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Rodent Carcinogenicity with the Thiazolidinedione Antidiabetic Agent Troglitazone

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Carcinogenic potential of the thiazolidinedione antidiabetic troglitazone was assessed in 104-week studies in mice and rats. Mice were given 50, 400, or 800 mg/kg, male rats 100, 400, or 800 mg/kg, and female rats 25, 50, or 200 mg/kg. Vehicle and placebo controls were included. Survival was significantly decreased in both sexes of both species at high doses, but was adequate for valid evaluation of carcinogenicity. Hypertrophy and hyperplasia of brown adipose tissue was observed in both species at all doses, and fatty change and hypocellularity of bone marrow was noted in mice at all doses and in female rats at 50 and 200 mg/kg. Hepatocellular vacuolation was observed in mice at 400 and 800 mg/kg, and centrilobular hepatocellular hypertrophy occurred in rats at ≥ 200 mg/kg. Ventricular dilatation, myocardial fibrosis, and atrial myocyte karyomegaly in male rats at 400 and 800 mg/kg and female rats at all doses were morphologically similar to spontaneous lesions, but incidence and severity were increased compared with controls. In mice, the incidence of hemangiosarcoma was increased in females at 400 mg/kg and in both sexes at 800 mg/kg. The incidence of hepatocellular carcinoma was increased in female mice at 800 mg/kg. Troglitazone exposure [$AUC_{(0-24)}$] at the lowest dose associated with increased tumor incidence in mice was 16 times human therapeutic exposure at 400 mg daily. No tumors of any type were increased in rats at exposures up to 47 times therapeutic exposure.

Key Words: **thiazolidinedione; troglitazone; Rezulin®; Noscal®; Romozin®; rodent carcinogenicity; hemangiosarcoma; hepatocellular carcinoma.**

Troglitazone is a novel thiazolidinedione that binds and activates the peroxisome proliferator-activated receptor-gamma (PPARγ), enhancing insulin action and inhibiting hepatic gluconeogenesis (Keller *et al.*, 1993; Saltiel and Horikoshi, 1995; Saltiel and Olefsky, 1996; Tontonoz *et al.*, 1994). Troglitazone was withdrawn from the market in March 2000 because of rare idiosyncratic hepatic effects (Gale, 2001). Since its introduction in early 1997, 60 patients have died, and 10 patients have received liver transplants. The incidence of troglitazone-induced acute liver failure has been estimated to be between 1 in 8000 and 1 in 20,000 patients. Prior to withdrawal, troglitazone was used concomitantly with a sulfonylurea or insulin, enabling many patients to achieve American Diabetes Association goals for glycemic control (Horton *et al.*, 1998) for the first time. Troglitazone undergoes first-pass metabolism and forms three major metabolites: a sulfate conjugate (M-1), a glucuronide conjugate (M-2), and a quinone (M-3) (Brodfuehrer *et al.*, 1993; Horikoshi *et al.*, 1994; Izumi *et al.*, 1996; Loi *et al.*, 1997). The recommended dose effective in most patients was 200 or 400 mg daily, with associated troglitazone exposures [$AUC_{(0-24)}$] up to 13.4 μg · h/ml.

Intended chronic administration of troglitazone in the diabetic population necessitated assessment of carcinogenic potential in 104-week studies in mice and rats. Carcinogenicity data with troglitazone have been presented previously in abstract form (Herman *et al.*, 1998; McGuire *et al.*, 1998). The objective of this manuscript is to present the full preclinical assessment of carcinogenic potential of troglitazone, the first published report of such data with the thiazolidinedione chemical class.

MATERIAL AND METHODS

Studies were conducted between 1993 and 1995 at MPI Research (formerly International Research and Development Corporation), Mattawan, Michigan, and were in compliance with U.S. Food and Drug Administration Good Laboratory Practice regulations. Animal use was in accordance with the Guide for the Care and Use of Laboratory Animals.

Test substance. Troglitazone (USAN; INN) is a member of the thiazolidinedione chemical class and is described systematically as (±)-5-[[4-[(3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H-1-benzopyran-2-yl)methoxy]phenyl]methyl]-2,4-thiazolidinedione (Fig. 1). Test article used in these studies was an amorphous coprecipitate of troglitazone with polyvinylpyrrolidone, polyethylene glycol 400, and polysorbate 80, the formulation used in the marketed product Rezulin®. Administered doses were based on active drug content of 56.7%. Over the concentration range used, troglitazone was homogeneous and stable in vehicle, 0.5% methylcellulose (Sigma Chemical Co., St Louis, MO, and Aldrich Chemical Co., Inc., Milwaukee, WI). Analysis of dosing preparations confirmed that intended concentrations of the test article were achieved. Bulk drug retained potency throughout the study.

Animals. Random-bred, barrier-raised B6C3Fl mice and Wistar rats were obtained from Charles River Laboratories, Portage MI, and Kingston NY,

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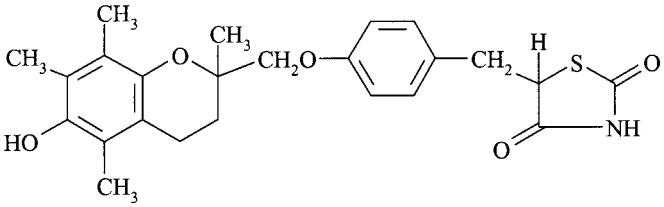

FIG. 1. Troglitazone, (±)-5-[[4-[(3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H-1-benzopyran-2-yl)methoxy]phenyl]methyl]-2,4-thiazolidinedione (CAS 97322-87-7).

respectively. These strains were selected as appropriate test systems because they are widely used as animal models for evaluating potential toxicologic and carcinogenic effects, and historical data are available for reference purposes when evaluating results. Animals were observed during a 2-week acclimation period for clinical signs of disease and were given a detailed physical examination. Mice were approximately 8 weeks old at study initiation, with males weighing 20–26 g and females 18–23 g. Rats were approximately 7 weeks old, with males weighing 231–274 g and females 162–197 g. Animals were individually housed in stainless steel wire-mesh cages in environmentally controlled rooms with a 12-h light/12-h dark cycle, a humidity range of 40–70%, and temperature ranges of 68–78°F for mice and 67–75°F for rats. Water and Certified Rodent Chow® #5002 (Purina Mills, Inc., St. Louis, MO) were available *ad libitum*.

