NONCLINICAL SAFETY EVALUATION OF A NOVEL HYPOGLYCEMIC AGENT PPAR-4; PARTIAL PPAR-γ AGONIST.

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Abstract—The toxicity of PPAR-4; partial peroxisome proliferator activated receptor (PPAR) agonist, was evaluated in a comprehensive nonclinical toxicology program that included single-dose oral toxicity studies in mice and rats; repeat-dose toxicity studies in rats; a battery of in vitro and in vivo genetic toxicity studies; carcinogenicity studies in mice and rats; reproductive and developmental toxicity studies in rats. Pharmacologically mediated changes, similar to those observed with other PPARγ agonists, were observed following chronic administration and included subcutaneous edema, hematologic/hematopoietic and serum chemistry alterations, and morphologic findings in the heart and adipose tissue in rats. PPAR-4 was nongenotoxic in the standard battery of genotoxicity studies. Gallbladder adenomas in male mice and adipocyte neoplasms in male and female rats were seen at suprapharmacologic exposures, whereas urinary bladder tumors occurred in male rats at lower exposures. PPAR-4 had no effects on reproductive function in male and female rats at high systemic exposures, was not teratogenic in rats and demonstrated no selective developmental toxicity. Overall, there were no nonclinical findings that precluded the safe administration of PPAR-4 to humans.

Key words: Hypoglycemic; lowering triglycerides; Partial PPAR-γ Agonist; toxicology profile; chronic toxicity.

1. INTRODUCTION

The incidence of type 2 diabetes, a chronic debilitating disease, is increasing rapidly in industrialized nations, and it is estimated that there will be 221 million diabetic patients worldwide by the year 2010.[1] Several agonists of the peroxisome proliferator–activated receptors (PPARs) have been developed for the treatment of type 2 diabetes based on their role in regulating lipid metabolism and insulin sensitization.[2,3,4] PPARs are ligand-activated transcription factors that modulate target gene expression by binding to specific peroxisome proliferator response elements (PPREs) in promotor regions of regulated genes.[2,5,6] Three receptor types have been identified in the family including alpha (α), gamma (γ), and delta (δ)—also called beta. PPARα agonists are primarily used to treat dyslipidemia,[7] PPARγ is mainly associated with adipose tissue, where it controls adipocyte differentiation and insulin sensitivity whereas the PPARδ agonists increase glucose utilization, reduce hepatic glucose production, and enhance insulin sensitivity.[8,9]

PPAR-4 showed a activation against human PPARγ without activating human PPARα and PPARδ. Overall, these studies suggest that PPAR-4 improves insulin resistance in such animal models through activation of PPARγ-mediated transcriptional activity and that it would be a new therapeutic candidate with potential for the treatment of type 2 diabetic patients.

The toxicity of PPAR-4 in animals was well characterized in a comprehensive nonclinical toxicology program to establish a safe starting dose for clinical trials and to identify potential adverse effects during clinical development. The objective of this article is to present the overall nonclinical toxicity profile of PPAR-4.

2. MATERIALS AND METHODS

2.1 Compounds

PPAR-4 was synthesized at Poona College of pharmacy, Pune, India. The compounds were dissolved in dimethyl sulfoxide (DMSO) and added to medium to a final DMSO concentration of 0.1% for toxicological studies. PPAR-4 is chemically described as (5Z)-5-[3, 4, 5 trimethoxy-phenyl) methylene] thiazolidine-2, 4-dione).

2.2 Animals

The species used in the toxicology studies included C57BL/6J mice and Sprague-Dawley rats. PPAR-4 was administered by oral gavage for both mice and rats.

2.3 Doses administered

Single-dose toxicity studies of PPAR-4 were conducted by the oral route at doses of 500 4000 mg/kg in mice (five per sex per group) and rats (five per sex per group).

Chronic oral toxicity studies were conducted over a wide

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range of doses in 20 rats per sex per group (0.3–300 mg/kg) for 6 months.

2.4 Dose selection in rat (chronic studies)

PPAR-4 was well tolerated when administered to rats at doses of 3–300 mg/kg for up to 1 month. Minimal to moderate decreases in erythrocyte count and hematocrit were observed at all doses; vacuolation and hyperplasia of adipocytes were observed at _ 30 mg/kg; and at 300 mg/kg, drug-related changes included increased liver and heart (> 30%) weights and multifocal fibroplasias /fibrosis of adipose tissue. Based on these results, 300 mg/kg was selected as the high dose in the definitive chronic study; additional doses of 0.3, 3, and 30 mg/kg were selected to investigate dose-response relationships. The doses selected for the 6-month oral toxicity study were associated with PPAR-4 plasma concentrations 0.7–312 times and 0.6–376 times human therapeutic exposure in male and female rats, respectively (Table 1).

