Effects of Pioglitazone, a Peroxisome Proliferator–Activated Receptor Gamma Agonist, on the Urine and Urothelium of the Rat

Shugo Suzuki,* Lora L. Arnold,* Karen L. Pennington,* Satoko Kakiuchi-Kiyota,* Min Wei,† Hideki Wanibuchi,†
and Samuel M. Cohen*[,1,2]

*Department of Pathology and Microbiology and the Eppley Institute for Cancer Research, University of Nebraska Medical Center, Omaha, Nebraska
68198-3135; and †Department of Pathology, Osaka City University Medical School, Osaka 545-8585, Japan

[1]Havlik-Wall Professor of Oncology.
[2]To whom correspondence should be addressed. Fax: (402) 559-9297. E-mail: scohen@unmc.edu.

Peroxisome proliferator–activated receptors (PPARs) are ligand-activated transcription factors, which belong to the nuclear receptor superfamily. Some PPARγ agonists, such as pioglitazone, and dual PPARγ/PPARα agonists, such as muraglitazar, induced urothelial bladder tumors in rats but not in mice. In this study, we investigated the early effects in the urine and bladder of rats treated with pioglitazone to evaluate the possible relation between urinary solids formation and urothelial cytotoxicity and regenerative proliferation. In a 4-week experiment, treatment of rats with 16 mg/kg pioglitazone induced cytotoxicity and necrosis of the urothelial superficial layer, with increased cell proliferation measured by bromodeoxyuridine labeling index and hyperplasia by histology. It also produced alterations in urinary solid formation, especially calcium-containing crystals and calculi. PPARγ agonists (pioglitazone and troglitazone) *in vitro* reduced rat urothelial cell proliferation and induced uroplakin synthesis, a specific differentiation marker in urothelial cells. Our data support the hypothesis that the bladder tumors produced in rats by pioglitazone are related to the formation of urinary solids. This strongly supports the previous conclusion in studies with muraglitazar that this is a rat-specific phenomenon and does not pose a urinary bladder cancer risk to humans treated with these agents.

Key Words: peroxisome proliferator–activated receptor; urinary bladder; urothelial cell cytotoxicity; urinary solids; differentiation.

Peroxisome proliferator–activated receptors (PPARs) are ligand-activated transcription factors, which belong to the nuclear receptor superfamily (Michalik *et al.*, 2004; Tachibana *et al.*, 2008; Yki-Jarvinen, 2004). Three major PPAR receptors have been identified, alpha, gamma, and delta (beta), with differing tissue distributions and effects (Berger and Moller, 2002). PPAR alpha (PPARα) is expressed predominantly in the liver, kidney, heart, and skeletal muscle and enhances free fatty acid oxidation, controls expression of multiple genes regulating lipoprotein concentration, and has anti-inflammatory effects (Berger and Moller, 2002; Tachibana *et al.*, 2008). PPAR delta

(PPARδ) (also referred to as PPAR beta) is expressed ubiquitously, is required for placental development, and is involved in the control of lipid metabolism (Berger and Moller, 2002; Michalik *et al.*, 2004). PPAR gamma (PPARγ) has two isoforms, PPARγ1 and PPARγ2. PPARγ2, which contains an additional 28 amino acids at the N-terminal compared to PPARγ1, is expressed exclusively in adipose tissue, whereas PPARγ1 is expressed in heart, skeletal muscle, kidney, pancreas, and several epithelial tissues, such as urothelium and intestine. PPARγ induces adipocyte differentiation and is involved in the control of inflammatory reactions and in glucose metabolism through enhanced insulin sensitivity (Berger and Moller, 2002; Tachibana *et al.*, 2008). Agonists have been developed for each of these receptors, with differing pharmacologic and toxicologic effects (Berger and Moller, 2002; Yki-Jarvinen, 2004).

In a tabulation of PPAR agonists under development as pharmaceuticals, El-Hage (2005) reported that five of six dual PPARγ/PPARα agonists and pioglitazone, a PPARγ agonist, induced urothelial bladder tumors in male rats but not in mice. Lubet *et al.* (2008) also reported that another PPARγ agonist, rosiglitazone, enhanced bladder tumors in rats pretreated with N-(4-hydroxybutyl)-N-(butyl)nitrosamine (BBN), a known DNA-reactive bladder carcinogen in several species. Muraglitazar, one of the five dual PPARγ/PPARα agonists listed by El-Hage (2005) that induced rat urinary bladder carcinogenesis in a 2-year bioassay, caused bladder tumors, which occurred predominantly in male rats compared to females and did not occur in mice (Tannehill-Gregg *et al.*, 2007). Evidence was presented that the mechanism of rat bladder carcinogenesis induced by muraglitazar involved a mode of action involving increased formation of urinary solids resulting in urothelial cytotoxicity and increased cell proliferation (Cohen, 2005; Dominick *et al.*, 2006; Tannehill-Gregg *et al.*, 2007). In contrast, naveglitazar, another dual PPARγ/PPARα agonist, induced bladder tumors but urinary tract solids were not detected (Long *et al.*, 2008). However, detection of urinary solids can be problematic because of methodological issues (Cohen *et al.*, 2007).

Since urothelial cells have PPARγ receptors, it has been suggested that a direct effect of the agonist on the urothelial receptor might be the cause of bladder carcinogenesis by these non–DNA reactive agents, possibly, in the case of dual PPARγ/PPARα agonists, by an interaction between the PPARα and PPARγ receptors (Varley and Southgate, 2008). However, PPARγ agonists inhibit cell proliferation or induce differentiation in various cancer cell lines, including urothelial cell carcinoma lines (Tachibana *et al.*, 2008). Additionally, the PPARγ agonist, troglitazone, inhibits cell proliferation and induces differentiation in human urothelial cells in culture (Varley *et al.*, 2004, 2009) rather than increasing cell proliferation as would be expected for a non–DNA reactive chemical's carcinogenic mode of action.

In this study, we investigated the early effects on the bladder and on the urine of rats treated with pioglitazone, a PPARγ agonist, to evaluate the possible relation between urinary solids formation and urothelial cytotoxicity and proliferation.

MATERIALS AND METHODS

Chemicals. Pioglitazone (purity: > 99%) and troglitazone (purity: 99.5%) were kindly provided by Bristol-Myers Squibb (Mount Vernon, IN). Pioglitazone was stored in the dark at ~4°C. Troglitazone was stored in the dark at room temperature. For the *in vitro* study, stock solutions of pioglitazone or troglitazone were prepared by dissolving the agonist in dimethyl sulfoxide (DMSO; Sigma, St Louis, MO). Working solutions of pioglitazone and troglitazone were prepared by diluting the stock solution in medium. The DMSO concentration of the working solutions was 0.1%.