Experimental design. Sixty mice per sex per group were given troglitazone by gavage at 50, 400, or 800 mg/kg for 104 weeks. A vehicle control group was given 0.5 % methylcellulose and a placebo control group received suspensions of the excipient mixture, polyvinylpyrrolidone, polyethylene glycol 400, and polysorbate 80, at a concentration equivalent to that given to mice in the high-dose group. Sixty rats per sex per group were also given troglitazone by gavage for 104 weeks, males receiving 100, 400, or 800 mg/kg and females 25, 50, or 200 mg/kg. Consistent with the mouse study, vehicle and placebo control groups were included. Dose volume in both studies was 10 ml/kg.

Animals were observed daily for clinical signs of toxicity. Individual body weights and food consumption were recorded weekly throughout the study. Ophthalmic examinations were performed on all animals at approximately 6-month intervals. Hematological evaluations were conducted on surviving animals at termination of the study, except for animals scheduled for plasma drug concentration analysis. Blood samples were collected from animals euthanized *in extremis* when possible. Bone marrow smears were prepared at necropsy and myeloid/erythroid (M/E) ratios were determined. In rats only, clinical biochemical parameters were evaluated at 104 weeks. All animals received a thorough postmortem examination. Organs were weighed at necropsy, and tissues from all animals except those from animals designated for plasma drug concentration analysis were processed for microscopic examination.

In the mouse carcinogenicity study, plasma concentrations of troglitazone and M-1 metabolite were measured in blood samples collected from 10 mice per sex per group at the approximate tmax of 1 h postdose during Weeks 52 and 104. In the rat study, plasma concentrations of troglitazone, M-1, and M-3 were measured in samples collected from 10 animals per sex per group at the approximate tmax of 2 h postdose during Weeks 13, 26, 52, 78, and 104.

Dose selection criteria. Doses administered in the mouse carcinogenicity study were based on a 13-week study; doses of 50–1200 mg/kg were evaluated (McGuire *et al.*, 1997). After 13 weeks of treatment, dose-related increases in heart and liver weights were observed at 400, 800, and 1200 mg/kg. Hepatocellular hypertrophy was observed predominately at ≥ 400 mg/kg. Administered doses were associated with plasma concentrations of troglitazone 2–23 times human therapeutic exposure at 400 mg daily (Table 1). Doses in the rat carcinogenicity study were based on results of a 13-week study with doses of 100-1200 mg/kg in males and 25–400 mg/kg in females (Herman *et al.*, 1997). Liver weight increased at doses of 400-1200 mg/kg in males and 50–400 mg/kg in females, and heart weight increased in males at 1200 mg/kg and in females at 400 mg/kg. Dose-related hepatocellular hypertrophy was observed in males at ≥ 400 mg/kg and in females at ≥ 50 mg/kg. The doses selected for the rat carcinogenicity study were associated with troglitazone plasma concentrations approximately 2–12 times and 5–47 times human therapeutic exposure in males and females, respectively (Table 2).

Histopathology and statistical analyses. Tissues from all animals were evaluated microscopically by study pathologists at MPI Research using established diagnostic criteria. Tumor diagnoses were peer reviewed. Diagnostic discrepancies were reexamined blindly by pathology working groups composed of the MPI Research and Pfizer pathologists as well as independent consulting pathologists. Consensus tumor diagnoses were compiled in the final tumor listings used for statistical analyses.

The number of animals in each group surviving to scheduled terminal sacrifice were compared using the product-limit method (Kaplan and Meier, 1958). Pairwise differences between each troglitazone-treated and control group were assessed using methods described by Tarone and Ware (1977). A

TABLE 1
Troglitazone and M-1 and M-3 Metabolite Toxicokinetic Parameters in Mice Given Troglitazone for 13 Weeks

Dose (mg/kg)	Troglitazone		M-1		M-3	
	Cmax	$AUC_{(0-24)}$	Cmax	$AUC_{(0-24)}$	Cmax	$AUC_{(0-24)}$
Male						
50	8.78	30.5	0.377	2.25	0.460	ND
400	23.3	195	1.90	27.3	1.35	10.0
800	27.9	216	3.45	30.2	2.07	7.48
1200	21.2	273	4.72	62.6	1.58	19.4
Female						
50	8.14	55.4	0.640	7.48	0.312	ND
400	35.3	242	5.27	64.0	1.87	9.81
800	38.4	304	5.29	60.1	2.61	10.7
1200	32.1	237	7.32	104	1.58	12.6

Note. Plasma samples obtained 1, 2, 4, 8, and 24 h postdose in Week 13; mean, $n = 5$/time point. Cmax, maximum plasma concentration (μg/ml); $AUC_{(0-24)}$, area under the plasma concentration-time curve from 0 to 24 h ($\mu g \cdot h/ml$); ND, not determined because of insufficient plasma concentration data.

TABLE 2
Troglitazone and M-1 and M-3 Metabolite Toxicokinetic Parameters in Rats Given Troglitazone for 13 Weeks

Dose (mg/kg)	Troglitazone		M-1		M-3	
	Cmax	$AUC_{(0-24)}$	Cmax	$AUC_{(0-24)}$	Cmax	$AUC_{(0-24)}$
Male						
100	3.35	21.1	70.6	413	BLQ	BLQ
400	8.93	77.7	266	3530	0.508	1.75
800	12.3	162	323	4060	4.91	66.2
1200	24.0	247	284	4170	4.94	28.4
Female						
25	13.4	61.4	1.26	3.44	BLQ	BLQ
50	21.2	192	2.79	12.1	BLQ	BLQ
200	66.0	631	16.2	138	1.67	24.7
400	73.6	1060	33.1	424	7.30	75.9

Note. Plasma samples obtained 1, 2, 4, 8, and 24 h postdose in Week 13; mean, $n = 5$/time point. Cmax, maximum plasma concentration (μg/ml); $AUC_{(0-24)}$, area under the plasma concentration-time curve from 0 to 24 h ($\mu g \cdot h/ml$); BLQ, below limit of quantification.

test for dose-response trend was also performed (Tarone, 1975). These tests were two-tailed at the 5% significance level. Statistical assessment of carcinogenic potential was based on differences in tumor incidence and time of tumor appearance. Briefly, each tumor category was analyzed using the Peto dose trend test to compare troglitazone-treated groups with both the vehicle and placebo control groups (Peto *et al.*, 1980). The analysis was performed using Haseman's rule, whereby significance level for testing rare tumor categories was two-tailed at the 5% level, and significance level for testing more common tumors was two-tailed at the 1% level (Lin and Ali, 1994). A one-tailed exact trend test using Haseman's rule was also used to analyze tumor types for which there were 12 or fewer tumor-bearing animals when summed across all groups. Tumor categories with positive dose trends were subsequently analyzed with an additional series of statistical tests including the Cochran-Armitage method, Fisher's exact test, and the Tarone trend and pairwise tests (Armitage, 1971; Gart *et al.*, 1979). Relevance of tumor data was based on the entire battery of statistical tests, with consideration given to actual tumor incidence and historical background tumor rates.

