Toxicology and Applied Pharmacology 251 (2011) 234–244

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

# Suppressive effects of acid-forming diet against the tumorigenic potential of pioglitazone hydrochloride in the urinary bladder of male rats

Keiichiro Sato [a,*], Yasuyuki Awasaki [a], Hitoshi Kandori [a], Zen-yo Tanakamaru [a], Hirofumi Nagai [a], David Baron [b], Masaki Yamamoto [a]

[a] Development Research Center, Takeda Pharmaceutical Company Limited, Osaka 532-8686, Japan
[b] Takeda Global Research & Development Center, Inc., Deerfield, IL 60015, USA

ARTICLE INFO

*Article history:*
Received 30 November 2010
Revised 6 January 2011
Accepted 7 January 2011
Available online 19 January 2011

*Keywords:*
Pioglitazone
Urinary bladder
Tumor
Hyperplasia
PPAR
Rats

ABSTRACT

Pioglitazone hydrochloride (PIO), a peroxisome proliferator-activated receptor gamma (PPARγ) agonist, was administered orally for 85 weeks at 16 mg/kg/day to male rats fed either a diet containing 1.5% ammonium chloride (acid-forming diet) or a control diet to investigate the effects of urinary acidification induced by the acid-forming diet on the tumorigenic potential of PIO in the urinary bladder. The surviving animals at the end of the administration period were followed to the end of the 2-year study period without changes in the diet and were subjected to terminal necropsy on Week 104. The number of urinary microcrystals, evaluated by manual counting with light microscopy and by an objective method with a laser diffraction particle size analyzer, was increased by PIO on Weeks 12 and 25 and the increases were markedly suppressed by urinary acidification. Urinary citrate was decreased by PIO throughout the study period, but no changes were seen in urinary oxalate at any timepoint. The incidences of PIO-treated males bearing at least one of the advanced proliferative changes consisting of papillary hyperplasia, nodular hyperplasia, papilloma or carcinoma were significantly decreased from 11 of 82 males fed the control diet to 2 of 80 males fed the acid-forming diet. The acid-forming diet did not show any effects on the toxicokinetic parameters of PIO and its metabolites. Microcrystalluria appears to be involved in the development of the advanced stage proliferative lesions in bladder tumorigenesis induced by PIO in male rats.


## Introduction

Peroxisome proliferator activated receptors (PPARs) are transcriptional factors belonging to the nuclear receptor superfamily, and 3 major isoforms, encoded by separate genes, have been identified as PPARγ, PPARα and PPARδ (also known as PPARβ). PPARγ is ubiquitously expressed throughout the body, including the tissues important for insulin sensitivity such as adipose tissue, skeletal muscle and the liver. Activation of PPARγ modulates the transcription of a number of insulin-responsive genes. Pioglitazone hydrochloride (PIO) is an oral antidiabetic agent acting through its potent and selective agonistic activity of PPARγ and has been widely used for the treatment of type 2 diabetes mellitus since 1999 (Drugs@FDA (Drug approval package), 1999).

The PPARγ agonists, such as PIO (Drugs@FDA, 1999) and rosiglitazone (Lubet et al., 2008), and the PPARα/γ dual agonists, such as ragaglitazar (Oleksiewicz et al., 2005), muraglitazar (Dominick et al., 2006) and naveglitazar (Long et al., 2008), have been reported to induce urothelial bladder tumors in rats, whereas treatment with various PPAR agonists was not associated with urothelial hypertrophy or hyperplasia in mice or monkeys (Hardisty et al., 2008). The urinary bladder is not an exceptional location of the ubiquitous expression of PPARγ in humans and laboratory animals (Chopra et al., 2008; Guan et al., 1997; Nakashiro et al., 2001); however, the direct effects of PPARγ agonists on bladder cell lines have always been decreased proliferation and/or accelerated differentiation in any of the human or animal cell lines (Guan et al., 1999; Kawakami et al., 2002; Suzuki et al., 2010; Varley and Southgate, 2008; Yoshimura et al., 2003). The paradox between the *in vivo* rat studies and the *in vitro* cell line studies has attracted scientific attention (Cohen, 2005), and the risk assessment in terms of the human relevancy of the tumorigenesis has been a major regulatory concern (El-Hage, 2005).

PIO was confirmed to have no genotoxicity or clastogenic potential in a standard panel of tests, no indication of tumorigenic or precancerous potential in a 2-year study in mice, or no proliferative lesions in the urinary bladder in dogs and monkeys given PIO for up to 1 year (Drugs@FDA, 1999). There was an increased incidence of transitional cell tumors in male rats administered doses of 4 mg/kg/day and higher for 2 years, with the highest incidence at 16 mg/kg/day. Thus PIO was associated with a neoplastic response in one species

* Corresponding author at: Development Research Center, Takeda Pharmaceutical Company Limited, 17-85, Jusohonmachi 2-chome, Yodogawa-ku, Osaka 532-8686, Japan. Fax: +81 6 6300 6917.
*E-mail address:* Sato_Keiichiro@takeda.co.jp (K. Sato).