Animal experiments. Five-week-old male Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Kingston, NY). On arrival, the animals were placed in a level-4 barrier facility accredited by the American Association for Accreditation of Laboratory Animal Care, in a room with a targeted temperature of 22°C, humidity of 50%, and a 12-h light/dark cycle (0600/1800 h). The level of care provided to the animals met or exceeded the basic requirements outlined in the Guide for the Care and Use of Laboratory Animals (NIH Publication #86-23, revised 1986). The animals were housed three per cage in polycarbonate cages, on dry corncob bedding, and fed basal diet (Certified Purina 5002; Dyets Inc., Bethlehem, PA). Food and tap water were available *ad libitum* throughout the study. Nylabones (Nylabone Products, Neptune, NJ) were added to the cages for environmental enrichment. Fresh diet was supplied to the animals at least once weekly. Food consumption and water consumption were measured during study week 3. Body weights of all animals were measured the day after arrival, once per week, and on the day of sacrifice. Detailed clinical observations of each animal were performed on day 0 and on the last day of the consumption period, including behavior and movement, respiratory function, ocular appearance, condition around the ears and mouth, condition of coat, and abdominal palpation.

Rats were ~6 weeks of age at the beginning of treatment. Following quarantine, animals were randomized using a weight stratification method (Martin *et al.*, 1984) into two groups of 15 rats each: group 1 was gavaged daily between 0800 h and 1000 h with 0.5% methyl cellulose in distilled water as vehicle and group 2 was similarly gavaged with pioglitazone (16 mg/kg body weight) in 0.5% methyl cellulose. All animals were sacrificed after 4 weeks of treatment by an overdose of Nembutal (150 mg/kg of body weight, ip). One hour prior to sacrifice, all rats were injected with l00 mg/kg bromodeoxyuridine (BrdU). The urinary bladder from 10 rats from each group was inflated *in situ* with Bouin's fixative, and after removal, the bladders were placed in Bouin's fixative. Following fixation, the bladders were rinsed in 70% ethanol, bisected

longitudinally, and weighed. The entire surface of one half of the bladder was examined by scanning electron microscopy (SEM) and classified in one of five categories as previously described (Cohen *et al.*, 1990). Briefly, class 1 bladders have flat polygonal superficial urothelial cells; class 2 bladders have occasional small foci of superficial urothelial necrosis; class 3 bladders have numerous small foci of superficial urothelial necrosis; class 4 bladders have extensive superficial urothelial necrosis, especially in the dome of the bladder; and class 5 bladders have necrosis and piling up (hyperplasia) of rounded urothelial cells. Normal rodent urinary bladders are usually class 1 or 2 or occasionally class 3. The other half of the bladder was cut longitudinally into strips and with a slice of intestinal tissue removed at the time of necropsy, was embedded in paraffin, stained with hematoxylin and eosin, and examined histopathologically (Cohen, 1983; Cohen *et al.*, 1990, 2007). A diagnosis of mild simple hyperplasia was made when there were four to five cell layers in the bladder epithelium and a diagnosis of severe simple hyperplasia was made when nine or more cell layers were present. Unstained slides of the bladder and intestinal tissue were used for immunohistochemical detection of BrdU (Cohen *et al.*, 2007). The intestinal tissue served as a positive control. Anti-BrdU (Millipore Corporation, Temecula, CA) was used at a dilution of 1:200. The number of BrdU-labeled cells in at least 3000 urothelial cells (all layers) was counted to determine a labeling index. Unstained slides of the bladder and intestinal tissue were used for periodic acid-Schiff (PAS) staining (Luna, 1968). The urinary bladder from the remaining five rats from each group was excised, and the epithelial cell layer was collected by scraping with a scapel blade. The epithelial cells were immediately immersed in TRIzol Reagent (Invitrogen, Carlsbad, CA) and stored at −80°C until processed for RNA extraction.

Evaluation of crystals in urine. After treatment for 14 and 22 days, fresh void urine samples were collected separately from each rat between 7:00–9:00 A.M. All urine samples were centrifuged at approximately 6400 *g* for 10 min. After removal of most of the supernatant, the urine sediment was resuspended in the remaining urine and filtered through a 0.22μM Millipore filter (Millipore, Billerica, MA) by vacuum. The crystals remaining on the filter were characterized morphologically by SEM, and their composition was determined by attached energy dispersive X-ray spectroscopy.

In vitro *experiments.* The MYP3 urinary rat bladder epithelial cell line was provided by Dr. Ryoichi Oyasu (Northwestern University, Chicago, IL). The MYP3 cell line was obtained from a small benign nodule that developed in a heterotopically transplanted rat urinary bladder after treatment with N-methyl-N-nitrosourea (Kawamata *et al.*, 1993). The cell line has retained the characteristics of epithelial cells in culture, expresses keratin 5 mRNA, does not exhibit anchorage-independent growth, and does not cause the development of tumors when inoculated sc in nude mice. The cells were grown in Ham's F-12 medium (Gibco-BRL, Grand Island, NY) supplemented with 10μM nonessential amino acids, 10 ng/ml epidermal growth factor, 10 μg/ml insulin, 5 μg/ml transferrin, 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Gibco), and 2.7 mg/ml dextrose and 1 μg/ml hydrocortisone (from Sigma). All cells were grown in an atmosphere of 95% air and 5% CO_2 at 37°C.

For determination of PPARγ agonist effects on the urothelial cells *in vitro*, cells were seeded at a concentration of 5.0×10^3 cells per well in eight-well Lab-Tek Chamber Slides (NUNC, Inc., Naperville, IL). Twenty-four hours later, treatment with pioglitazone (5μM) or troglitazone (5μM) was begun and continued for 3 days without changing the medium. For immunohistochemistry, after fixation with 10% formalin, sections were autoclaved in 10mM sodium citrate buffer (pH 6.0) at 120°C for 5 min. The sections were then treated with anti-Ki-67 antibody (MIB-5; Dako, Carpinteria, CA) at a dilution of 1:50 or anti-asymmetrical unit membrane (AUM) antibody kindly provided by Dr. T. T. Sun, which consisted of rabbit antiserum made against highly purified bovine AUM (Wu *et al.*, 1990), at a dilution of 1:200. The number of Ki-67–labeled cells in at least 500 urothelial cells was counted to determine a labeling index.