RESULTS

Mouse Carcinogenicity Study

Clinical and hematologic effects. There were no drug-related clinical signs or effects on body weight, food consumption, or ophthalmic parameters (data not shown). No clinically significant drug-related hematologic changes occurred, although minor decreases in erythrocyte indices (mean corpuscular volume, mean corpuscular hemoglobin concentration, and hemoglobin concentration) and minor changes in erythrocyte morphology were observed at 400 and/or 800 mg/kg.

Toxicokinetics. Troglitazone plasma concentrations during Weeks 52 and 104 increased with dose from 50 to 800 mg/kg, but the increases were less than dose proportional. There were no significant differences between males and females. Troglitazone and metabolite exposures during Weeks 52 and 104 were comparable and similar to exposures in the 13-week study (Fig. 2), suggesting that the toxicokinetic profile did not change appreciably during the 104-week study.

Nonneoplastic pathology. There were no gross pathologic changes in mice euthanized for plasma drug concentration analysis at 52 weeks. After 104 weeks, enlargement of interscapular brown fat was observed in males at 400 and 800 mg/kg and in females at all doses. Lipid vacuoles in the brown fat increased in size and coalesced. Liver weight increased 18% in males at 400 and 800 mg/kg, and 33 and 55% in females at 400 and 800 mg/kg, respectively. Males given $\geq$ 400 mg/kg and females given $\geq$ 800 mg/kg had hepatocellular vacuolation. Heart weight increased 17 and 22% in males and 24 and 29% in females at 400 and 800 mg/kg, respectively. No histopathologic findings correlated with increased heart weight. The incidence of fatty change and hypocellularity in bone marrow was increased in both sexes at all doses. Decreased kidney weights (12%) and a decreased incidence of

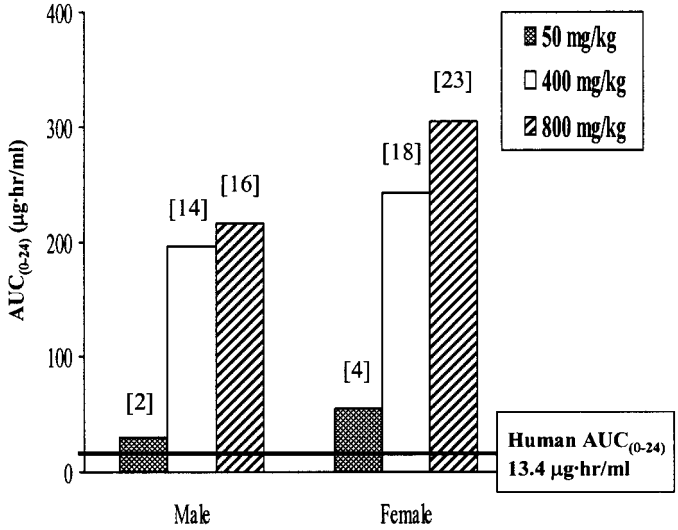

FIG. 2. Plasma $AUC_{(0-24)}$ in mice given troglitazone for 13 weeks. Values in brackets are multiples of human $AUC_{(0-24)}$ at 400 mg daily.

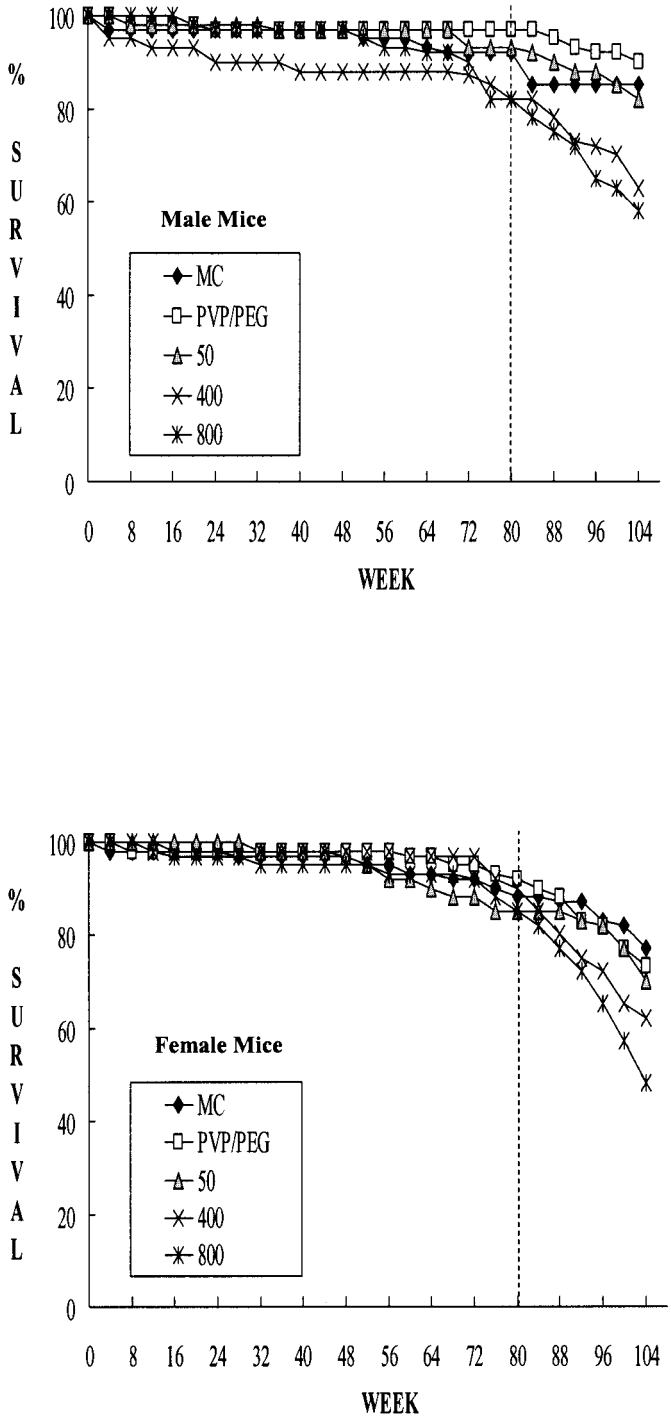

FIG. 3. Kaplan-Meier survival curves in male and female mice given troglitazone for 104 weeks.

renal tubular degeneration were observed in males at 800 mg/kg.

