thalidomide-related lesions. Physical examinations of all dogs were within normal limits. Heart rate, respiration rate, and body temperature of the dose groups were statistically similar to those of the control.

Neurological examinations and nerve conduction. No consistent neurological deficits were observed. None of the abnormalities noted during a single examination was noted in the same animal at subsequent examinations. No neurological abnormalities can be ascribed to the administration of thalidomide.

Hematology, blood chemistry, and urinalysis. Although most hematological parameters remained within normal limits throughout the 12-month dosing and recovery periods, thalidomide significantly decreased erythrocyte count and MCHC ($p < 0.05$, 0.01 , respectively) and increased MCH ($p < 0.05$)

TABLE 1
Changes in Hematology and Blood Chemistry between Control and 1000 mg/kg Dose

	Least squares means \pm SEM		
	0 mg/kg	1000 mg/kg	% Change
Hematology			
Erythrocyte counts ($\times 10^6$)	7.02 \pm 0.12	6.65 \pm 0.12	5.3 \downarrow
MCHC (g/dl)	35.39 \pm 0.10	34.53 \pm 0.09	2.4 \downarrow
MCH (pg)	23.23 \pm 0.18	23.80 \pm 0.18	2.5 \uparrow
Blood Chemistry			
AP (U/l)	83.68 \pm 7.94	117.72 \pm 7.94	40.7 \uparrow
BUN (mg/dl)	13.87 \pm 0.66	17.60 \pm 0.66	26.9 \uparrow
Chloride (meq/dl)	114.00 \pm 0.41	117.68 \pm 0.41	3.2 \uparrow
Sodium (meq/dl)	146.69 \pm 0.28	148.85 \pm 0.28	1.5 \uparrow
Albumin (g/dl)	3.88 \pm 0.05	3.64 \pm 0.05	6.2 \downarrow
Calcium (mg/dl)	10.33 \pm 0.07	10.03 \pm 0.07	2.9 \downarrow
Glucose (mg/dl)	98.76 \pm 1.96	92.34 \pm 1.96	6.5 \downarrow
Potassium (meq/dl)	4.42 \pm 0.04	4.19 \pm 0.04	5.1 \downarrow
Total bilirubin (mg/dl)	0.18 \pm 0.01	0.13 \pm 0.01	27.5 \downarrow

(Table 1). There was greater effect of thalidomide on blood chemistry. Alkaline phosphatase, BUN, chloride, and sodium levels increased ($p < 0.01$, 0.05), whereas albumin, calcium, glucose, potassium, and total bilirubin decreased ($p < 0.01$, 0.05) with thalidomide dose. Potassium levels were also significantly lower ($p < 0.01$) at 43 and 200 mg/kg. Sodium levels in females were slightly greater ($p < 0.05$) than males. At various time points, statistically significant differences between the control and thalidomide-dosed groups were observed for all hematology and blood chemistry parameters except MCHC, MCH and creatinine. Apart from the chromaturia described earlier, urinalysis results from the thalidomide-dosed and control dogs were comparable. All parameters measured in the recovery dogs were within normal limits 5 weeks after the last thalidomide dose.

Thyroid and other endocrine functions. Thalidomide had no significant effect on serum concentrations of TSH and T_3 while significantly ($p < 0.05$) reducing T_4 (data not shown). T_4 concentration decreased in a dose-dependent manner, it was 34.8% lower at 1000 mg/kg compared to control. Administration of thalidomide did not produce any differences in levels of other endocrine hormones at week 24. Statistically significant differences were observed at various time points for all hormones except aldosterone, prolactin, T_3 , and TSH.

Absolute and relative organ weights. No thalidomide-related differences in the absolute and relative organ weights were observed at the 26-, 53-, or 58-week recovery necropsy (Table 2). The 2 females exhibiting increased uterine weights at the 6-month interim euthanization were in estrus at the time of necropsy.

Gross Pathology

Twenty-six-week (interim) euthanization. Yellow discoloration of the cranial bones was observed in 2 out of 4 mid-dose

dogs and 3 out of 4 high-dose dogs. In addition, one of the affected high-dose animals exhibited similar discoloration of the rib, femur, and orbit. These gross findings are possibly related to the administration of thalidomide; however, no microscopic correlate was observed. Other gross lesions were an ovarian focus in a low-dose dog and a small testicle in a mid-dose dog. These were considered incidental.