In the 6-month oral toxicity study, rats were observed daily for clinical signs of toxicity; detailed physical examinations were performed pretest and weekly; individual body weights and food consumption were recorded weekly; ophthalmic examinations were performed on all animals pretest and after a daily dose during weeks 13 and 26; water intake (fasted) over an 18-h period was determined during weeks 14 and 27; blood analyses were collected from the tail vein (fasted animals) at weeks 12 and 24 for hematology and clinical chemistry and from the heart at study termination for blood coagulation testing; urinalyses were conducted on urine samples collected over an 18-h fasted period during weeks 14 and 27; all animals were necropsied at study termination and subjected to a thorough gross and microscopic evaluation of tissues.

Table 1. Multiples of Human exposure for PPAR-4 in Pivotal Toxicity Studies

Species	Type of study (sampling time)	Dose (mg/kg/day)	Exposure multiple[a]	
			Male	Female
Mouse	2-year oral carcinogenicity (day 177)	1	2	4
		5	13	23
		20	60	81
		40	144	158
Rat	6-month oral toxicity (day 168)	0.3	0.4	0.7
		3	5	7
		30	45	52
		300	310	368
Rat	2-year oral carcinogenicity (day 178)	1	2	3
		5	6	5
		30	33	40
		50	44	53
Pregnant rat	Toxicokinetics[b] (DG 15)	1.8	--	4
		18	--	51
		180	--	403
Lactating rat	Toxicokinetics[c] (DL 4)	1.5	--	3
		5	--	12
		15	--	33
		45	--	121

[a]AUC(TAU) at steady state at 5 mg/day doses in a multiple ascending dose study in type 2 diabetic human subjects (CV168002): 4.884 lg_h/ml. AUC exposure in animals at steady state (animal exposure divided by human exposure).

[b]Supports embryo-fetal development study in rats or rabbits.

[c]Supports pre- and postnatal development studies in rats.

Note. ''--'' Indicates not applicable for male.

2.5 Genotoxicity

The potential genotoxicity of PPAR-4 was investigated in a standard battery of in vitro and in vivo genetic toxicity studies including an exploratory bacterial mutagenicity assay, Ames reverse mutation assay in Salmonella and Escherichia coli, cytogenetics study using primary human lymphocytes, and oral micronucleus study in rats.

2.6 Reproductive toxicity studies

A battery of reproductive and developmental toxicity studies was conducted in rats with PPAR-4. These studies evaluated the effects on fertility of PPAR-4 in male and female rats (25 per sex per group), as well as the potential embryo-fetal toxicity and teratogenicity in 22 presumed pregnant rats and prenatal/postnatal toxicity in 25 presumed pregnant rats per group.

2.7 Carcinogenicity studies

Traditional lifetime carcinogenicity studies (up to 2 years) were conducted in mice and rats with PPAR-4. In mice, two studies were conducted—doses of 1, 5, or 20 mg/kg were evaluated in the first (initial) and 40 mg/kg in the second (Supplementary). The first study contained two vehicle control groups, whereas one vehicle control group was included in the second study; all groups, including controls, contained 60 mice per sex per group. In addition, 19 mice per sex per group were designated for toxicokinetic analysis in each study and received PPAR-4 by oral gavage once daily at 1, 5, 20, or 40 mg/kg for 26 weeks.

In the carcinogenicity study in rats, 65 rats per sex group were

given PPAR-4 by oral gavage once daily at 1, 5, 30, or 50 mg/kg for up to 105 weeks. Two control groups (65 rats per sex per group) were given the same vehicle used in mice, dimethyl sulfoxide (DMSO solution. The dose volume for all studies was 5 ml/kg. In the rat carcinogenicity study, toxicokinetic analyses were conducted on blood samples collected from study animals rather than from separate satellite groups.

3. RESULTS

The systemic exposures (AUC) achieved in mice, rats (nongravid, gravid, lactating). Table 1 provides the animal exposure achieved in pivotal toxicology studies. Since PPAR-4 is highly bound (> 99%) to mouse, rat serum proteins, exposure were calculated using total concentration of the drug. These demonstrate that adequate doses were utilized in the toxicity studies to ensure a comprehensive evaluation of the toxicity of PPAR-4 and to assess potential risk.