Survival and tumor analysis. Statistically significant dose-related decreases in survival occurred in both sexes (Fig. 3, Table 3). Pairwise comparisons to vehicle controls indicated mortality rates were statistically significantly increased in males at 400 and 800 mg/kg and in females at 800 mg/kg. Most deaths occurred during the final 6 months of the study. Overall survival at 104 weeks was 83, 90, 82, 63, and 58% in males and 77, 73, 70, 62, and 48% in females in vehicle and placebo controls, and at 50, 400, and 800 mg/kg, respectively. The mortality rate in high-dose mice confirmed that a maximum-tolerated dose was evaluated. Causes of death included vascular and nonvascular tumors and spontaneous intercurrent diseases; no one cause predominated.

An overall summary of tumor incidence in mice is presented in Table 3. In both sexes, total malignant tumors, malignant tumor-bearing animals, and malignant tumors/animals starting indicated a compound-related carcinogenic response. Nineteen distinct tumor types in males and 37 tumor types in females were identified, most with a frequency of 20 or fewer tumor-bearing mice. Compared with vehicle control, Peto analysis identified statistically significant positive dose trends for two tumor categories in males and eight tumor categories in females (Table 4).

Statistically significant positive dose trends for hemangiosarcomas and all vascular tumors (hemangioma plus hemangiosarcoma) were identified in both sexes by Peto analysis. Supplemental Cochran-Armitage and Tarone trend tests confirmed statistical significance for these categories. Pairwise comparisons to vehicle controls using Tarone's trend test were statistically significant in males at 800 mg/kg and in females at 400 and 800 mg/kg. Incidence of hemangioma was comparable in treated and both control groups, indicating that increases in all vascular tumors was attributable to increases in hemangiosarcoma. In females, increases in all tumors and all malignant tumors were due in part to hemangiosarcoma. Of the 84 hemangiosarcomas observed, 35 were multicentric and 49 were single tumors. There were no primary vascular tumors in the lung and no pulmonary metastases. Onset (time of earliest appearance) of hemangiosarcoma was shortened in females at 400 and 800 mg/kg, and latency (average time of appearance) was decreased in females at 400 mg/kg and in both sexes at 800 mg/kg. Onset of hemangiosarcoma was 31, 104, 93, 63, and 35 weeks in males and 94, 91, 104, 47 and 56 weeks in females in vehicle and placebo controls, and at 50, 400, and 800 mg/kg, respectively. A hemangiosarcoma was first identified in a vehicle control male during Week 31; remaining hemangiosarcomas in this group were not detected until Week 104. Latency of hemangiosarcoma was 92, 104, 99, 95, and 86 weeks in males and 101, 102, 104, 87, and 84 weeks in females in vehicle and placebo controls, and at 50, 400, and 800 mg/kg, respectively.

In females only, dose trends for hepatocellular carcinoma and for hepatocellular adenoma plus carcinoma combined (all hepatocellular tumors) were statistically significantly positive by Peto analysis. The Tarone trend test confirmed statistical significance for these categories. Pairwise comparisons to vehicle and placebo controls using the Tarone test were statisti-

TABLE 3
Tumor Incidence Summary in Mice Given Troglitazone for 104 Weeks

	Dose (mg/kg)				
	Vehicle	Placebo	50	400	800
Male					
Animals starting	60	60	60	60	60
Animals necropsied at termination	50	54	49	38	35
Unscheduled necropsies	10	6	11	22	25
Non–tumor-bearing animals	22	24	26	32	28
Total tumors[a]	56	49	47	41	47
Tumor-bearing animals	38	36	34	28	32
Tumors/animals starting	0.9	0.8	0.8	0.7	0.8
Tumors/tumor-bearing animal	1.5	1.4	1.4	1.5	1.5
Total malignant tumors	23	13	18	20	28
Malignant tumor-bearing animals	21	12	16	20	24
Malignant tumors/animals starting	0.4	0.2	0.3	0.3	0.5
Malignant tumors/malignant tumor–bearing animal	1.1	1.1	1.1	1.0	1.2
Female					
Animals starting	60	60	60	60	60
Animals necropsied at termination	46	44	42	37	29
Unscheduled necropsies	14	16	18	23	31
Non–tumor-bearing animals	18	21	21	16	11
Total tumors[a]	69	55	57	67	71
Tumor-bearing animals	42	39	39	44	49
Tumors/animals starting	1.2	0.9	1.0	1.1	1.2
Tumors/tumor-bearing animal	1.6	1.4	1.5	1.5	1.1
Total malignant tumors	29	18	28	35	49
Malignant tumor–bearing animals	23	15	25	30	37
Malignant tumors/animals starting	0.5	0.3	0.5	0.6	0.8
Malignant tumors/malignant tumor–bearing animal	1.3	1.2	1.1	1.2	1.3

[a]Does not include multiple sites per tumor type.

cally significant only at 800 mg/kg. Incidence of hepatocellular adenoma was comparable in treated and both control groups, indicating that increases in all hepatocellular tumors was attributable to increases in hepatocellular carcinoma. Increases in all tumors and all malignant tumors were due in part to hepatocellular carcinoma.

A significant increase in the incidence of alveolar-bronchiolar carcinoma, also in females, was identified by Peto analysis. Statistical significance of this tumor category was not confirmed by Cochran-Armitage, Tarone, or exact trend tests, or by Fisher's exact test. In addition, the 3.3% (2/60) incidence of alveolar-bronchiolar carcinomas in high-dose females in this study was within the historical range of 0–4% in female control B6C3F1 mice ($n = 475$) from eight carcinogenicity studies conducted at MPI Research from 1983 to 1994. Incidence of uterine adenocarcinoma compared with vehicle control was also significantly increased by Peto analysis. Statistical significance for this tumor category was not confirmed by supplemental Cochran-Armitage, Tarone, or exact trend tests, or by Fisher's exact test. The 3.3% (2/60) incidence of uterine adenocarcinoma in high-dose females was slightly higher than the historical range at MPI Research (0–2.0%). A single uterine adenocarcinoma in the placebo control group negated the statistically significant positive Peto dose trend.