Fifty-three-week (terminal) euthanization. Yellow discoloration of the femur, calvarium (skull), and/or rib was observed for 5 of the 8 high-dose dogs, and 1 of 8 dogs of each of the low- and mid-dose groups. The yellow discoloration leached into the formalin fixative. Similar discoloration of the bones was not observed in any of the control dogs. These findings suggest a thalidomide effect; however, no microscopic correlate was present. All other gross lesions, a vaginal mucosa cyst, glans nictitans prolapse (present at study initiation), enlarged tonsils, and gelatinous subcutaneous tissue, were considered incidental and not related to thalidomide administration.

Fifty-eight-week (recovery) euthanization. All high-dose recovery dogs exhibited a leachable yellow discoloration of bone without a microscopic correlate. The significance of the yellow discoloration of the right cardiac lobe of the lung of a high-dose female is not known. All other findings during necropsy were considered incidental and not related to thalidomide.

Histopathology

Twenty-six-week (interim) euthanization. No thalidomide-related microscopic changes were observed in any of the collected tissues or organs. The kidney, mammary, bone, and nerve tissues of all the dogs necropsied at the interim euthanization were determined to be normal when observed under light microscopy.

TABLE 2
Absolute and Relative Group Mean Organ Weights in Male (M) and Female (F) Dogs after 53 Weeks
of Oral Thalidomide Exposure

Dose (mg/kg)	Sex	Brain	Heart	Liver	Thymus	Spleen	Thyroids/ parathyroids	Epididymides/ uterus	Kidneys	Adrenals	Testes/ ovaries
Absolute mean organ weight											
0	M	77.8 ± 7.0	97.7 ± 17.2	314 ± 108	9.8 ± 1.5	29.3 ± 2.6	1.20 ± 0.26	4.07 ± 0.27	52.8 ± 14.2	1.609 ± 0.207	15.5 ± 4.0
	F	76.8 ± 3.6	83.4 ± 7.6	286 ± 23	10.9 ± 2.7	28.7 ± 7.4	1.60 ± 0.54	7.55 ± 5.49	47.8 ± 4.0	1.498 ± 0.128	1.15 ± 0.41
43	M	85.4 ± 6.1	102.6 ± 10.2	373 ± 67	12.0 ± 4.7	48.6 ± 32.9	1.238 ± 0.199	18.622 ± 27.154	52.0 ± 2.0	1.461 ± 0.316	17.4 ± 2.9
	F	76.9 ± 5.3	72.8 ± 8.1	299 ± 38	8.0 ± 3.0	36.5 ± 8.9	1.070 ± 0.111	7.06 ± 6.25	39.6 ± 4.1	1.533 ± 0.347	0.99 ± 0.52
200	M	80.7 ± 4.6	102.1 ± 14.0	335 ± 49	13.1 ± 2.1	40.4 ± 10.5	1.754 ± 0.686	4.274 ± 0.846	53.7 ± 7.5	1.376 ± 0.190	16.5 ± 3.2
	F	74.7 ± 6.9	76.0 ± 16.2	274 ± 30	9.0 ± 1.5	32.4 ± 6.3	1.289 ± 0.396	7.12 ± 3.04	38.7 ± 2.7	1.417 ± 0.208	0.71 ± 0.09
1000	M	75.6 ± 6.8	92.3 ± 10.3	354 ± 40	8.8 ± 1.9	39.9 ± 6.0	1.054 ± 0.061	4.048 ± 0.967	53.4 ± 12.2	1.402 ± 0.108	13.7 ± 1.3
	F	74.8 ± 4.4	82.4 ± 23.2	340 ± 67	11.0 ± 5.6	32.4 ± 9.9	1.162 ± 0.389	6.54 ± 2.45	45.3 ± 7.3	1.558 ± 0.296	0.87 ± 0.28
Relative mean organ weight (%)											
0	M	0.70 ± 0.15	0.85 ± 0.15	2.7 ± 0.5	0.09 ± 0.02	0.26 ± 0.06	0.011 ± 0.004	0.036 ± 0.007	0.4 ± 0.1	0.014 ± 0.003	0.2 ± 0.1
	F	0.75 ± 0.06	0.81 ± 0.03	2.8 ± 0.1	0.11 ± 0.03	0.28 ± 0.06	0.015 ± 0.004	0.07 ± 0.05	0.5 ± 0.1	0.015 ± 0.001	0.01 ± 0.01
43	M	0.64 ± 0.13	0.76 ± 0.12	2.8 ± 0.5	0.09 ± 0.02	0.33 ± 0.15	0.009 ± 0.002	0.115 ± 0.149	0.04 ± 0.1	0.011 ± 0.001	0.1 ± 0.1
	F	0.74 ± 0.11	0.71 ± 0.14	2.8 ± 0.2	0.08 ± 0.03	0.35 ± 0.07	0.010 ± 0.001	0.06 ± 0.05	0.4 ± 0.1	0.015 ± 0.002	0.01 ± 0.00
200	M	0.63 ± 0.05	0.79 ± 0.04	2.6 ± 0.4	0.10 ± 0.02	0.32 ± 0.09	0.014 ± 0.006	0.033 ± 0.007	0.4 ± 0.1	0.011 ± 0.002	0.1 ± 0.1
	F	0.74 ± 0.05	0.75 ± 0.14	2.8 ± 0.6	0.09 ± 0.01	0.33 ± 0.07	0.013 ± 0.005	0.08 ± 0.04	0.4 ± 0.1	0.014 ± 0.002	0.01 ± 0.00
1000	M	0.64 ± 0.08	0.77 ± 0.11	3.0 ± 0.3	0.07 ± 0.2	0.34 ± 0.06	0.009 ± 0.001	0.033 ± 0.004	0.4 ± 0.1	0.012 ± 0.002	0.1 ± 0.00
	F	0.75 ± 0.16	0.79 ± 0.09	3.4 ± 0.5	0.10 ± 0.03	0.31 ± 0.07	0.012 ± 0.005	0.07 ± 0.04	0.05 ± 0.1	0.016 ± 0.003	0.01 ± 0.00