0041-008X/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2011.01.006

(rat), one sex (male), one organ (urinary bladder), and one cell type (transitional epithelium). The bladder tumors were considered to result from a proliferative response to chronic irritation following the formation and retention of calculi and/or other mineralized particulate matter because a positive correlation between the tumors and calculi or related lesions such as mineralization and corpora amylacea was confirmed, and the majority of the proliferative lesions were located on the ventral surface of the urinary bladder, an area of predilection for deposition of solids in horizontal quadrupeds.

A considerable variety of nongenotoxic chemicals produce urinary calculi or microcrystals and consequently cause bladder tumors in a rat-specific manner including the sodium salts of saccharin, ascorbate, glutamate, aspartate, citrate, erythorbate, bicarbonate and chloride (Cohen et al., 1995b; IARC Working Group, 1999). This mode of action has been believed to show little human relevance for biochemical and anatomical reasons. The osmolality and protein concentration of human urine are far less favorable, than those of male rodents, for urolithiasis (Cohen, 1995; Hard, 1995; Olson et al., 1990; Rodent Bladder Carcinogenesis Working Group, 1995). Even if calculi might be formed, the bipedal posture of humans and the effect of gravity may favor early discharge (DeSesso, 1995) or produce complete urinary obstruction and pain with consequent removal, so the exposure is usually brief. In a limited number of case-control epidemiological studies, there was a weak statistical significance that urinary/kidney stone formation may be related to development of bladder cancer but a precise relationship was not revealed, and the association between inflammation and tumors applies more often to squamous cell cancers in humans, whereas most tumors are transitional cell neoplasms in rodents (Burin et al., 1995; Oyasu, 1995).

Among the PPAR agonists which were developed after PIO, the results of the mechanistic study for muraglitazar were consistent with a mode of action involving urinary solids (Dominick et al., 2006), but evidence for urolithiasis could not be identified in the mechanistic study for another PPAR agonist, naveglitazar (Long et al., 2008). This discrepancy may be due to physiologically dynamic variations in urine composition and many technical issues at collection, manipulation and evaluation of urine and urinary bladders (Cohen et al., 2007). Because crystallization of magnesium ammonium phosphate and calcium-containing solids, major components of urinary solids of rats related to the administration of various chemicals, can be prevented through urinary acidification to a pH <6.5 (Cohen et al., 1995a; Wall and Tiselius, 1990), evaluation of urinary solids as a mode of action can be most directly assessed by treating the rats with ammonium chloride to produce acidic urine.

For this purpose, PIO was administered chronically by oral gavage to male rats fed either a normal diet or a diet containing ammonium chloride (acid-forming diet) and the effects of urinary acidification on the tumorigenic potential of PIO in the urinary bladder were investigated. The study was designed to have 2 scheduled necropsies — at 18 months (interim necropsy) and at 24 months (terminal necropsy). Urinary composition, including pH, electrolytes, and number, morphology and elemental composition of any microcrystals was examined during the course of the experiment. Morphologic changes in the urinary bladder mucosal cells were evaluated along with the labeling index in BrdU immunostained cell nuclei as a measure of DNA synthesis and scanning electron microscopic (SEM) evaluation of the surface at the interim sacrifice. Light microscopy (hematoxylin and eosin staining) were conducted separately for the ventral and dorsal parts of the urinary bladder to check whether the predominant location of the proliferative lesions was the ventral part, consistent with the area of predilection for the deposition of solids in horizontal quadrupeds. Moreover, mRNAs were collected from the urinary bladder mucosa to characterize the gene expression associated with cell proliferation and PPARs. The plasma exposure levels of PIO and its metabolites were investigated in satellite groups of animals to determine if diet or repeated daily dosing affected their toxicokinetics and to confirm reproducibility of the exposure levels in comparison with the original carcinogenicity study (Drugs@FDA, 1999).

## Material and methods

*Animals and husbandry conditions.* SPF male Crl:CD(SD) rats were purchased from Charles River Laboratories (Japan). Females were not used because there were no treatment-related bladder tumors in females at dosage levels up to 64 mg/kg/day in the original 2-year carcinogenicity study in rats (Drugs@FDA, 1999). They were 6 weeks old and their body weights ranged from 187 to 224 g at the commencement of treatment. They were individually housed in suspended stainless-steel wire-bottom cages in rooms maintained on a 12-hour light–dark cycle (light phase: 7 AM to 7 PM) at a temperature range of 19 to 25 °C and a humidity range of 35% to 75%. The rats were allowed free access to food and tap water throughout the study period. This study was conducted in compliance with the Good Laboratory Practice Standards "Standards for Conduct of Nonclinical Studies on the Safety of Drugs" (Ministry of Health, Labor and Welfare, Japan, Ordinance number 21, March 26, 1997). The study protocol was reviewed by the Institutional Animal Care and Use Committee and was approved by the General Manager according to an internal guideline for animal welfare in the test facility. The level of animal care met the basic requirements in the "Act on Welfare and Management of Animals" (Ministry of the Environment, Japan, Act No. 105 of October 1, 1973).