Rat Carcinogenicity Study

Clinical and hematologic effects. Male rats were given 100, 400, or 800 mg/kg; females were given 25, 50, or 200 mg/kg. Enlargement of interscapular brown fat was observed within the first 13 weeks in high-dose females, and by 104 weeks almost all animals at this dose were affected. Similar findings were seen in mid-dose females and high-dose males during the last 12 months of the study. Increased ventral and anogenital staining was seen in mid- and high-dose females and high-dose males; mid-dose males were also affected during the last 6 months. Labored breathing was noted in both sexes at the high dose during most of the study. Body weight of drug-treated males and low- and mid-dose females were similar to controls throughout the study. In high-dose females, body weight increased during the first 6 months and decreased during the last 12 months of the study. At 104 weeks, body weight of females at 200 mg/kg decreased approximately 11% compared with vehicle control. Food consumption increased at all doses, approximately 4–6% in males and 5–10% in fe-

TABLE 4
Tumor Categories in Mice Given Troglitazone with Statistically Significant Positive Peto Dose Trend Analyses

Tumor category	Dose (mg/kg)[a]					Trend test probability level			
	Vehicle	Placebo	50	400	800	Peto[b]	Tarone	Cochran-Armitage	Exact
Male versus vehicle control									
All vascular tumors	8	10	3	8	17	<0.001	0.002	0.008	ND
Hemangiosarcoma	6	6	2	7	16	<0.001	<0.001	0.002	ND
Female versus vehicle control									
All tumors	42	39	39	44	49	<0.001	<0.001	0.051	ND
All malignant tumors	23	15	25	30	37	<0.001	<0.001	0.004	ND
All vascular tumors	8	7	7	19	18	<0.001	<0.001	0.003	ND
Hemangiosarcoma	5	7	6	16	13	0.002	0.002	0.007	ND
All hepatocellular tumors	8	5	5	8	16	0.002	0.002	0.034	ND
Hepatocellular carcinoma	3	1	3	2	10	0.007	0.006	0.031	ND
Alveolar-bronchiolar carcinoma[c]	0	0	0	0	2	0.012	0.03	0.029	0.034
Uterine adenocarcinoma[b]	0	1	0	0	2	0.012	0.03	0.029	0.034
Male versus placebo control									
All malignant tumors	23	12	16	20	24	<0.001	<0.001	0.008	ND
All vascular tumors	8	10	3	8	17	<0.001	0.005	0.034	ND
Hemangiosarcoma	6	6	2	7	16	<0.001	<0.001	0.002	ND
Female versus placebo control									
All tumors	42	39	39	44	49	<0.001	<0.001	0.008	ND
All malignant tumors	23	15	25	30	37	<0.001	<0.001	<0.001	ND
All vascular tumors	8	7	7	19	18	<0.001	<0.001	<0.001	ND
Hemangiosarcoma	5	7	6	16	13	0.006	0.007	0.026	ND
All hepatocellular tumors	8	5	5	8	16	<0.001	<0.001	0.003	ND
Hepatocellular carcinoma	3	1	3	2	10	<0.001	<0.001	0.002	ND
Alveolar-bronchiolar carcinoma[c]	0	0	0	0	2	0.012	0.031	0.030	0.035

[a]Number of animals affected, $n = 60$.
[b]$p < 0.01$ for common tumors; $p < 0.05$ for rare tumors (Haseman's rule).
[c]Rare tumor based on <1% incidence in controls.

males, compared with vehicle controls. No drug-related ophthalmic findings were observed.

Mean erythrocyte counts decreased 14% in high-dose females compared with vehicle control. Increased incidence of alterations in erythrocyte morphology was observed in mid- and high-dose males, and in females at all doses. Decreased myeloid and increased erythroid counts and a resulting decreased myeloid/erythroid ratio in bone marrow were noted in females at all doses and mid- and high-doses males.

Toxicokinetics. Plasma troglitazone and metabolite concentrations 2 h postdose increased with increasing dose, but were not dose proportional. Troglitazone concentrations were higher in females than males, and M-1 metabolite concentrations were higher in males. Concentrations of all three analytes in this study were similar to those observed at tmax in the 13-week study (Fig. 4), suggesting the toxicokinetic profile did not change appreciably during the 104-week study.

Nonneoplastic pathology. Nonneoplastic changes were observed in heart, brown fat, liver, and bone marrow. Heart weight increased significantly (16–25% in males and 15–51% in females) at the mid and high doses compared with vehicle controls. Increased incidence and severity of ventricular dila-

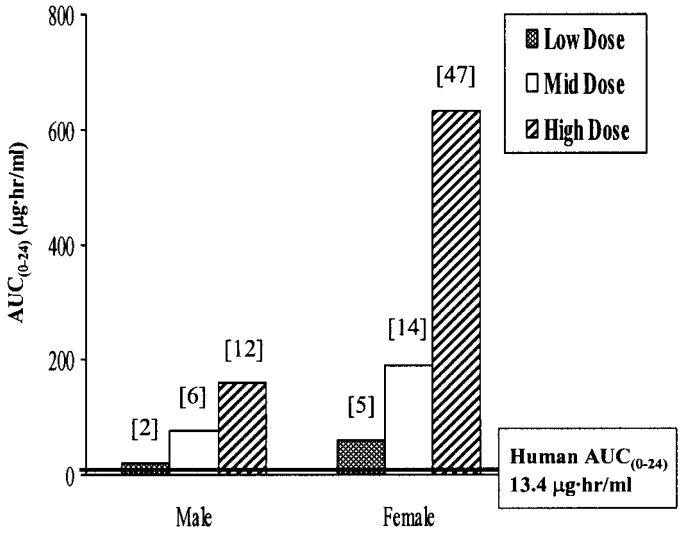

FIG. 4. Plasma $AUC_{(0-24)}$ in rats given troglitazone for 13 weeks. Values in brackets are multiples of human $AUC_{(0-24)}$ at 400 mg daily.