3.1 PPAR-4 modulation of ppar target gene expression in mice

The peroxisome proliferator activated receptors (PPARs) are nuclear receptors that play key roles in the regulation of lipid metabolism and differentiation. In transient transactivation assay in NIH3T3 cells, PPAR-4 showed activation against human PPARγ with an EC_{50} of 1.01 μM without activating human PPARα and PPARδ.

3.2 Acute toxicity studies

PPAR-4 demonstrated a low order of acute toxicity; the minimum lethal dose was 2000 mg/kg in mice, greater than 4000 mg/kg in rats. Single doses of 2000 mg/kg and greater caused decreased activity and death in mice; a single dose of 4000 mg/kg resulted in unkempt appearance, loose feces, and decreased body weights in rats.

3.3 Chronic toxicity studies (Pharmacologically mediated effects)

Edema

Swelling (edema) of the limbs, jaw, abdomen, head, and/or whole body (generalized) was observed at the overtly toxic dose of 300 mg/kg in rats administered PPAR-4 for up to 6 months (Table 1). Due to the severity of the edema observed at 10 mg/kg in the 9-month study, the high dose was reduced to 5 mg/kg during week 17. However, the severity of the edema was not appreciably different after the dose was reduced. Over the course of the 9-month and 1-year studies, the frequency of edema, its severity, and the affected area varied for the animals.

Hematologic /hematopoietic effects

Dose-dependent hematologic and/or hematopoietic changes occurred in rats after chronic treatment with PPAR-4. At doses of ≥30 mg/kg, hematologic changes in rats included generally minimal to mild decreases in red blood cell count, hematocrit and hemoglobin (3–20%) after 6 months of treatment with no associated effect on bone marrow (Table 2). Additionally, in response to the decreased erythrocytic parameters, there was evidence of an erythrocytic regenerative response characterized by increased polychromasia, anisocytosis, reticulocyte counts, and splenic extramedullary hematopoiesis at the overtly toxic dose of 300 mg/kg in the 6-month study (Table 1).

Table 2. PPAR-4-Related Erythrocytic Changes in Rats

Species	Control	3 mg/kg	30 mg/kg	300 mg/kg
Rat; Males				
Red blood cells (3106/ll) month 6	9.1	9.1	8.3 **	7.1**
Hematocrit (%) month 6	48.5	48.0	45.6**	40.1**
Hemoglobin (g/dl) month 6	15.6	15.3	14.7*	13.2**
Rat; Females				
Red blood cells (3106/ll) month 6	7.8	7.9	7.1**	6.0**
Hematocrit (%) month 6	43.0	43.1	42.0	37.2**
Hemoglobin (g/dl) month 6	14.4	14.4	13.6*	11.8**

Note : Mean, n = 9–20 (rats). Statistically significant, *p < 0.05, **p < 0.01; statistical comparison to the concurrent control groups (Dunnett test).

Serum chemistry effects

Expected pharmacologically mediated serum chemistry changes occurred across the dose range tested in rats (0.3–300 mg/kg) following chronic treatment (≥6 months) with PPAR-4. In rats, these changes generally included decreases in serum glucose, triglycerides, and total cholesterol at doses ≥3 mg/kg (Table 3). Other pharmacologically mediated serum chemistry changes included minimal decreases in albumin (4–12%) and protein (4–18%) at 30 and 300 mg/kg in rats (Table 3).

Table 3. Selected PPAR-4-Related Changes in Serum Chem-

istry in Rats at 6 months

Species	Control	0.3 mg/kg	3 mg/kg	30 mg/kg	300 mg/kg
Rat; Males					
Cholesterol (mg/dl)	130.3	132.5	112.2*	90.7**	104.5*
Glucose (mg/dl)	141.2	151.7**	140.4	142.0	130.2
Triglycerides (mg/dl)	86.7	96.9	70.4*	58.8**	50.8**
Total protein (g/dl)	7.02	6.75*	6.80	6.63**	5.68**
Albumin (g/dl)	4.41	4.37	4.22	4.0**	3.70**
Rat; Females					
Cholesterol (mg/dl)	139.7	137.8	130.5	124.3*	121.8
Glucose (mg/dl)	150.0	147.4	143.1*	137.9**	130.2**
Triglycerides (mg/dl)	70.3	67.0	78.7	65.1*	44.4**
Total protein (g/dl)	6.86	6.77	6.81	6.40**	6.10**
Albumin (g/dl)	4.57	4.60	4.61	4.18**	4.34**

Note: Mean, n = 9–20. Statistically significant, *p < 0.05, **p < 0.01; statistical comparison to the concurrent control groups (Dunnett test).