Fifty-three-week (terminal) euthanization. A thalidomide-related effect was observed in the mammary glands of females administered thalidomide. Dilatation of the ducts with distention by eosinophilic proteinaceous fluid and/or hyperplasia of the glandular epithelium were observed at all dose levels. For the control group, minimal severity dilatation occurred in 1 of 4 dogs. No hyperplasia was seen in any control dog. The control female exhibiting postmortem dilatation was the only control female described during the in-life phase of the study with blue coloration of the mammary tissue. At the low and mid doses, 1 of 4 dogs each exhibited dilatation with moderate and mild severity, respectively. Minimal to mild hyperplasia was observed in 2 and 1 of 4 dogs for the low and mid doses, respectively. At the high dose, 3 of 4 dogs exhibited minimal to moderate dilatation and 2 of 4 dogs had mild to moderate hyperplasia. The high-dose dogs with hyperplasia had a relatively late onset of clinically observed enlargement of mammary glands. The increased incidence of ductal dilatation in treated animals was considered related to thalidomide administration, despite its presence in a control animal, due to the greater incidence and severity in the thalidomide-dosed dogs. Accumulation of bile pigment in the canaliculi of the liver was observed in all high-dose male dogs and was of minimal severity and diffuse in distribution. There was no widespread stasis of bile and the change was minimal. This lesion was not seen in the high-dose females or in any other dose group and was considered to be a result of thalidomide administration. No brain or pituitary abnormalities were found.

Fifty-eight-week (recovery) euthanization. Dilatation of the mammary ducts with eosinophilic proteinaceous fluid was

observed in 1 of 2 control females and in 1 of 2 high-dose females. This same high-dose female also exhibited hyperplasia of the glandular epithelium. The hepatic bile pigment seen previously in the high-dose males was absent in all dogs at the recovery euthanization.