tation, myocardial fibrosis, and karyomegaly of atrial myocytes in males at mid and high doses and in females at all doses were associated with increased heart weight. An increased incidence of atrial thrombosis was noted in mid-dose males and in both sexes at the high dose. These lesions were morphologically similar to age-related spontaneous lesions that occur in this rat strain. In contrast, no histopathologic changes lesions were observed in B6C3F1 mice, a strain that does not exhibit spontaneous age-related cardiomyopathy. Mortality attributed to myocardial lesions was 9, 15, 12, 21, and 49% in males and 4, 10, 9, 5, and 36% in females in the vehicle and placebo controls, and at the low, mid, and high doses, respectively. Changes secondary to myocardial lesions included diffuse centrilobular hepatocellular necrosis in both sexes at the high dose; an increased incidence of alveolar macrophages in low-dose females and in both sexes at mid and high doses; and subcutaneous edema/thoracic effusion in mid-dose males and both sexes at the high dose. In contrast with increased heart weight, skeletal muscle weights (plantaris and gastrocnemius/soleus muscles) were not significantly different from vehicle control, and there were no accompanying histopathologic observations.

Dose-related enlarged brown fat in the interscapular region was noted at necropsy in mid- and high-dose males and in females at all doses, and correlated with firm areas of subcutaneous tissue in the dorsal thorax noted clinically. Increased size and coalescence of fat droplets and increased fibrosis and/or fibroplasia in intra- and interlobular septa were observed microscopically. Similar changes in brown fat in other locations, including paravertebral, thoracic and lumbar, mediastinal, and/or perirenal sites, were observed in high-dose males and in females at all doses.

No gross pathologic changes accompanied 22–32% increases in liver weight and body- and brain-weight ratios in males at mid and high doses. Centrilobular hepatocellular hypertrophy in mid-dose males and in both sexes at the high dose correlated with the organ weight changes. Bone marrow fatty change and hypocellularity were noted in mid- and high-dose females.

Survival and tumor analysis. Survival at high doses was reduced in both sexes during the last 6 months of the study (Fig. 5, Table 5). At termination, survival was 47, 45, 45, 45, and 25% in males and 53, 65, 62, 68, and 17% in females in the vehicle and placebo controls, and at the low, mid, and high doses, respectively. There were no significant drug-related effects on overall tumor incidence in either sex (Table 5). There were 56 distinct tumor types diagnosed in males and 59 in females, most with a frequency of 15 tumor-bearing animals or less. Seventy-one tumor categories (each specific tumor type, tumors grouped by organ, all benign or malignant tumors, and all tumors) in males and 77 tumor categories in females were analyzed statistically. Of the 148 tumor categories analyzed, 3 in males and 8 in females had significant dose trends in the Peto test when compared with controls (Table 6). Tumors with negative dose trends were pituitary adenoma in males, and all tumors of mammary gland, fibroadenoma of mammary gland, and all benign tumors in females.

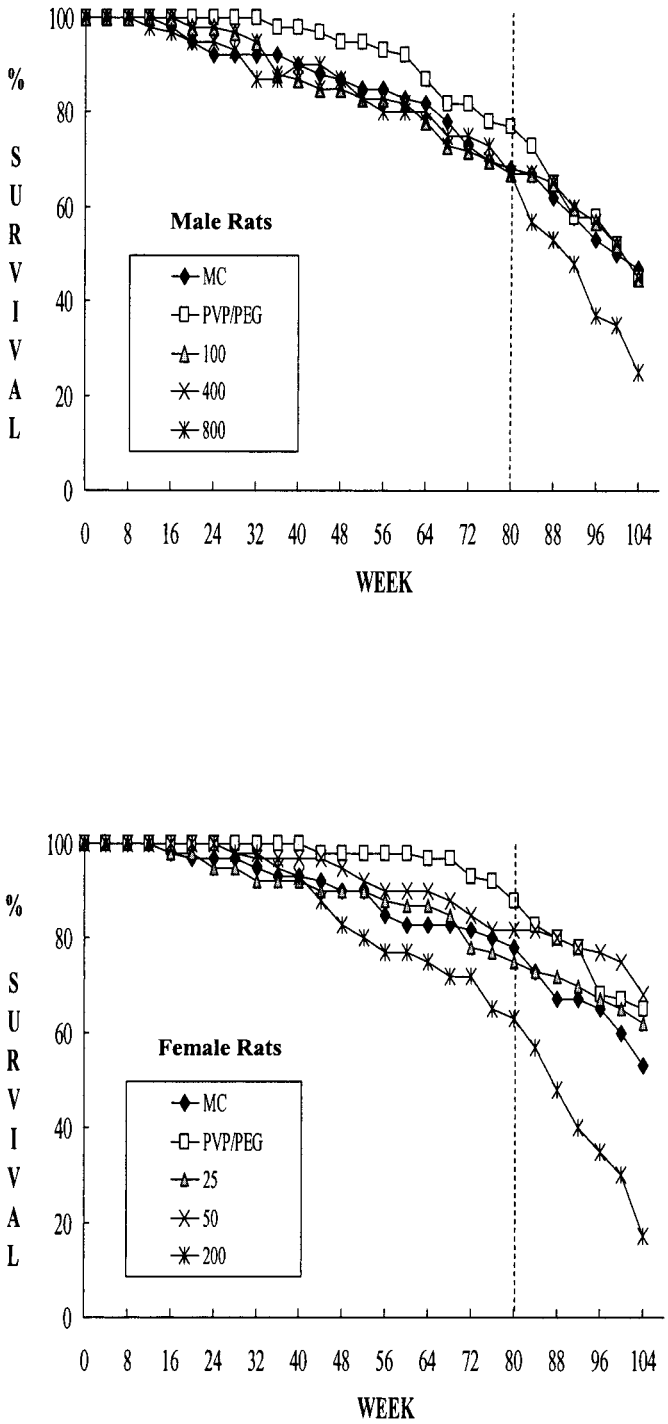

FIG. 5. Kaplan-Meier survival curves in male and female rats given troglitazone for 104 weeks.