To determine expression of RNA, cells were seeded at a concentration of 1.0×10^4 cells per well in a 24-well plate. Twenty-four hours later, treatment with 5μM pioglitazone or 5μM troglitazone was begun and continued for

TABLE 1
Effects of Treatment with Pioglitazone on Body, Bladder, and Heart Weights and on the Bladder Urothelium

	Control	Pioglitazone
BW, g (n)[a]	393 ± 6 (14)	387 ± 6 (15)
Bladder weight (n)[a]		
Absolute, g	0.111 ± 0.013 (9)	0.108 ± 0.006 (10)
Relative, mg/g BW	0.28 ± 0.03 (9)	0.28 ± 0.02 (10)
Heart weight (n)[a]		
Absolute, g	1.26 ± 0.03 (14)	1.32 ± 0.02 (15)
Relative, mg/g BW	3.2 ± 0.1(14)	3.4 ± 0.1 (14)[b]
Bladder histopathology		
Normal	9	5
Hyperplasia	0	5[b,c]
Bladder labeling index, % (n)[a]	0.16 ± 0.03 (9)	0.43 ± 0.07 (10)[b]
Bladder SEM classification[d]		
1	1	3
2	4	—
3	3	2
4	—	—
5	—	1

Note. BW, body weight; (n), number of rats.

[a]Values expressed as the mean ± SE.

[b]Significantly different from control group, $p < 0.05$.

[c]Four of five bladders with mild simple hyperplasia; one of five bladders with severe simple hyperplasia.

[d]Unable to classify one bladder in the control group and four bladders in the pioglitazone-treated group by SEM due to the presence of an unknown substance on the bladder surface.

3 days without a change of medium. After the treatment, cells were treated with TRIzol Reagent (Invitrogen) and stored at −80°C until processed for RNA extraction.

RNA extraction and detection of RNA expression. Total RNA was isolated with TRIzol Reagent according to the manufacturer's instructions. Sequence-specific primers and probes (Taqman Gene Expression Assay) were purchased from Applied Biosystems, Inc. (Foster City, CA). β-Actin was employed as an internal control. Briefly, complementary DNA (cDNA) synthesis was performed with 600 ng of RNA using an Advantage RT-for-PCR kit (Takara Bio, Inc., Shiga, Japan) and then cDNA solutions were diluted to a final volume of 100 μl by adding 80 μl diethylpyrocarbonate-treated H_2O. PCRs were performed in a 20 μl reaction mixture containing 5 μl cDNA, 1 μl of Taqman Gene Expression Assay Mix, and 10 μl TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Inc.) under the following conditions: 95°C for 20 s, then 40 cycles at 95°C for 3 s, and 60°C for 30 s using a 7500 Fast Real-Time PCR System (Applied Biosystems, Inc., Tokyo, Japan). Serially diluted standard cDNA was included in each Taqman PCR to create a standard curve. The amount of gene products in the test samples was estimated relative to the respective standard curves. Values for target genes were normalized to those for β-actin.

Statistics. For the *in vivo* studies, group means for body weights, consumptions, tissue weights, and labeling indices were evaluated using analysis of variance followed by Duncan's multiple range test for group-wise comparisons. Histopathology was compared using the two-tailed Fisher's exact test. SEM data were analyzed using one-way nonparametric procedures followed by a chi square test. p Values < 0.05 were considered significant. These statistical analyses were performed using SAS for Windows (Version 9.1).

For the *in vitro* studies, the difference between control and treatment group was compared by the unpaired *t*-test. For multiple groups, differences between control and treated were compared by ANOVA, which, when significant ($p < 0.05$), was followed by Dunnett's test (GraphPad Prism 5; GraphPad Software, Inc., La Jolla, CA).

RESULTS

Body and Heart Weights

Administration of pioglitazone to male rats caused little or no depression in body weight gain. However, it induced a significant increase in the relative weight of the heart (Table 1), a characteristic effect of PPARγ and dual agonists (El-Hage, 2005). It had no effect on food and water consumption (control and pioglitazone-treated rats [mean ± SE]: water consumption, 40.3 ± 1.9 and 41.3 ± 1.7 g/rat/day and food consumption, 26.9 ± 1.2 and 28.5 ± 1.0 g/rat/day, respectively).

Urothelial Effects

Administration of pioglitazone caused no effects on the absolute and relative bladder weights (Table 1). By light microscopy, simple hyperplasia of the bladder urothelium was significantly increased in pioglitazone-treated rats (Table 1) and was not present in control rats (Fig. 1A). Additionally, one of five pioglitazone-treated rats had severe simple hyperplasia (Fig. 1C), whereas the other instances of hyperplasia were mild simple hyperplasia (Fig. 1B). Administration of pioglitazone also caused a significant increase in the BrdU labeling index of the urothelium compared to the control group (Table 1). Administration of pioglitazone tended to induce cytotoxicity and necrosis in the bladder epithelium (Figs. 1E and 1F), although the SEM classification in the group-administered pioglitazone was not statistically significantly different from the control group (Table 1). The pioglitazone-treated rat with severe urothelial hyperplasia was also the rat diagnosed as class 5 by SEM. Examination by SEM showed that the bladder surface of the rats in one of the control and four of the pioglitazone-treated rats was covered with a coarse substance (Fig. 2A), which made it difficult to visualize the bladder surface in many areas. The morphology of the substance by SEM was similar to mucin (Balish *et al.*, 1982). An eosinophilic substance was also detected by light microscopy on the luminal surface of these same bladders (Fig. 2B) and it stained positive by PAS stain (Fig. 2C). Therefore, this covering likely corresponds to the glycosaminoglycan layer described for the normal urinary bladder (Soler *et al.*, 2008), which is usually lost during routine processing for histology or SEM examination.