*Diets.* Powdered diet sterilized by radiation (PMI5002, Purina Mills, USA) was used as the control diet. The control diet was given to all animals before group allocation and to the control animals after group allocation. Ammonium chloride ($NH_4Cl$, Wako Pure Chemical Industries, Japan) was added to the control diet at the concentration of 1.5%, and this acid-forming diet was fed to half of the rats from the day before the start of dosing with PIO or placebo. The concentration of $NH_4Cl$ in diet was determined to be high enough to suppress the urinary pH to 6.5 or below throughout a 24-hour period according to results of a preliminary study, where rats were given 16 mg/kg/day of PIO orally and fed 3 concentrations (1.0%, 1.25% and 1.5%) of $NH_4Cl$ in their respective diets for 4 weeks. In another preliminary study using a positive control compound, acetazolamide (a carbonic anhydrase inhibitor) (Durand-Cavagna et al., 1992), the 1.5% $NH_4Cl$ diet effectively eliminated microcrystalluria induced by a 7-day oral treatment with acetazolamide at 200 mg/kg/day, and suppressed any histopathological changes of epithelial injury or proliferation in the urinary bladder (data not shown). The concentration of $NH_4Cl$ was kept at 1.5% throughout the 2-year study period and frequent urinalysis for pH had shown that the experimental conditions in the present study were adequate to provide a constantly acidified urinary milieu. The values of urinary pH in the groups fed the 1.5% $NH_4Cl$ diet were within the expected range: 5.1–6.6 as individual values and 5.3–6.0 as a group mean value, while group mean values of urinary pH in the control group fed the control diet were around 7.

*Test substances.* Pioglitazone hydrochloride (PIO) was synthesized by the Takeda Pharmaceutical Company Limited (purity: 98.2%) and was provided as a citric acid granule to improve oral absorption. Citric acid granules not containing PIO were used for the placebo control groups. The granules were suspended in distilled water, and the dosing preparations were administered orally by gavage at a dosage volume of 10 mL/kg once daily in the morning. The dosage level of PIO was set at 16 mg/kg/day as the hydrochloride salt (14.5 mg/kg/day as free base), a dose at which urinary bladder tumors were noted in male rats with the highest incidence among all treatment groups in the original 2-year carcinogenicity study (dosage levels: 1, 4, 8, 16 and 63 mg/kg/day as the hydrochloride salt) (Drugs@FDA, 1999).

*Study groups.* Animals were randomly allocated to the 4 main groups [90 males per group, placebo control + control diet (CC), placebo control + acid-forming diet (CA), PIO + control diet (PC) and PIO + acid-forming diet (PA)] and the 2 satellite groups for toxicokinetics (10 males per group for PC and PA). At the initiation of the study, 40 of 90 males per main group

were assigned to the interim necropsy scheduled on Weeks 78–79, and 25 of the 40 males per group were selected for histopathological examination including BrdU index and scanning electron microscopy (SEM) while the remaining 15 males per group were kept for mRNA analysis. 50 of 90 males in the main group were assigned for the terminal necropsy on Week 104 and were used for in-life urinalysis and light microscopic examination.

*Animal room examinations.* The initial day and the initial week of the dosing period were designated as Day 1 and Week 1, respectively. All animals were observed twice daily for mortality and clinical conditions during the treatment period. Body weights and food consumption were measured once weekly.

*Urinalysis using fresh urine samples.* Freshly voided urine samples were collected at about 4 AM, 8 AM, 4 PM and 8 PM on Weeks 12–13, 25–26, 38–39, 51–52, 64–65, 77–78 and 90–91. The forced urination was performed for each rat once daily for 4 successive days during a sampling week point, 4 AM on the 1st day, 8 AM on the 2nd day, 4 PM on the 3rd day and 8 PM on the 4th day to set intervals of 24 h and longer. The animals were allowed free access to the diet and tap water before urine collection to avoid any effects of fasting on urinary composition (Cohen et al., 2007). The urinary pH was measured within 10 min after urination using a pH meter (ISFET pH Meter KS723, Shindengen Electric Manufacturing, Japan), and the number of microcrystals in homogeneously mixed urine samples was manually counted by light microscopy. The urine samples were also quantitatively analyzed using a particle size analyzer (SALD2000, SHIMADZU, Japan) for the intensity of light scattering as an indicator of the microcrystal counts and the median particle size of the urinary particles. Part of the urine sample was filtered through 0.22 μm filters (Nihon Millipore, Japan) using a suction pump to collect the insoluble solid residues. Then 0.1 N hydrochloric acid was added to the solid materials on the filter to dissolve the microcrystals. The supernatant of the solution was analyzed for oxalate, inorganic phosphorus, calcium and magnesium concentrations using chemical analyzers (FOM-110A, Hokuto Denko, Japan and TBA-200FR, Toshiba, Japan). The microcrystal samples were also examined with SEM (VE-8800, KEYENCE, Japan). The other filter with the trapped microcrystals, the one not used for the above urinalysis, was attached to a sample holder with double-coated adhesive tape and was treated with gold vapor for SEM. The elemental composition of typical urinary solids was analyzed with an energy dispersive X-ray diffractometer (Genesis 2000, EDAX, Japan).