In males, angiolipoma of skin had a statistically significant positive dose trend in the Peto test applying Haseman's rule, irrespective of control group used in the analyses. No signifi-

TABLE 5
Tumor Incidence Summary in Rats Given Troglitazone for 104 Weeks

	Males					Females				
Dose (mg/kg)	Vehicle	Placebo	100	400	800	Vehicle	Placebo	25	50	200
Animals starting	60	60	60	60	60	60	60	60	60	60
Animals necropsied at termination	28	27	27	27	15	32	39	37	41	10
Unscheduled necropsies	32	33	33	33	45	28	21	23	19	50
Non–tumor-bearing animals	21	13	20	17	28	8	5	22	8	23
Total tumors[a]	69	75	74	82	54	99	110	67	78	53
Tumor-bearing animals	39	47	40	43	32	52	55	38	52	37
Tumors/animals starting	1.2	1.2	1.2	1.4	0.9	1.6	1.8	1.1	1.3	0.9
Tumors/tumor-bearing animal	1.8	1.6	1.8	1.9	1.7	1.9	2.0	1.8	1.5	1.4
Total malignant tumors	15	18	13	19	15	19	12	13	13	14
Malignant tumor–bearing animals	15	16	12	17	13	18	10	11	13	14
Malignant tumors/animals starting	0.2	0.3	0.2	0.3	0.2	0.3	0.2	0.2	0.2	0.2
Malignant tumors/malignant tumor–bearing animal	1.0	1.1	1.1	1.1	1.2	1.1	1.2	1.2	1.0	1.0

[a]Does not include multiple sites per tumor type.

cant trends were noted with the Tarone or Armitage trend tests, the exact trend test applying Haseman's rule, or in pairwise comparisons using Fisher's exact or Tarone tests. Also in males, liposarcoma of skin had significant positive dose trends in the Peto test applying Haseman's rule, irrespective of control group used in the analyses. A significant positive dose trend in the Peto test was observed for liposarcoma of skin in females when compared with either control group. No significant trends in either sex were noted in the Tarone or Armitage trend tests, the exact trend test applying Haseman's rule, or in pairwise comparisons using Fisher's exact or Tarone tests. Also in females, a significant positive dose trend in the Peto test was observed for fibrosarcoma of skin. No significant trends were noted in the Tarone or Armitage trend tests or in pairwise comparisons using Fisher's exact or Tarone tests. A significant positive dose trend was observed in the exact trend

TABLE 6
Tumor Categories in Rats Given Troglitazone with Statistically Significant Positive Peto Dose Trend Analyses

	Dose[a]					Trend test probability level			
Tumor category	Vehicle	Placebo	Low	Mid	High	Peto[b]	Tarone	Cochran-Armitage	Exact
Male versus vehicle									
Angiolipoma of skin[c]	0	0	0	1	2	0.014	0.031	0.033	0.026
Liposarcoma of skin[c]	0	0	0	1	2	0.044	0.060	0.072	0.053
Female versus vehicle									
All hepatocellular tumors	2	3	0	0	4	0.006	0.065	0.082	0.007
Adenocarcinoma of large intestine[c]	0	0	0	0	1	0.046	0.137	0.136	0.189
Fibrosarcoma of skin[c]	0	0	1	0	4	0.003	0.021	0.028	0.007[d]
Liposarcoma of skin[c]	0	0	0	0	2	0.003	0.030	0.045	0.029
Male versus placebo									
Angiolipoma of skin[c]	0	0	0	1	2	0.014	0.029	0.031	0.026
Liposarcoma of skin[c]	0	0	0	1	2	0.040	0.055	0.066	0.049
Female versus placebo									
Adenocarcinoma of large intestine[c]	0	0	0	0	1	0.040	0.128	0.127	0.180
Fibrosarcoma of skin[c]	0	0	1	0	4	0.002	0.018	0.025	0.006[d]
Liposarcoma of skin[c]	0	0	0	0	2	0.003	0.027	0.040	0.027
Schwannoma of uterus (malignant)	1	0	0	1	2	0.004	0.022	0.067	0.015[d]

[a]Number of animals affected, $n = 60$. Low, mid, and high doses were 100, 400, and 800 mg/kg in males, respectively, and 25, 50, and 200 mg/kg in females, respectively.

[b]$p < 0.01$ for common tumors; $p < 0.05$ for rare tumors (Haseman's rule).

[c]Rare tumor based on <1% incidence in controls.

[d]$p < 0.005$ for common tumors; $p < 0.025$ for rare tumors (Haseman's rule).

test in comparison with both the vehicle and placebo controls. Fibrosarcoma was not observed in concurrent female controls but have been noted in 2–4% of controls in carcinogenicity studies conducted at MPI Research. The incidence in females at the high dose (6.7%) was slightly higher than the historical background incidence.

The category of all hepatocellular tumors in female rats exhibited a positive dose trend in the Peto test when compared with vehicle control but not when compared with placebo control. No significant differences were seen in additional analyses of this category. Further, no significant differences in any statistical test versus either control group were seen in hepatocellular adenoma or hepatocellular carcinoma, tumor types comprising the category all hepatocellular tumors. In females, adenocarcinoma of the large intestine exhibited a positive dose trend in the Peto analysis, irrespective of control group used for comparison. No significant differences were observed in additional statistical tests used, and the incidence was within the historical control range in this strain. Additionally, an adenocarcinoma of the small intestine was observed in a vehicle control male, but not in drug-treated groups. In the Peto test, a significant positive dose trend was observed for schwannoma of the uterus compared with placebo control, but not when compared with vehicle control. A significant positive dose trend was observed in the exact trend test using Haseman's rule when compared with placebo control, but not when compared with vehicle control.

DISCUSSION

For valid evaluation of tumorigenic potential in rodents, survival in all groups ideally should be at least 50% at Week 80 (Lin and Ali, 1994). In these studies with troglitazone, there was adequate survival in both mice and rats. In mice, survival at Week 80 was $\geq$ 82%, with at least 49 surviving animals per group. In rats, survival at Week 80 was $\geq$ 63%, with at least 38 surviving animals in each group. With the exception of hemangiosarcoma in male and female mice and hepatocellular carcinoma in female mice, there were no significant increases in tumor incidence in either species. Other tumor types occurred at very low incidence; incidences were comparable to concurrent controls and/or historical background rates; there was a lack of dose response; and/or there was no statistical evidence of significance based on the inferential test battery.