Evaluation of Urinary Sediments

In the urine, the normally present $MgNH_4PO_4$ crystals (Fig. 3A) were observed in both control and pioglitazone-treated groups in similar amounts (Table 2). Aggregates of
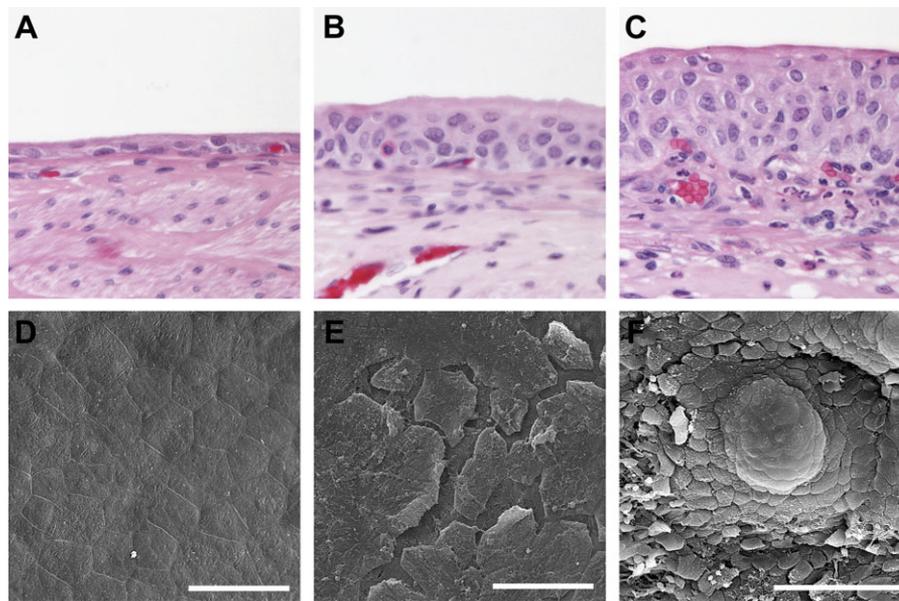
FIG. 1. Histopathology, SEM of rat bladder. (A–C) hematoxylin and eosin. (D–F) SEM. Normal epithelium in control rat (A and D). Mild (B) and severe (C) simple hyperplasia in pioglitazone-treated rats. Abrasion of superficial cells (E) and piling up of rounded urothelial cells (F) in pioglitazone-treated rats. White bar: 100 μm.

$MgNH_4PO_4$ crystals occurred in similar amounts, sizes, and number of rats in the two groups. Thin rod-like crystals were only detected in one control group rat at day 14, and they appeared to be $MgNH_4PO_4$. Calcium phosphate–containing amorphous precipitate was not observed in the urinary sediment from any of the rats from either group at either time point. Calcium-containing crystals (Figs. 3B and 3C) were only detected in pioglitazone-treated rats at day 14. However, they were detected in both control and pioglitazone-treated rats at day 22. At day 14, the calcium-containing crystals also contained oxygen in the sediment from three rats, most likely representing calcium oxalate crystals, and one rat had calcium phosphate crystals. Carbon and hydrogen are not clearly distinguished by our instrument. At day 22, similar calcium oxalate crystals were observed in controls and in the pioglitazone-treated rats, but pioglitazone-treated rats also had calcium phosphate crystals and crystals containing calcium, oxygen, and sulfur. Furthermore, a calcium phosphate

calculus was detected in one pioglitazone-treated rat at day 22 (the rat with severe hyperplasia).

PPARγ Effects on Rat Urothelial Cells In Vitro

Treatment with pioglitazone (5μM) induced enlargement of cytoplasm and binucleated and multinucleated cells (Figs. 4B and 4D) compared to control cells (Figs. 4A and 4C). Additionally, treatment with pioglitazone significantly ($p < 0.001$) reduced the Ki-67 labeling index ($24.7 \pm 2.1\%$; Fig. 4F) compared to controls ($49.5 \pm 3.3\%$; Fig. 4E). Anti-AUM antibody diffusely stained the nucleus and cytoplasm of large cells present in both control and treated wells but small cells present in the wells did not stain. In the control wells, the number of small cells and large cells was similar. In pioglitazone-treated wells, the ratio of large cells to small cells was higher than in the control wells. Therefore, the ratio of AUM-positive staining cells to negative cells in pioglitazone-treated wells (Fig. 4H) was higher than
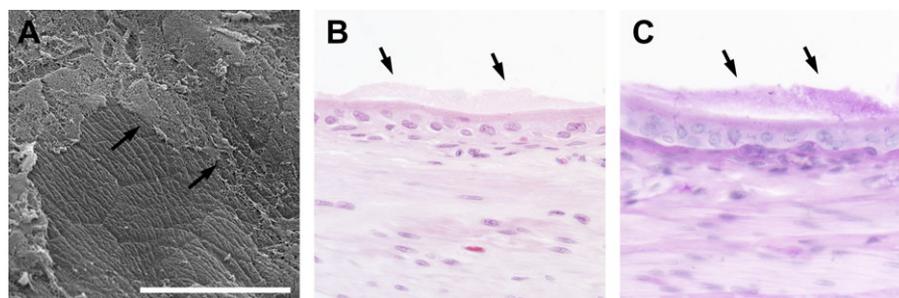

FIG. 2. Rough substance on superficial layer of urothelium. (A) SEM, (B), and hematoxylin and eosin. (C) PAS staining. Rough substance was covered on superficial cells (A and B) and stained with PAS staining (C). White bar: 100 μm.
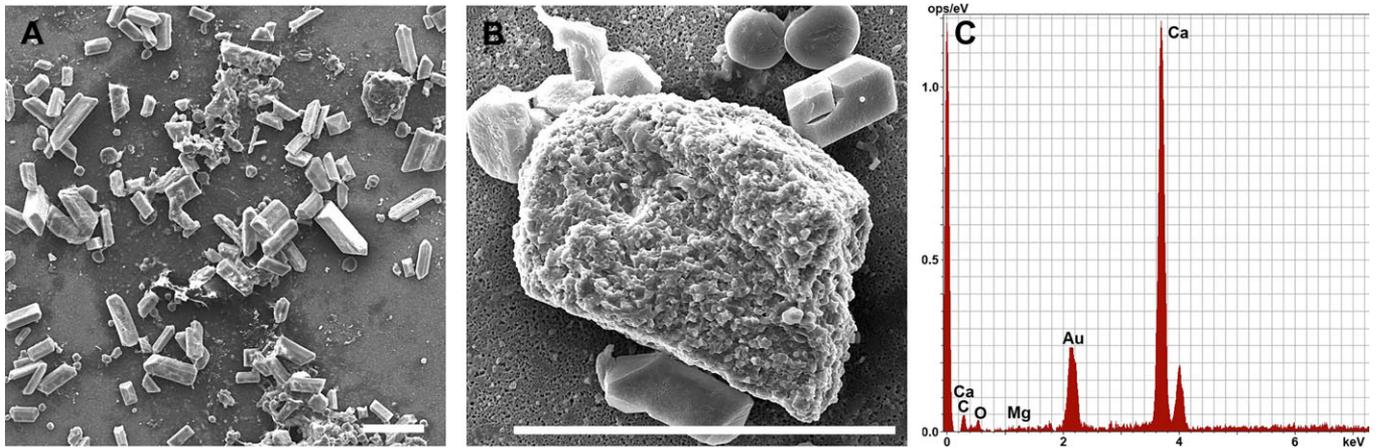
FIG. 3. Crystals in rat urine. (A and B) SEM. (C) energy dispersive X-ray spectroscopy (EDS). $MgNH_4PO_4$ crystals (A) and calcium-containing crystal (B) in urine of pioglitazone-treated rat. Composition of calcium-containing crystal (B) by EDS (C). White bar: 100 μm.

in control wells (Fig. 4G). Treatment with troglitazone (5μM) induced the same morphology and significant reduction ($p < 0.001$) of the Ki-67 labeling index ($23.3 \pm 2.4\%$) compared to control ($45.2 \pm 4.7\%$). The effects of both pioglitazone and troglitazone on MYP3 cells were the same in this study.