*Urinalysis using 6-hour accumulated urine samples.* After the collection of fresh urine samples, 10 animals per group were placed in metabolic cages to collect urine samples for about 6 h after dosing on Weeks 13, 26, 39, 52, 65, 78 and 91. The animals were not deprived of diet and water during collection. The urine samples were centrifuged and the supernatants were analyzed for the concentrations of oxalate, citric acid, inorganic phosphorus, calcium, magnesium, creatinine, sodium, potassium and chloride using specific analyzers (FOM-110A, Hokuto Denko, Japan, TBA-200FR, Toshiba, Japan, and PVA-αIII, A&T, Japan) and for urine osmotic pressure using an osmometer (OM-801, VOGEL, Germany).

*Necropsy.* The interim necropsy on Weeks 78–79 was conducted 3 h after lights-on without preceding fasting. The remaining animals in the main group were subjected to terminal necropsy on Week 104. The urinary bladder was collected under inhalation anesthesia with diethyl ether, and then the animals were necropsied after euthanasia by exsanguination via severing the abdominal aorta.

*Histopathological examination by light microscopy.* The urinary bladders were collected from all surviving animals in the main groups (excluding the animals for mRNA analysis) at the interim and terminal necropsies and from the animals that were found dead or euthanized moribund. They were inflated with Bouin's solution in situ and immersed in the same solution for 1 to 4 h. The bladders were then divided into 2 hemispheres by sectioning at the median plane, and were fixed and preserved in 10 vol% neutral phosphate-buffered formalin. The right hemisphere at the interim necropsy or both hemispheres at the terminal necropsy were longitudinally divided into 4 strips per hemisphere, and 1 section per strip (2 sections each for the ventral and dorsal parts in each hemisphere) was processed by the standard method to prepare hematoxylin–eosin stained sections for light microscopic examination. The original histopathological findings recorded by a study pathologist were reviewed by a sponsor's pathologist for the findings from the tissues obtained up to the end of the interim necropsy and by a third party pathologist for the findings from all animals. Following the peer review, significant differences of opinion between the study and reviewing pathologists were discussed and resolved by mutual agreement as to the final diagnosis.

*Evaluation of cell proliferation in urinary bladder mucosal cells.* BrdU (bromodeoxyuridine) labeling index measurements and SEM examinations were conducted for the animals euthanized at the interim necropsy. BrdU was injected intraperitoneally at a dose of 100 mg/kg about 1 h before necropsy. After fixation with Bouin's solution and immunostaining for BrdU with mouse monoclonal antibody of clone Bu20a (diluted to 100 times, DAKO Japan), the numbers of BrdU positive cells per all mucosal epithelial cells in the right hemisphere of the bladder (BrdU index) were counted under light microscopy for each of the dorsal or ventral parts. The number of urinary bladder mucosal epithelial cells to be counted was set at 1500 for each part (totally 3000 cells/urinary bladder). The left hemisphere was dehydrated in ethanol, replaced with isoamyl acetate, dried, attached to a sample holder and coated with gold vapor. The gold-coated urinary bladder was examined by SEM (JSM-5200, JEOL, Japan).

*Analysis of mRNA from the urinary bladder mucosal cells.* The urinary bladder from all surviving animals (8–12 animals/group) for mRNA analysis in each main group was collected at the interim necropsy, inverted and washed with phosphate buffer and RLT buffer containing 0.01 vol% 2-mercaptoethanol (QIAGEN, RNeasy Total RNA Isolation Kit, Germany). The urinary bladder mucosal cells were isolated by rubbing against a tube wall in 0.5 mL of RLT buffer, and then the mRNA was eluted into the RLT buffer from the mucosal cells. The total RNA was purified from the RLT solutions (RNeasy Mini Kit, QIAGEN, Germany), and the purity, concentration and quality of total RNA were checked using a spectrophotometer (U-3310, Hitachi High-Technologies, Japan), a micro-cuvette (130-0623, Hitachi High-Technologies, Japan), and a 2100 BioAnalyzer and an RNA6000 Nano LabChip (Agilent Technologies, USA).

Expression of mRNAs of the 7 genes (CDK1 and Cyclin B as candidate genes for markers of cell proliferation, PPARα, PPARγ, PPARδ and RXRα as PPAR-related genes, and AFABP as a PPARγ target gene) were measured by quantitative RT-PCR using a 7900HT Real-Time PCR System and the Sequence Detection System ver. 2.3 (Applied Biosystems, USA). The TaqMan probes and the primers used for the mRNA measurement are shown in Table 1. The relative amount of each mRNA was estimated by the relative standard curve method and the calculated mRNA quantity for the 7 genes was normalized using the mRNA quantity of GAPDH.