DISCUSSION

This study assessed the systemic toxicity of thalidomide in dogs after daily oral dosing for 53 weeks at 43, 200, or 1000 mg/kg. The dose for an ENL patient ranges between 200 and 400 mg/day or 3 and 6 mg/kg/day. The high dose of 1000 mg/kg/day was therefore more than 300 times the therapeutic dose of 3 mg/kg/day. Daily administration of thalidomide at all 3 doses for 53 weeks was generally well tolerated. The lack of tumors is consistent with previous studies showing that thalidomide is not genotoxic (Ashby *et al.*, 1997; Teo *et al.*, 2000a). In contrast to the thalidomide-related changes found in dogs, nonteratogenic human toxicity at therapeutic doses is manifested by peripheral neuropathy, with adverse effects including lightheadedness, constipation, lethargy, skin rashes, and sedation (Teo *et al.*, 1999b, 2000b, 2001). These findings suggest a species difference in the toxicity of thalidomide. The presence of unabsorbed thalidomide in the feces is an indication of thalidomide's low solubility and absorption rate-limited kinetics (flip flop) as seen in humans (Teo *et al.*, 1999b, 2000b, 2001). Similar kinetics was also seen in the dogs (manuscript in preparation). In a recent study, low toxicity was seen in rodents at oral doses of up to 3000 mg/kg over 13 weeks. Based on body weight changes, the no-observed-adverse effect

levels (NOAEL) for mice and female rats were 3000 mg/kg and 30 mg/kg for male rats (Teo *et al.*, 1999a). In a later study mice dosed at the same dose over 4 months had centrilobular hepatocellular hypertrophy and karyomegaly (manuscript in preparation). Although 1000 mg/kg was generally well tolerated after 53 weeks of oral dosing, 200 mg/kg was conservatively regarded as the NOAEL for this study due to the occurrence of several thalidomide-related effects at the 1000 mg/kg high-dose level. The low toxicity in rodents and dogs is also evident in humans, where no mortality from overdoses or attempted suicide has ever been recorded, even in doses of up to 14 g (Bresnahan, 1961). The end result of these cases was a moderate deep sleep without need for any supporting therapy upon awakening.

The dilatation and distention of mammary gland ducts and hyperplasia of glandular epithelium in female dogs correlated with the enlarged mammary glands and is consistent with lactation. The significance of the mammary gland lesions in the recovery euthanization dogs could not be definitely ascertained. The presence of bile pigment in the livers of high-dose male dogs did not correlate with any changes in clinical chemistry. Although it is not known if thalidomide is present in the mammary proteinaceous fluid and bile, a recent study found thalidomide in the semen of HIV-seropositive patients (manuscript in preparation). We also found thalidomide in cerebrospinal fluid of rabbits after oral administration (data not shown), and an earlier study detected its presence in semen (Lutwak-Mann *et al.*, 1967). It is therefore probable that thalidomide is secreted in major bodily fluids.

The etiology of the persistent proestrus/estrus in some of the thalidomide-dosed females is unknown. Generally, mature female dogs come into estrus approximately 2 times a year. These 5 females could have been in estrus just prior to the start of the study. Persistent proestrus/estrus is generally demonstrated by the presence of an enlarged vulva, vaginal bleeding, and attraction of males for longer than 21 days in any one ovarian cycle. The causes may vary, but the final common denominator is continued exposure to increases in serum estrogen concentration (Feldman and Nelson, 1996). Proestrus/estrus in the presence of mammary changes consistent with lactation appears to implicate an endocrine effect, possibly at the pituitary-hypothalamic axis.

Acute studies in mice and rats have shown little effect and no consistent effects on hematology and clinical chemistry. The minor changes seen were mainly due to biological variation (Teo *et al.*, 1999a). Most of the significant hematologic and clinical chemistry changes seen in the beagle dogs were also small in magnitude. No morphological and histopathological correlates could be associated with these transient changes, and all parameters returned to normal in the recovery dogs. Apart from T_4 , there was no effect of thalidomide on endocrine function. In contrast, studies in immature rats of both sexes showed that thalidomide at 5–10 mg/kg/day decreased TSH and increased ACTH without affecting the levels of FSH, LH,

ADH, and oxytocin. This suggested some involvement of the hypothalamus (Locker *et al.*, 1971). This study, however, was based only on indirect organ weight measurements and histological examinations. In more recent rat studies, there was an apparent dose-dependent decrease in total and free T_4 that was more consistent in females (Teo *et al.*, 1999a). Levels of other hormones were not measured in the rat study. In humans, thalidomide was shown to normalize hyperthyroid states (Esser and Heinzler, 1956) and decrease iodine uptake by the thyroid gland (Murdoch and Campbell, 1958). More studies are needed to corroborate these findings.