In addition to tumor incidence, an important consideration in assessment of carcinogenic potential is genotoxicity. The genotoxic potential of troglitazone was evaluated in a series of *in vitro* and *in vivo* assays that indicated no genotoxic risk (Hirano *et al*., 1993; Rezulin Package Insert). Troglitazone was neither mutagenic in bacteria nor clastogenic in bone marrow of mice or rats. Equivocal increases in chromosome aberrations were observed in an *in vitro* assay in Chinese hamster lung cells, but only at cytotoxic doses. In mouse lymphoma gene-mutation assays, results were equivocal when conducted with a microtiter technique, but were negative with the more reliable agar plate technique. An *ex vivo* rat liver unscheduled DNA synthesis assay was negative. Chemical structure-activity assessment using the computational predictive system Deductive Estimation of Risk from Existing Knowledge (DEREK™; Lhasa Ltd.) indicates that the novel chemical structure of this thiazolidinedione, with a tetramethyl benzopyran functional group that confers antioxidant properties to the molecule, does not contain relevant structural alerts associated with known carcinogens.

Also important in assessing carcinogenic risk are biologic characteristics of chemically induced tumors. With respect to hepatocellular carcinoma induced in mice with troglitazone, liver weight increase in the carcinogenicity study is consistent with increased liver weights in shorter duration studies occurring concurrently with microsomal enzyme induction. Promotion of hepatocarcinogenesis in rodents appears to be related to microsomal enzyme induction, particularly in mice (McClain, 1990; Popp and Cattley, 1993). The incidence of liver tumors in rats was not increased at troglitazone exposures up to 47-times the human therapeutic level. The relevance of mouse liver tumors to human drug therapy is questionable (Butler and Newberne, 1975; McClain, 1990; Newberne, 1975), and such tumors generally are not considered indicators of human risk. Troglitazone exposure in female mice at 800 mg/kg, the only dose associated with an increased incidence of hepatocellular carcinoma, was 23 times the human therapeutic level.

Little is known about the tumor biology of hemangiosarcoma in rodents, and metastatic potential of these tumors is poorly understood. When multiple vascular tumors appear in the same animal, it is not possible to establish the primary site by routine histopathological examination. Evidence suggests the widely distributed neoplasms arise concurrently (Yamate *et al.*, 1988). Vascular tumors can develop as phenotypically benign tumors in one site and malignant tumors in other sites. This combination of benign and malignant phenotypes within the same animal suggests a multicentric derivation rather than a primary origin followed by metastasis.

Species specificity in troglitazone-associated hemangiosarcoma incidence was not pharmacokinetically based, as drug exposure in high-dose female rats was twice that of high-dose male and female mice, yet an increased incidence of vascular tumors was observed only in mice. These data suggest that relative to rats, mice are more susceptible to vascular neoplasms. Hemangiosarcomas in troglitazone-treated mice and concurrent controls were located in the same sites, suggesting the drug exacerbated the incidence of a spontaneously occurring tumor.

Hemangiosarcomas occur spontaneously, particularly in B6C3F1 mice, typically at an incidence of 3–5% (Haseman *et al.*, 1984; Sheldon *et al.*, 1995). Contemporary carcinogenicity studies at MPI Research suggest a time-related increase in background incidence such that spontaneous hemangiosarcomas are more prevalent than reported in the literature. The

differences in tumor incidence in this study (8–12%), other studies conducted at MPI Research (0–13%) and at Pfizer Ann Arbor Laboratories (0–9.2%), as well as published ranges, reflect the broad range of variation in B6C3F1 mice.

The biologic characteristics of hemangiosarcoma in mice are considerably different from those in humans. Benign and malignant neoplasms occur in two distinct patient populations (Enzinger and Weiss, 1995). Hemangiomas develop principally in children and generally involve the skin. Multifocal and parenchymal lesions develop in a small subset of patients. Lesion regression is spontaneous; time to complete regression can be up to 10 years. In the liver, these vascular tumors can act as space-occupying masses with potential to induce portal hypertension or abdominal hemorrhage. Multiple hemorrhagic and thrombotic episodes involving vascular tumors can lead to thrombocytopenia and hemorrhagic crises. In contrast, hemangiosarcomas occur principally as cutaneous lesions of the head and neck, particularly in the scalp of the elderly. These tumors have the potential to metastasize to bone marrow, liver, and lung. Hemangiosarcomas are uncommon vascular tumors in humans, representing less than 1% of all sarcomas (Enzinger and Weiss, 1995). Moreover, malignant transformation of benign vascular tumors in humans is unusual.

In contrast, vascular tumors in mice appear as a morphologic continuum from benign to malignant, with similar onset and latency, and occur primarily in the second year of life. With troglitazone, multicentric and single vascular tumors occurred in all groups, and most multicentric multiorgan tumors were hemangiosarcomas. There were no primary or metastatic hemangiosarcomas in lung. Thus, the biologic behavior of troglitazone-induced hemangiosarcomas in mice differ from spontaneous vascular tumors in humans.

Because troglitazone is not a genotoxicant, an indirect epigenetic mechanism of vascular tumor-induction is likely. None of the 165 vascular tumors in mice had inactivating p53 mutations, and only a low frequency ($< 5\%$) had mutations of Ki- and Ha-*ras* protooncogenes (Duddy *et al*., 1999a,b). One potential mechanism of vascular tumor induction is based on constitutive synthesis of angiogenic growth factors such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) in adipocytes (Bastagli *et al.*, 1995; Claffey *et al.*, 1992; Folkman and Klagsbrun, 1987; Kardami *et al.*, 1995). As adipose tissue mass is significantly increased in mice by troglitazone, drug-induced stimulation could lead to sustained increases in the local concentration of endogenous angiogenic factors. These angiogenic factors, in turn, could exert trophic effects on endothelial cells, leading to angiogenesis, endothelial cell proliferation, increased mutational frequency, and eventual neoplastic transformation of endothelial cells, leading to clonal expansion and tumor formation (Aaronson, 1991; Ames *et al.*, 1995).

In summary, troglitazone increased the incidence of hemangiosarcoma in mice at $AUC_{(0-24)}$ values at least 16 times therapeutic $AUC_{(0-24)}$ in humans given 400 mg daily. The incidence of hepatocellular carcinoma was increased in female mice at 23 times human therapeutic exposure. Because troglitazone is not genotoxic, the mechanism of tumor formation was considered epigenetic. No carcinogenic effects were observed in mice at exposures up to 14 times human therapeutic exposure. No tumors of any type were increased in rats at exposures up to 47 times therapeutic exposure.

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