*Toxicokinetics.* Plasma concentrations of PIO and its metabolites, M-II, M-III and M-IV were determined to assess their systemic exposure levels. The blood samples were collected from the subclavian vein of the satellite animals before dosing and at 0.5, 1, 2, 4, 8, 12 and 24 h after dosing in Weeks 1, 12, 25, 51 and 77. Plasma concentrations were determined by

**Table 1**
TaqMan probes and the primers used for the mRNA measurement.

| Gene name | | Sequence (5′ to 3′) |
|---|---|---|
| CDK1 | TaqMan probe | TCCAAGCCGTTTTCATCCAGGTTCTTG |
| | Forward primer | GGCCAGAAGTAGAGTCCCTGC |
| | Reverse primer | GACCAGCATTTTCGAGAGCAA |
| Cyclin B | TaqMan probe | TTCTTCGGCAGGTGCACATCCAGA |
| | Forward primer | TTATGGAAGTGAAACACTCTCCTGA |
| | Reverse primer | CGGAGAAAGCCTGACACAGAT |
| PPARα | TaqMan probe | CTGGAGGGTTCGTGGAGTCCTGGA |
| | Forward primer | GCGTTCGCAGCTGTTTTGT |
| | Reverse primer | GACCCAGCGTCGCTTCAG |
| PPARγ | TaqMan probe | TGCGGAAGCCCTTTGGTGA |
| | Forward primer | ACCAGGGAGTTCCTCAAAAGC |
| | Reverse primer | GCAAACTCAAACTTAGGCTCCATAA |
| PPARδ | TaqMan probe | TGCACCCCCTGCTCCAGGAGATCTA |
| | Forward primer | GAAGAAGACGGAGAGTGAGACCTT |
| | Reverse primer | TGCGTGCAGCCTTAGTACATG |
| RXRα | TaqMan probe | CCATAGCTGTGAAAGACGGCATCCTCCT |
| | Forward primer | AGCTGCTGATTGCCTCCTTCT |
| | Reverse primer | TGTGAGCGCTGTTCCTGTGTA |
| AFABP | TaqMan probe | TGGCCGGTATGGCCAAGCCC |
| | Forward primer | GGCTTCGCCACCAGGAA |
| | Reverse primer | CCCTTCTACGCTGATGATCAAGT |

high performance liquid chromatography (LC-10A system, Shimadzu, Japan) with electrospray ionization tandem mass spectrometry (API3000, AB/MDS SCIEX, USA).

*Statistical analysis.* Statistical analysis was conducted for the 3 paired comparisons between the CC and CA, CC and PC, and PC and PA groups. The interval scale data in urinalysis, BrdU index and mRNA expression were analyzed by the F test followed by the Student's *t* test or the Aspin–Welch *t* test. The F test was conducted at the significance level of 0.20 and the other tests were conducted at the two-tailed significance level of 0.05. The rate of proliferative lesions of the urinary bladder (hyperplasia, tumor and their combinations) was tested by the Peto test (asymptotic test). When the total number of animals with a proliferative lesion in the 2 groups to be compared was 10 or less, the rate was tested by the Peto exact test. For the Peto and Peto exact tests, the study was partitioned into 5 periods: Weeks 1–52, Weeks 53–78, scheduled interim necropsy on Weeks 77–78, Weeks 79–104 and scheduled terminal necropsy on Week 104, and these tests were conducted at the one-tailed significance levels of 0.05. Toxicological Data Processing System (MiTOX, Mitsui Zosen Systems Research, Japan) and SAS Preclinical Package (SAS Institute Japan) were used for the above statistical analyses.

## Results

### *Mortality*

The numbers of animals that were found dead or euthanized moribund in the PC and PA groups were increased compared with those in the CC and CA groups (Table 2). After the interim sacrifice, the survival rate in the CA group was higher than the CC group and that in the PA group was higher than the PC group. The mortality rates in the CC and PC groups were higher than those in the corresponding groups in the original 2-year carcinogenicity study of PIO. Since the mortality in the male rats receiving 16 mg/kg/day in the original study was 63.3% at the end of 2-year administration, the protocol of the mechanistic study had specified that dosing would be ceased at the time when the mortality in the groups given PIO reached about 60%. Consequently, the dosing was ceased at Week 85 when the mortality in the PC group exceeded 60%. The surviving animals were followed to the end of the 2-year study period without changes in the diet and were subjected to the examinations scheduled at the terminal necropsy on Week 104. The causes of death or hyposthenia as far as could be identified at necropsy were considered to be pulmonary edema, increased pleural and/or abdominal fluid, enlargement and atrial congestion of the heart and a nodule in the pituitary, but there were large numbers of animals showing no gross abnormalities that could be related to the death or hyposthenia. None of the lesions in the urinary bladder could be regarded as a cause of death.

### *Animal room examinations*

No treatment-related findings related to toxicity were evident in the clinical signs, nor were palpable masses or nodules noted in any group except for unspecific findings generally seen in a moribund state. The body weights were increased with PIO and were suppressed by the acid-forming diet from the early to middle phases of the study; however, the effects were not constant at the late phase because of larger individual variations in body weights at the late phase (see Table 3 for typical values). The body weights in the PA group were lower than those in the PC group but were generally equivalent to those in the CC and CA groups during the first half of the study. Food consumption was increased by PIO and the increase was suppressed by the acid-forming diet; however, the effects disappeared at the late phase of the study (data not shown). The food consumption in the CA group was the same as that in the CC group, showing no palatability issue for the acid-forming diet. Food consumption in the groups other than the PC group was almost the same as that in the CC group throughout the experimental period.