Thalidomide did not produce any sedation in dogs. Recent studies have also shown the lack of sedation in mice and rats at doses of up to 2–3 g/kg/day (Teo *et al.*, 1999a) and rabbits at 100 mg/kg/day (Schroder and Mattiesen, 1985). This is in contrast to humans, where it was once used as a sedative. Possible explanations include species differences in sensitivity and metabolism. As thalidomide is a racemate, studies have been performed to determine the specific enantiomer and metabolites responsible for the various effects. However, this has been hampered by chiral interconversion of thalidomide in humans after oral dosing (Eriksson *et al.*, 1995). More recent studies have helped to elucidate the metabolism of thalidomide in various species. In a 28-day dog study (data not shown), green and brown urine was obtained in nearly every treatment day after dosing with 1000–2000 mg/kg of thalidomide. The discoloration was thought to be due to the presence of various thalidomide metabolites. The present study confirms this finding and suggests a species difference in the metabolism of thalidomide. In previous single dose studies, one-fifth of the mice and none of the rats given oral doses (200–2000 and 1000 mg/kg, respectively) exhibited red-orange colored urine (data not shown). Hemoglobin tests on the mice urine confirmed that the color was not due to the presence of blood. In 13-week oral studies in mice and rats (30–3000 mg/kg), orange-pink and normal colored urine, respectively, were obtained (Teo *et al.*, 1999a). Healthy human volunteers were similar to rats in not producing any discolored urine after repeated oral dosing at between 4 and 10 mg/kg (Teo *et al.*, 1999b, 2000b, 2001).

The main route of thalidomide metabolism appears to be nonenzymatic hydrolysis (Schumacher *et al.*, 1965b), although a cytochrome P-450-mediated hepatic metabolism (Braun *et al.*, 1986) or other enzyme-facilitated hydrolysis (Williams *et al.*, 1965) has been postulated. Recent studies using pooled human microsomes, microsomes containing cloned human CYP isozymes and Hansen's disease patients showed little to no hepatic metabolism (Teo *et al.*, 2000c). Thalidomide has been shown to be unstable in aqueous media and undergoes spontaneous hydrolysis at pH 6.0 or higher to produce at least 12 hydrolysis products. Schumacher *et al.* (1965a) isolated α -aminoglutarimide, a hydrolysis product of thalidomide, from the urine of rabbits following oral doses of between 350 and 500 mg/kg and observed that the extract and purified crystalline substance were red. In a related paper, Schumacher *et al.*

(1965b) showed that α -aminoglutarimide changed color from red to blue after it was allowed to stand in an aqueous medium. The discolored and normal-colored urines from various species are therefore thought to be due to the metabolic/hydrolytic formation of different chromophores. Humans could resemble the rat in terms of metabolite/hydrolysis product profile. Ancillary studies showed that unlike humans, beagle dogs and rats did not develop peripheral neuropathy (Teo *et al.*, 1999a, 2000d). This could be due to the action of species-specific hydrolysis products or other unknown factors.

Some of the clinical and histological findings in this study could be considered as adaptive responses to high doses of a relatively nontoxic xenobiotic. For instance, a high-dose male dog weighing 15 kg would receive a daily thalidomide dose of 15,000 mg compared to 200–800 mg for humans. The response of the male dogs to such a high dose appears to be minimal accumulation of bile pigments in liver, excretion of some of the thalidomide unabsorbed, and conversion of some of the absorbed material to colored metabolites that were eliminated in the urine. Given the molecular weight of thalidomide and its most frequently found hydrolysis products, it is quite probable that biliary excretion of conjugated products was responsible for the bile accumulations observed in the livers of the high-dose males. To date, none of these processes have produced changes indicative of thalidomide-mediated toxicity. Nonadaptive responses probably related to thalidomide were mammary enlargement and blue coloration around the nipples in female dogs. These responses were transient and were not seen during the recovery period. Both observations were probably the result of a greater than normal physiological reaction to thalidomide. The white watery mammary discharge observed in 1 mid-dose and 1 high-dose female dog may have been associated with false pregnancy and unrelated to thalidomide.

In summary, beagle dogs orally dosed with 43, 200, or 1000 mg/kg thalidomide per day for 53 weeks did not show major systemic toxicity or tumors. The NOAEL was 200 mg/kg. The potential for lifetime tumor development is currently being assessed in 2-year rodent oncogenicity assays.

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