Gene Expression Differences in Urothelium of PPARγ Agonist–Treated Rats and Urothelial Cells

In vivo, there was no difference in the mRNA expression of PPARγ in the urothelium of pioglitazone-treated rats compared to control rats (Fig. 5A). *In vitro*, PPARγ mRNA in both pioglitazone- and troglitazone-treated cells was significantly reduced compared to control (Fig. 5B). The mRNA expression of cyclin D1 in both pioglitazone- and troglitazone-treated cells was also reduced compared to control (Fig. 5C).

DISCUSSION

PPARγ and dual PPARα/γ agonists frequently increase the incidence of bladder cancer in rats in 2-year bioassays but not in mice (El-Hage, 2005). Pioglitazone, a thiazolidinedione, is a PPARγ agonist that induced a relatively low incidence of bladder tumors in the 2-year bioassay and only in male rats (El-Hage, 2005; Physicians Desk Reference, 2008). Rosiglitazone, another thiazolidinedione PPARγ agonist, has not been reported to produce bladder tumors in a 2-year bioassay but did produce an increased incidence of bladder tumors in rats pretreated with BBN, a known DNA-reactive bladder carcinogen in several species (Lubet *et al.*, 2008). Troglitazone, a third PPARγ agonist of the thiazolidinedione class, has not been reported to induce effects on the urothelium of rats or mice (Herman *et al.*, 2002). In a summary of the carcinogenic effects of various PPAR agonists under development as

TABLE 2
Effects of Treatment with Pioglitazone on Urinary Sediments

| Treatment (n) | Normal $MgNH_4PO_4$ crystals | | | Aggregates of $MgNH_4PO_4$ crystals | | | | | | Calcium-containing crystals | | | Calcium phosphate calculi | | |
| | | | | < 100 μm | | | > 100 μm | | | | | | | | |
	0	+1	+2	0	+1	+2	0	+1	+2	0	+1	+2	0	+1	+2
A: Day 14															
Control (10)	3	7	–	7	3	–	8	2	–	10	–	–	10	–	–
Pioglitazone (9)	5	4	–	7	2	–	7	2	–	5	4[a]	–	9	–	–
B: Day 22															
Control (5)	–	4	1	1	4	–	4	1	–	1	4[b]	–	5	–	–
Pioglitazone (11)	2	8	1	5	6	–	9	2	–	7	4[c]	–	10	1	–

[a]Ca- and O-containing crystals on three filters and two Ca- and P-containing crystals on one filter.
[b]One to two Ca- and O-containing crystals per filter.
[c]Ca- and O-containing crystals on two filters; Ca- and P-containing crystals on one filter; and Ca-, O-, and S-containing crystal on one filter.
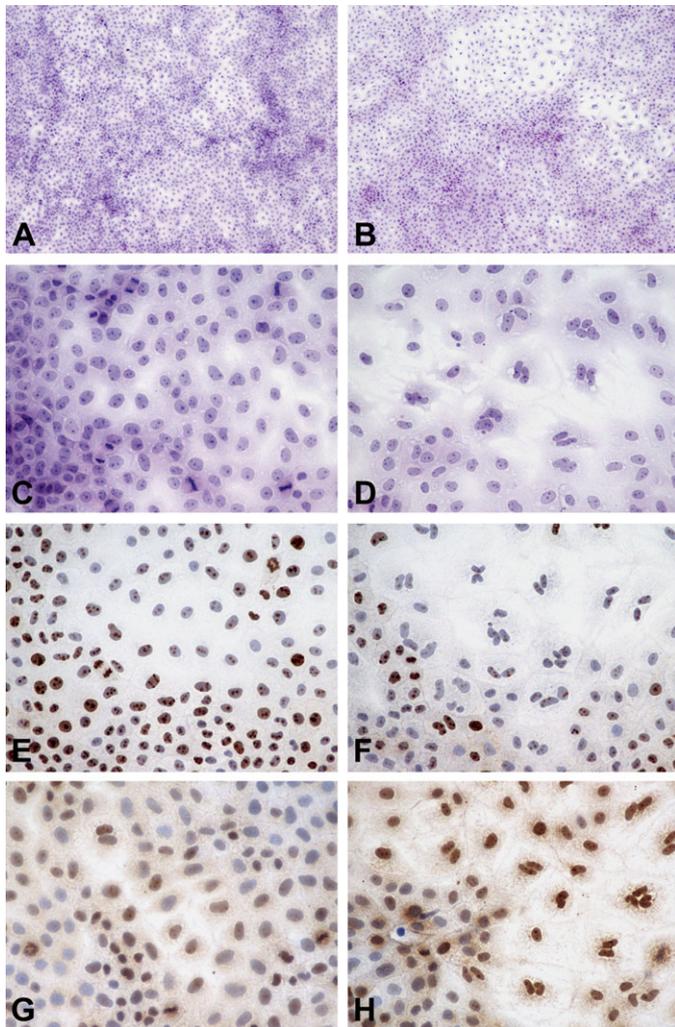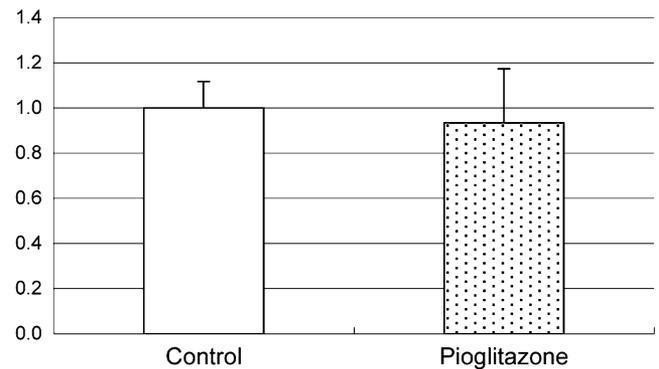
FIG. 4. Histology and immunohistochemistry of rat bladder cell line. (A–D) hematoxylin and eosin. (E and F) Ki-67 antibody. (G and H) AUM antibody. Control cells (A, C, E, and G) and pioglitazone-treated cells (B, D, F, and H).
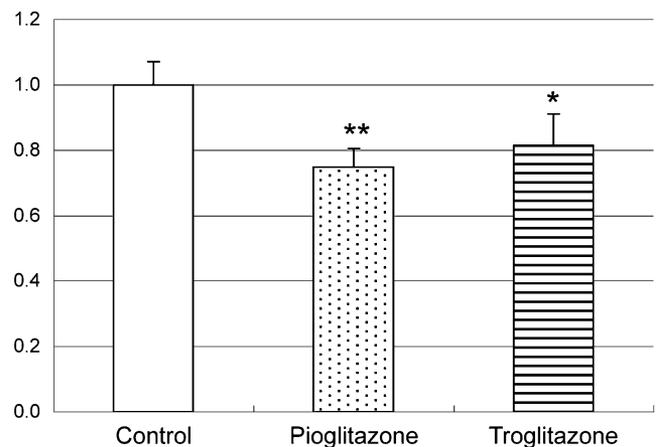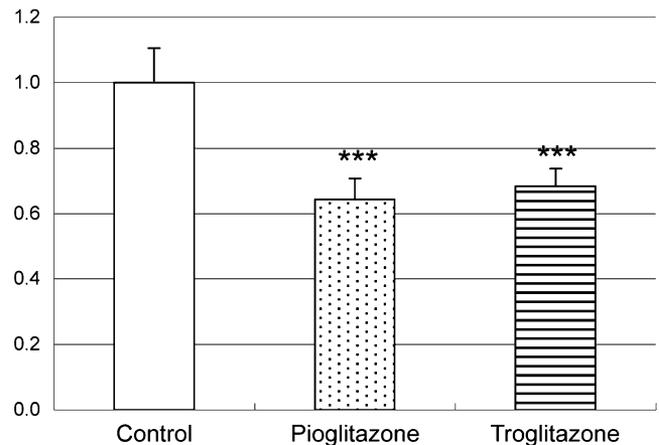