**Table 2**
Cumulative mortality.

| Study | | Mechanistic study | | | | Original study (e) | |
|---|---|---|---|---|---|---|---|
| Group | | CC | CA | PC | PA | CC | PC |
| Dose (mg/kg) | | 0 | 0 | 16 | 16 | 0 | 16 |
| Diet | | Control | Acidified | Control | Acidified | Control | Control |
| Week | 13 | 0/90 (0) | 0/90 (0) | 2/90 (2) | 0/90 (0) | 0/60 (0) | 3/60 (5) |
| | 26 | 0/90 (0) | 0/90 (0) | 3/90 (3) | 1/90 (1) | 1/60 (2) | 5/60 (8) |
| | 39 | 0/90 (0) | 0/90 (0) | 8/90 (9) | 2/90 (2) | 7/60 (12) | 7/60 (12) |
| | 52 | 0/90 (0) | 1/90 (1) | 15/90 (17) | 10/90 (11) | 7/60 (12) | 9/60 (15) |
| | 79 (a) | 17/90 (19) | 15/90 (17) | 40/90 (44) | 35/90 (39) | 15/60 (25) | 23/60 (38) |
| | 79 (b) | 11/50 (22) | 6/50 (12) | 24/50 (48) | 20/50 (40) | 15/60 (25) | 23/60 (38) |
| | 85 (c) | 20/50 (40) | 12/50 (24) | 31/50 (62) | 24/50 (48) | 16/60 (27) | 25/60 (42) |
| | 104 (d) | 33/50 (66) | 27/50 (54) | 43/50 (86) | 41/50 (82) | 36/60 (60) | 38/60 (63) |

Number in parentheses shows mortality rate (%): animals that died or were found dead / total number of animals × 100. CC, placebo control + control diet; CA, placebo control + acid-forming diet; PC, pioglitazone + control diet; PA, pioglitazone + acid-forming diet. (a), before the interim sacrifice; (b), after the interim sacrifice; (c), at the end of administration; (d), before the terminal sacrifice period; (e), the original 2-year carcinogenicity study consisted of 7 groups.

**Table 3**
Representative values of body weight.

| Study | | Mechanistic study | | | | Original study (e) | |
|---|---|---|---|---|---|---|---|
| Group | | CC | CA | PC | PA | CC | PC |
| Dose (mg/kg) | | 0 | 0 | 16 | 16 | 0 | 16 |
| Diet | | Control | Acidified | Control | Acidified | Control | Control |
| Week | 1 | 208 | 204 | 207 | 204 | 226 | 229 |
| | 13 | 566 | 542 | 594 | 557 | 491 | 507 |
| | 26 | 666 | 633 | 719 | 661 | 560 | 602 |
| | 38 | 722 | 679 | 771 | 715 | 610 | 650 |
| | 52 | 773 | 718 | 823 | 755 | 639 | 691 |
| | 78 (a) | 809 | 770 | 811 | 778 | 666 | 695 |
| | 80 (b) | 809 | 773 | 805 | 779 | 669 | 693 |
| | 86 (c) | 847 | 763 | 770 | 758 | 677 | 694 |
| | 104 (d) | 825 | 745 | 757 | 725 | 631 | 658 |

Shown as group mean body weights (g) (see Table 2 for the number of rats examined at each timepoint). CC, placebo control + control diet; CA, placebo control + acid-forming diet; PC, pioglitazone + control diet; PA, pioglitazone + acid-forming diet. (a), before the interim sacrifice; (b), after the interim sacrifice; (c), after the end of administration; (d), before the terminal sacrifice period; (e), the original 2-year carcinogenicity study consisted of 7 groups.

*Urinalysis using fresh void urine samples*

The values of urinary pH in the CC and PC groups and the CA and PA groups ranged from about 6 to 8 and from about 5 to 6, respectively, and the rats fed the acid-forming diet always showed lower pH values compared with the rats fed the non-acid-forming control diet throughout the study period. The number of microcrystals counted by light microscopy in the PC group was increased at 4 AM and 4 PM in Week 12 and at 4 AM and 8 PM in Week 25 compared with those in the CC group (Fig. 1), although statistical significance was not always reached due to the large inter-individual variability. Thereafter, the numbers tended to be decreased age-dependently in all groups with less clear inter-group differences in the absolute values. The numbers of microcrystals in the CA and PA groups were decreased compared with those in the CC and PC groups. The quantitative data for the microcrystal counts estimated with a laser diffraction particle size analyzer showed the same tendency as with the manual counts using light microscopy at most of the timepoints examined. The median size of the microcrystals was not consistently affected by PIO in Weeks 12, 25 and 38, and $NH_4Cl$ tended to reduce the median size in the CA and PA groups regardless of the test article in Week 12. The effects of PIO and $NH_4Cl$ on the size of microcrystals could not be evaluated by a laser diffraction particle size analyzer at most of the later timepoints because of lack of data due to the small number of particles captured. Chemical analysis for electrolytes in the filter-trapped microcrystals revealed a decrease in calcium with PIO and decreases in inorganic phosphate, magnesium and oxalate with the acid-forming diet (Fig. 2).