Adipose tissue effects

Dose- and time-dependent adipose tissue changes were observed in rats following chronic treatment with PPAR-4. In rats after 6 months of dosing at 30 and 300 mg/kg, PPAR-4-related gross pathologic changes consisted of subcutaneous fat masses beneath the skin of the flank, axillary, and interscapular regions (Table 1). Microscopically at ≥3 mg/kg, there was generally dose-related hyperplasia of brown and white, adipose cells in adipose tissue throughout the body. The hyperplasia was most apparent in samples collected from the macroscopically apparent masses in the skin (Table 4). Additionally, there was increased vacuolation, fibroplasia/ fibrosis, and subacute inflammation of subcutaneous brown adipose tissue masses from the interscapular regions at 30 and/or 300 mg/kg (Table 4). These included dose-related increases in white adipocytes and decreases in brown adipocytes in representative fat depots (subcutaneous and perirenal) at all doses; increased white adipocytes in the bone marrow, pancreas, adrenal cortex, and thyroid gland (females) at 2 and 5 mg/kg; and increases in white adipocytes in the parathyroid glands in females at 5 mg/kg.

Table 4. PPAR-4 Related Adipose Tissue Findings in Rats at 6 months

Finding	Control M/F	0.3 mg/kg M/F	3 mg/kg M/F	30 mg/kg M/F	300 mg/kg M/F
Hyperplasia, brown adipose tissuea					
Mild	---	---	---	---	4/2
Moderate	---	---	---	---	16/18
Fibroplasia/fibrosis, brown adipose tissuea					
Minimal to mild	---	---	---	---	18/11
Moderate	---	---	---	---	2/9
Vacuolation, brown adipose cellsa					
Minimal to mild	---	---	---	7/15	1/2
Moderate	---	---	---	12/5	16/16
Marked	---	---	---	---	3/1
Inflammation, sub-acute, brown adipose tissuea					
Minimal	---	---	---	---	3/2
Hyperplasia, white adipose tissue Minimal to mild	---	---	0/16	0/19	20/19

Note. ''—'' Indicates absence of finding/severity in the group.

Heart effects

Dose- and time-dependent heart weight increases and correlative morphologic changes occurred in both rats (Male and Female) following administration of high doses of PPAR-4. In rats, absolute heart weights were increased 16 and 51% in males and 17 and 35% in females at 30 and 300 mg/kg, respectively, after 6 months of treatment.

Liver effects

Following 6 months of treatment, liver weights in rats were minimally to moderately increased in males at 300 mg/kg (27% absolute weight) and in females at 30 and 300 mg/kg (15–64% absolute weight; Supplementary Table 1). These liver weight increases were not accompanied by increased serum transaminase activities (ALT, AST).

3.4 Chronic toxicity studies
(Nonpharmacologically mediated effects)

Central nervous system effects

There were no microscopic alterations in the brain/spinal cord following treatment with PPAR-4 in mice or rats for up to 2 years.

Testicular effects

There was no evidence of testicular lesions found with PPAR-4 treatment in mice or rats for up to 2 years.

Genotoxic assessment

PPAR-4 demonstrated no mutagenic activity in the bacterial reverse mutation assay and no clastogenic activity in primary human lymphocytes. In addition, PPAR-4 was not clastogenic

in the in vivo micronucleus test up to a maximum tolerated dose (1200 mg/kg) in female rats or the limit dose (2000 mg/kg) in male rats.

Carcinogenic assessment

In male mice, administered PPAR-4 at 20 and 40 mg/kg with systemic exposures ≥62 times that seen at a clinical dose of 5 mg/day, a low incidence of benign gallbladder adenoma (incidences of 1/60 and 2/60 mice, respectively) occurred and was considered drug-related[10].

In rats, PPAR-4-related tumors were observed in the urinary bladder and adipose tissue. An increased incidence of transitional cell papillomas and carcinomas of the urinary bladder were noted in males at 5, 30, and 50 mg/kg (8, 37, and 48 times, respectively, the mean human exposure at 5 mg/day). In addition to the urinary bladder tumorigenic response, there was an increased incidence of subcutaneous liposarcoma (malignant tumor of adipose cells) in male rats (48 times human exposure at 5 mg/day) and lipoma (benign tumor of adipose cells) in female rats (59 times human exposure at 5 mg/ day) at 50 mg/kg [10].