FIG. 5. Gene expression analysis of PPARγ and cyclin D1. PPARγ expression *in vivo* (A) and *in vitro* (B) in rat urothelial cells. Cyclin D1 expression *in vitro* (C). The expression data were adjusted to each control as 1.0. *, **, ***Significantly different from each control at $p < 0.05$, 0.01, 0.001, respectively.

pharmaceuticals, El-Hage (2005) reported that five of six dual PPARα/γ agonists induced bladder tumors in rats but again were without effect in mice. Those that have been reported specifically to cause bladder tumors include muraglitazar (Dominick *et al.*, 2006), naveglitazar (Long *et al.*, 2008), and ragaglitazar (Egerod *et al.*, 2005). Since rats treated with these agents at doses that produce bladder tumors have blood levels that frequently are similar to the blood levels achieved in patients treated with these drugs, bladder tumors in rats have become a significant issue for the potential approval of these agents by regulatory agencies for clinical use.

Two modes of action have been hypothesized for the possible induction of bladder tumors in rats by PPAR agonists, with very different implications for potential human cancer risk. Since PPARγ receptors are plentiful in the urothelium, one hypothesis is based on the direct interaction of the agonist with the receptor, producing an effect that ultimately leads to the induction of cancer. Although this possibility has not been completely excluded, there are several arguments against it (Cohen, 2005). Foremost among these are the observation that

the PPARγ receptor is expressed at similar levels in rat and mouse urothelium and yet the agents produce bladder tumors only in the rat not in the mouse (Cohen, 2005). Most importantly, the effects of PPARγ agonists *in vitro* appear to be an inhibition of cell proliferation and potentiation of differentiation when the epidermal growth factor receptor has been inhibited (Varley and Southgate, 2008; Varley *et al.*, 2004). These studies utilized human urothelial cell lines, and the decreased proliferation along with urinary differentiation was corroborated in the present experiment using a rat urothelial cell line. Thus, a direct mitogenic effect to the urothelium by the PPAR agonist is unlikely. Biologically, this is the opposite effect of what would be expected for a non–DNA reactive carcinogen. Furthermore, these agonists have frequently been shown to inhibit the proliferation of a variety of cancer cell lines, including urothelial carcinoma cell lines (Berger and Moller, 2002; Tachibana *et al.*, 2008). An additional factor is the small percentage of administered drug being excreted in the urine since PPAR agonists are highly lipophilic.

Previous studies with the thiazolidinedione class of drugs have suggested that they induce differentiation and inhibit proliferation of urothelial cells in culture. These studies investigated the effects of troglitazone and rosiglitazone on human cell lines derived from distal ureters obtained from kidney transplant patients (Varley and Southgate, 2008; Varley *et al.*, 2004). Pioglitazone is the only thiazolidinedione so far reported, which actually produced bladder tumors by itself in a 2-year bioassay (El-Hage, 2005). Thus, we examined the effect of pioglitazone *in vitro* to determine if it also had cell-differentiating properties, using a rat urothelial cell line since that is the target species for bladder carcinogenicity. Troglitazone was used as a positive control based on its reported effects on human urothelial cells. We were able to show that treatment with either pioglitazone or troglitazone *in vitro* utilizing a rat urothelial cell line reduced cell proliferation and there was evidence of cell differentiation in this culture system (Fig. 4). Our observations in the rat cell line (Fig. 4) support the previously reported observations utilizing human urothelial cells. Although we did not prove that this was a PPARγ-specific mechanism molecularly, we detected no difference in gene expression of PPARγ between control and pioglitazone-treated rats *in vivo* and found reduction of PPARγ in both pioglitazone- and troglitazone-treated cells *in vitro*. Reduction of PPARγ expression by muraglitazar *in vivo* was reported by Achanzar *et al.* (2007). For detection of PPARγ activity, it is necessary to use another methodology, which would also detect phosphorylation of PPARγ. Again, our results provide support for the previous observations utilizing troglitazone in human urothelial cell culture systems. Pioglitazone and troglitazone had the same effects on the rat urothelial cells *in vitro* but only pioglitazone has been reported to produce bladder urothelial tumors in rats (Physicians Desk Reference, 2008), whereas troglitazone does not affect the lower urinary tract of the rat.