In the analysis using SEM and an energy dispersive X-ray diffractometer, microcrystals with various shapes and sizes were categorized into “small”, “usual”, “large”, “rod-like”, “scale-like”, “needle-like”, “unclassified (irregular shape)” and their degree of aggregation. When the results were compared between the CC and PC groups, the incidence and/or grade (amounts) of various shapes and sizes of microcrystals and their 3-dimensional radial aggregation were occasionally increased by PIO (Table 4). The major types of microcrystals and their aggregation were confirmed to be magnesium ammonium phosphate crystals in most cases. Marked reductions of all types of microcrystals were also confirmed by SEM in both the CA and PA groups at the majority of timepoints examined.

*Urinalysis using 6-hour accumulated urine samples*

Increases in calcium and inorganic phosphorus and decreases in sodium, potassium and chloride were noted age-dependently in the CC group. As effects of PIO, decreases in citrate (all phases), calcium (late phase), sodium (late phase), potassium (late phase), magnesium (late phase) and osmotic pressure (mid-phase) were seen at multiple timepoints when compared with the age-matched CC group values. As effects of the acid-forming diet, increases in chloride (all phases), calcium (early phase) and inorganic phosphorus (early phase) and decreases in citrate (all phases), calcium (late phase) and inorganic phosphorus (late phase) were noted in comparison with the age-matched CC group values. Among these changes, decreases in citrate with PIO and the acid-forming diet were most marked and constant, but no changes were seen in oxalate at any timepoint (see Table 5 for these 2 parameters).

*Histopathology*

The single or combined incidences of carcinoma (malignant tumor), papilloma (benign tumor), nodular hyperplasia, papillary hyperplasia and/or simple hyperplasia in the mucosal epithelium of the urinary bladder are summarized in Table 6. The final incidence of male rats bearing either papilloma or carcinoma in the PC group was 7 of 82 males (9%) in the present study which was similar to that in the 16 mg/kg group in the original 2-year carcinogenicity study (7 of 60 males, 12%). The final incidence of rats bearing at least one of the advanced proliferative lesions, i.e. papillary hyperplasia, nodular hyperplasia, papilloma and/or carcinoma in the present study (11 of 82 males, 13%) was also reproduced at similar incidences to that in the original study (9 of 60 males, 15%). These data indicate that the tumorigenicity by PIO alone was reproduced at the similar incidences to those in the original 2-year carcinogenicity study in rats although the dosing of PIO was ceased on Week 85 because of the high mortality rates in the PC group.

Carcinoma was noted in the ventral section in 1 of 82 males in the PC group only and was not seen in the dorsal section in any animal in any group, resulting in no statistical significance for any comparison. Papilloma was noted in 0 of 78, 0 of 78, 7 of 82 and 1 of 80 males in the CC, CA, PC and PA groups, respectively. Statistically significant differences in the incidence of transitional cell papilloma in any section were noted between the CC and PC groups and between the PC and PA groups. The latency of the tumors in our study was relatively long, that is, papilloma was seen only in 2 of 56 and 1 of 50 rats found dead or euthanized by the end of the interim necropsy in Weeks 78–79 in the PC and PA groups, respectively, and carcinoma was not identified by time of the interim necropsy, whereas the incidences of transitional cell papilloma and carcinoma in rats receiving 50 mg/kg of muraglitazar, a PPARα/γ dual agonist, with a control diet were 1 and 14 out of 25 rats at Month 15 and 1 and 10 out of 17 rats at Month 18, respectively (Dominick et al., 2006).

Focal nodular hyperplasia was noted in 0 of 78, 0 of 78, 4 of 82 and 1 of 80 males in the CC, CA, PC and PA groups, respectively. Nodular hyperplasia in most of the rats in the PC group was noted as multiple lesions with focal distribution predominantly in the ventral section; however, the lesion in 1 rat in the PA group was seen as a single lesion in the ventral section. Statistically significant differences in the incidence of the nodular hyperplasia in the ventral section were noted between the CC and PC groups. Focal papillary hyperplasia was noted in the ventral section in 5 of 82 males in the PC group only, but not in any section from any animal in any other group. Two of these 5 animals had multiple (4 or 6) lesions. A statistically significant increase in the incidence of papillary hyperplasia in the ventral section was noted in the PC group compared with that in the CC group, and a statistically significant decrease was noted in the PA group compared with that in the PC group. Suppressive effects of the acidified diet were already seen before the treatment stoppage on Week 85, that is, incidences of animals bearing at least one of papillary hyperplasia, nodular hyperplasia and/or papilloma by the interim sacrifice in Weeks 78–79 were 6 of 56 males (11%) in the PC group and 2 of 50 males (4%) in the PA group (data not shown).

Simple hyperplasia was diagnosed in 12 of 78, 13 of 78, 28 of 82 and 40 of 80 males in the CC, CA, PC and PA groups, respectively, although the majority of changes was minimal in severity. A statistically significant difference was noted between the CC and PC groups.