Reproductive effects

In the study on fertility and early embryonic development in rats, PPAR-4 had no effect on reproductive function in males at 600 mg/kg (maximum dose tested) or in females at 60 mg/kg. In female rats, altered estrous cycling as well as reductions in ovulation, fertility, implantation, and litter size was observed only at 600 mg/kg, a dose that caused overt toxicity (excess salivation, soft feces, perioral substance, rales, reduced body weight gain/body weight loss, and reduced food consumption during gestation).

In the studies of pre- and postnatal development in rats, decreased pup viability and observations suggestive of subcutaneous hemorrhage and poor physical condition of pups at birth and/or during lactation occurred at doses of 5–135 mg/kg.

4. DISCUSSION

The administration of PPAR-4, resulted the potential toxicity was investigated and as such provided a comprehensive toxicity evaluation in animals.

The edema observed in rats treated chronically with PPAR-4 is a common finding in animals administered other PPAR dual and/or gamma agonists.[11] Although expanded plasma volume is generally considered a major factor in its development.[12,13,14] The persistent minimal to mild decreases in the erythrocytic parameters (RBC counts, hematocrit, and hemoglobin) in PPAR-4 treated rats and monkeys, along with reductions in serum total protein and albumin (rats) were consistent with hemodilution as a consequence of increased plasma volume.[13,14,15]

Alterations in brown and white adipocytes similar to those observed with PPAR-4 have been described in rats.[15,16] The adipose changes have been attributed to differentiation of preadipocytes (adipocyte precursor cells) into adipocytes in response to dietary lipids by activation of genes involved in lipid synthesis and storage pathways.[17,18,19]

Heart weight increases with PPAR-4 have been observed in rats treated with other dual and gamma PPAR agonists.[11,20,21,22,23] The increases in heart weights associated with these drugs have been attributed to cardiac hypertrophy secondary to plasma volume expansion and associated hemodilution. [11,14,15,24]

The liver weight increases in nonclinical toxicology studies in mice and rats treated with troglitazone.[20,21,22,23] In rats administered PPAR-4 has been attributed to induction of hepatic drug-metabolizing enzyme activity. PPAR-4 failed to induce hepatocellular peroxisome proliferation was potentially due to its less robust PPARα activation relative to other PPARα agonists.

PPAR-4 increased the incidence of benign gallbladder adenomas in male mice and adipocyte tumors in male and female rats.[12] In comparison, increased incidences of benign gallbladder adenomas have not been described in mouse carcinogenicity studies with PPARα agonists[25] and have been seen with only one of the seven PPARδ agonists reviewed by the FDA,[11] suggesting that the gallbladder adenomas in PPAR-4-treated mice were not likely mediated by a direct pharmacologic effect on the gallbladder epithelium. Importantly, there was no evidence of increased cholelithiasis or hepatobiliary disease in phase III clinical trials with PPAR-4.[26]

The subcutaneous adipocyte tumors in rats treated with PPAR-4 likely were a consequence of chronic pharmacologic stimulation of preadipocytes at high systemic exposures.

In male rats given PPAR-4, the incidences of transitional cell papilloma and carcinoma of the urinary bladder were increased.[10] Similarly, an increased incidence of urinary bladder tumors was reported for pioglitazone-treated male rats. In a subsequent 21-month investigative study of PPAR-4 in male rats, chronic mucosal injury proliferation secondary to pharmacologically mediated increases in urinary solids (calcium- and magnesium-containing microcrystalline precipitates, crystals, aggregates, and/or microcalculi) was shown to be the nongenotoxic mechanism for the male rat–specific urinary bladder tumorigenic response.[27]

The reproductive and developmental changes that occurred with PPAR-4 were similar to those observed with PPARγ agonists. Therefore PPAR-4 altered estrous cyclicity and reduced fertility in female rats.

5. CONCLUSION

In conclusion, PPAR-4 demonstrated good oral tolerability and a spectrum of clinical, clinical pathology, and anatomic chang-

es in rats that were pharmacologically mediated and, with the exception of edema, generally occurred at high doses and exposures. PPAR-4 was neither genotoxic nor teratogenic. In carcinogenicity studies, it induced urinary bladder and adipocyte tumors in rats, and a low incidence of gallbladder adenoma in mice. Overall, the toxicity profile of PPAR-4 is comparable to that described for PPARγ agonist.

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