A second, indirect mode of action such as reaction to urinary solids has been postulated by Cohen (2005) and has been demonstrated for the dual PPARγ/PPARα agonist muraglitazar (Dominick *et al.*, 2006). This involves alteration of the urine resulting in abnormal urinary solids leading to cytotoxicity, necrosis, and regenerative proliferation of the bladder epithelium. Alteration of the urine composition appears to be due to an inhibition by muraglitazar of citrate synthesis leading to hypocitratemia and consequent hypocitraturia (Dominick *et al.*, 2006). Citrate is the major chelating substance for divalent cations, such as calcium, in the urine keeping them in solution. When the citrate level is lowered significantly, the calcium salts, which are at supersaturated levels in the urine, precipitate. This ultimately induces tumors. Cytotoxicity followed by regenerative hyperplasia as a mode of action has been described for a wide variety of agents in the rat and occasionally in the mouse (Cohen, 1998). For reasons that are not entirely clear, the rat appears to be more susceptible to this effect. Most importantly, humans appear to be completely resistant to the urothelial cytotoxic effects of urinary amorphous precipitate and crystals but do have a toxic and regenerative response to the presence of calculi. Calculi, when formed, are present in the human urinary bladder for brief periods of time because of their propensity to cause obstruction and consequent severe pain leading to their removal clinically (DeSesso, 1995).

For muraglitazar, the sequence of key events has been demonstrated in extensive detail (Achanzar *et al.*, 2007; Dominick *et al.*, 2006; Tannehill-Gregg *et al.*, 2007). In these experiments, muraglitazar induced urinary bladder carcinomas and also induced calcium-containing solids and reduced citrate and soluble calcium concentrations in the urine. Also, coadministration of ammonium chloride with muraglitazar produces an acidified urine, which inhibits the formation of the calcium-containing crystals and consequently inhibits the bladder toxicity, regenerative proliferation, and tumorigenicity of muraglitazar. In contrast, studies with naveglitazar and ragaglitazar have reported that increased urinary solids have not been found in rats administered these drugs (Egerod *et al.*, 2005; Long *et al.*, 2008). However, there are several potential technical difficulties that must be addressed when examining urine for the presence of these solids. Foremost is the requirement that the animals not be fasted prior to collection of the urine. It is also best for fresh void collections to be used rather than using overnight or 24-h collections. The technical difficulties of urine collection and examination for solids have been described in detail elsewhere (Cohen *et al.*, 2007). The time of day of urine collection, the strain of the rat, where the rat was purchased, the type of diet used, and a variety of other details appear critical in the detection of the formation of urinary solids in response to PPAR agonists and also in response to other agents.

In the present series of experiments, we were able to demonstrate that pioglitazone produced calcium-containing

urinary solids, although limited, which were associated with increased urothelial cytotoxicity, necrosis, and regenerative proliferation (Table 2). The more extensive the urinary solids formation was, the more extensive the toxic and proliferative response was. In one of the rats we examined, calculi were present, which were associated with severe simple hyperplasia, even in as short a period as 4 weeks. Overall, there were considerably less urinary solids detected in these rats administered pioglitazone than previously observed for muraglitazar (Dominick *et al.*, 2006). Correspondingly, muraglitazar induced a significantly higher incidence of bladder tumors than the small number induced by pioglitazone. Furthermore, the amount of calcium-containing crystals varied between times of collection, even for this short experiment. Experiments with other agents have demonstrated the variability in formation of urinary solids over time, even with the continued administration of the agent (Clayson *et al.*, 1995). The reversibility and relatively low amount of crystals seen with pioglitazone is likely directly related to its weak overall effect on the rat bladder in contrast to the more plentiful and frequent urinary solids associated with muraglitazar, which also had more urothelial proliferation and neoplastic lesions. The sporadic nature of these urinary crystals in rats administered pioglitazone might partly explain the difficulty of detecting the crystals in rats administered pioglitazone or other PPARγ or dual agonists, and this intermittent nature might also explain the difficulty in detecting hyperplasia in short-term studies with pioglitazone and some of the other PPAR agonists.

Most commonly with these agents, the extent of cytotoxicity involves only the superficial urothelial cell layer, which can be difficult to observe by light microscopy but is readily observable by SEM. Although we did observe superficial cytotoxicity in some of the rats administered pioglitazone in the present study, several of the bladders could not be examined by SEM because of the overlying apparent glycosaminoglycan layer, which had adhered to the luminal surface of the epithelium (Fig. 2). This precludes the examination of the surface characteristics of the epithelium. Nevertheless, the combination of findings by light microscopy, SEM, and increased BrdU labeling index is supportive of the mode of action of cytotoxicity with consequent regenerative proliferation. The evidence from our urinalysis examinations supports the hypothesis that the cytotoxicity is produced by the formation of urinary solids, namely calcium-containing crystals and calculi.

In summary, we have demonstrated evidence that pioglitazone, a PPARγ agonist which induced bladder tumors in male rats, produces alterations in urinary solid formation, albeit in small amounts, especially calcium-containing crystals and calculi, which could lead to cytotoxicity and consequent regenerative proliferation. We have also confirmed that the thiazolidinediones, including troglitazone and pioglitazone, inhibit urothelial cell proliferation and potentiate differentiation

of rat urothelial cells in culture. Our data support but do not prove the hypothesis that the bladder tumors produced in rats by pioglitazone are related to the formation of urinary solids. The quantitative levels of the crystals appear to correlate with the extent and incidence of urothelial lesions occurring in rats administered PPAR agonists, few solids and few lesions with pioglitazone in contrast to numerous solids and lesions associated with muraglitazar. Since clinical trials with PPARγ and dual agonists have not been associated with urinary calculus formation (Dominick *et al.*, 2006; Dormandy *et al.*, 2005), the findings in the present experiment with pioglitazone strongly support the previous conclusion from studies with muraglitazar that this is a rat-specific phenomenon and does not pose a urothelial cancer risk to humans treated with these agents.

REFERENCES

Achanzar, W. E., Moyer, C. F., Marthaler, L. T., Gullo, R., Chen, S. J., French, M. H., Watson, L. M., Rhodes, J. W., Kozlosky, J. C., White, M. R., *et al.* (2007). Urine acidification has no effect on peroxisome proliferator-activated receptor (PPAR) signaling or epidermal growth factor (EGF) expression in rat urinary bladder urothelium. *Toxicol. Appl. Pharmacol.* **223,** 246–256.

Balish, M. J., Jensen, J., and Uehling, D. T. (1982). Bladder mucin: a scanning electron microscopy study in experimental cystitis. *J. Urol.* **128,** 1060–1063.

Berger, J., and Moller, D. E. (2002). The mechanisms of action of PPARs. *Annu. Rev. Med.* **53,** 409–435.

Clayson, D. B., Fishbein, L., and Cohen, S. M. (1995). The effect of stones and other physical factors on the induction of rodent bladder cancer. *Food Chem. Toxicol.* **33,** 771–784.

Cohen, S. M. (1983). Pathology of experimental bladder cancer in rodents. In *The Pathology of Bladder Cancer* (S. M. Cohen and G. T. Bryan, Eds.), Vol. 2, pp. 1–40. CRC Press, Boca Raton, FL.

Cohen, S. M. (1998). Urinary bladder carcinogenesis. *Toxicol. Pathol.* **26,** 121–127.

Cohen, S. M. (2005). Effects of PPARgamma and combined agonists on the urinary tract of rats and other species. *Toxicol. Sci.* **87,** 322–327.

Cohen, S. M., Fisher, M. J., Sakata, T., Cano, M., Schoenig, G. P., Chappel, C. I., and Garland, E. M. (1990). Comparative analysis of the proliferative response of the rat urinary bladder to sodium saccharin by light and scanning electron microscopy and autoradiography. *Scanning Microsc.* **4,** 135–142.

Cohen, S. M., Ohnishi, T., Clark, N. M., He, J., and Arnold, L. L. (2007). Investigations of rodent urinary bladder carcinogens: collection, processing, and evaluation of urine and bladders. *Toxicol. Pathol.* **35,** 337–347.

DeSesso, J. M. (1995). Anatomical relationships of urinary bladders compared: their potential role in the development of bladder tumours in humans and rats. *Food Chem. Toxicol.* **33,** 705–714.

Dominick, M. A., White, M. R., Sanderson, T. P., Van Vleet, T., Cohen, S. M., Arnold, L. E., Cano, M., Tannehill-Gregg, S., Moehlenkamp, J. D., Waites, C. R., *et al.* (2006). Urothelial carcinogenesis in the urinary bladder of male rats treated with muraglitazar, a PPAR alpha/gamma agonist: evidence for urolithiasis as the inciting event in the mode of action. *Toxicol. Pathol.* **34,** 903–920.

Dormandy, J. A., Charbonnel, B., Eckland, D. J., Erdmann, E., Massi-Benedetti, M., Moules, I. K., Skene, A. M., Tan, M. H., Lefebvre, P. J., Murray, G. D., *et al.* (2005). Secondary prevention of macrovascular events in patients with type 2 diabetes in the PROactive study (PROspective pioglitAzone clinical trial in macroVascular events): a randomised controlled trial. *Lancet* **366,** 1279–1289.

Egerod, F. L., Nielsen, H. S., Iversen, L., Thorup, I., Storgaard, T., and Oleksiewicz, M. B. (2005). Biomarkers for early effects of carcinogenic dual-acting PPAR agonists in rat urinary bladder urothelium in vivo. *Biomarkers* **10,** 295–309.

El-Hage, J. (2005). Peroxisome proliferator-activated receptor agonists: carcinogenicity findings and regulatory recommendations. International Atherosclerosis Society Symposium on PPAR. Monte Carlo.

Herman, J. R., Dethloff, L. A., McGuire, E. J., Parker, R. F., Walsh, K. M., Gough, A. W., Masuda, H., and de la Iglesia, F. A. (2002). Rodent carcinogenicity with the thiazolidinedione antidiabetic agent troglitazone. *Toxicol. Sci.* **68,** 226–236.

Kawamata, H., Kameyama, S., Nan, L., Kawai, K., and Oyasu, R. (1993). Effect of epidermal growth factor and transforming growth factor beta 1 on growth and invasive potentials of newly established rat bladder carcinoma cell lines. *Int. J. Cancer* **55,** 968–973.

Long, G. G., Reynolds, V. L., Lopez-Martinez, A., Ryan, T. E., White, S. L., and Eldridge, S. R. (2008). Urothelial carcinogenesis in the urinary bladder of rats treated with naveglitazar, a gamma-dominant PPAR alpha/gamma agonist: lack of evidence for urolithiasis as an inciting event. *Toxicol. Pathol.* **36,** 218–231.

Lubet, R. A., Fischer, S. M., Steele, V. E., Juliana, M. M., Desmond, R., and Grubbs, C. J. (2008). Rosiglitazone, a PPAR gamma agonist: potent promoter of hydroxybutyl(butyl)nitrosamine-induced urinary bladder cancers. *Int. J. Cancer* **123,** 2254–2259.

Luna, L. G. (Ed.) (1968). In *The Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology,* pp. 158–160. McGraw Hill, New York.

Martin, R. A., Daly, A. M., DiFonzo, A. J., and de la Iglesia, F. A. (1984). Randomization of animals by computer program for toxicology studies. *J. Am. Coll. Toxicol.* **3,** 1–11.

Michalik, L., Desvergne, B., and Wahli, W. (2004). Peroxisome-proliferator-activated receptors and cancers: complex stories. *Nat. Rev. Cancer* **4,** 61–70.

Physicians Desk Reference. (2008). *Actos,* 62nd ed. pp. 3156–3161, Thomson Healthcare, Montvale, NJ.

Soler, R., Bruschini, H., Martins, J. R., Dreyfuss, J. L., Camara, N. O., Alves, M. T., Leite, K. R., Truzzi, J. C., Nader, H. B., Srougi, M., *et al.* (2008). Urinary glycosaminoglycans as biomarker for urothelial injury: is it possible to discriminate damage from recovery? *Urology* **72,** 937–942.

Tachibana, K., Yamasaki, D., Ishimoto, K., and Doi, T. (2008). The role of PPARs in cancer. *PPAR Res.* **2008,** 102737.

Tannehill-Gregg, S. H., Sanderson, T. P., Minnema, D., Voelker, R., Ulland, B., Cohen, S. M., Arnold, L. L., Schilling, B. E., Waites, C. R., and Dominick, M. A. (2007). Rodent carcinogenicity profile of the antidiabetic dual PPAR alpha and gamma agonist muraglitazar. *Toxicol. Sci.* **98,** 258–270.

Varley, C. L., Bacon, E. J., Holder, J. C., and Southgate, J. (2009). FOXA1 and IRF-1 intermediary transcriptional regulators of PPARgamma-induced urothelial cytodifferentiation. *Cell Death Differ.* **16,** 103–114.

Varley, C. L., and Southgate, J. (2008). Effects of PPAR agonists on proliferation and differentiation in human urothelium. *Exp. Toxicol. Pathol.* **60,** 435–441.

Varley, C. L., Stahlschmidt, J., Lee, W. C., Holder, J., Diggle, C., Selby, P. J., Trejdosiewicz, L. K., and Southgate, J. (2004). Role of PPARgamma and EGFR signalling in the urothelial terminal differentiation programme. *J. Cell Sci.* **117,** 2029–2036.

Wu, X. R., Manabe, M., Yu, J., and Sun, T. T. (1990). Large scale purification and immunolocalization of bovine uroplakins I, II, and III. Molecular markers of urothelial differentiation. *J. Biol. Chem.* **265,** 19170–19179.

Yki-Jarvinen, H. (2004). Thiazolidinediones. *N. Engl. J. Med.* **351,** 1106–1118.