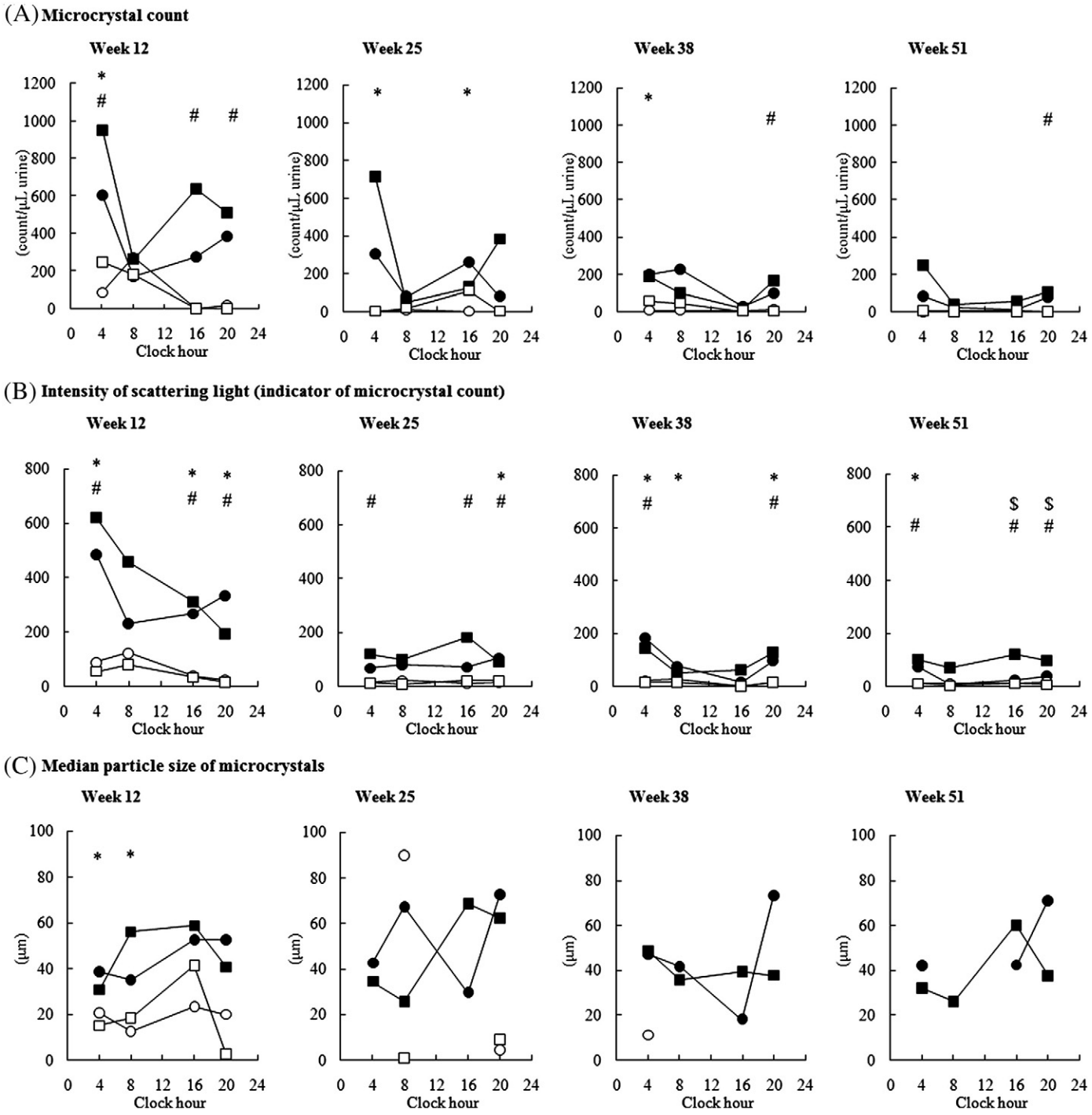


**Fig. 1.** Number and particle size of microcrystals in the freshly voided urine samples. Freshly voided urine was collected from 10 rats/group/point. Urinary microcrystals were counted by light microscopy (A), and the number (B) and particle size (C) were quantitatively analyzed with a laser diffraction particle size analyzer using the intensity of light scattering as an indicator of microcrystal counts. The median particle size could not be evaluated by a particle size analyzer at most late timepoints because of lack of data (shown as intermittent or no lines in the lower panels) due to the small number of particles captured by this device. ●, placebo control + control diet; ○, placebo control + acid-forming diet; ■, pioglitazone + control diet; □, pioglitazone + acid-forming diet. *$#, Statistical significance in comparisons between ● and ○ (*), ● and ■ ($), and ■ and □ (#) ($p \leq 0.05$).

*BrdU index of urinary bladder mucosal cells*

Statistically significant increases in the BrdU labeling indices in both the ventral and dorsal sections were noted in the PC group compared with the CC group, and the increase was more remarkable in the ventral sections than in the dorsal sections (Table 7). Although there was no statistical significance, the BrdU labeling indices tended to be decreased in the PA group compared with the PC group, and the tendency for suppression of the index was more remarkable in the ventral sections than in the dorsal sections.

*Scanning electron microscopy (SEM)*

Swelling of the urothelial cells was noted in the ventral area in 1 of 15 males in the PA group, the dorsal area in 1 of 15 males in the PA group, and both the ventral and dorsal areas in 0 of 22, 1 of 19, 8 of 17

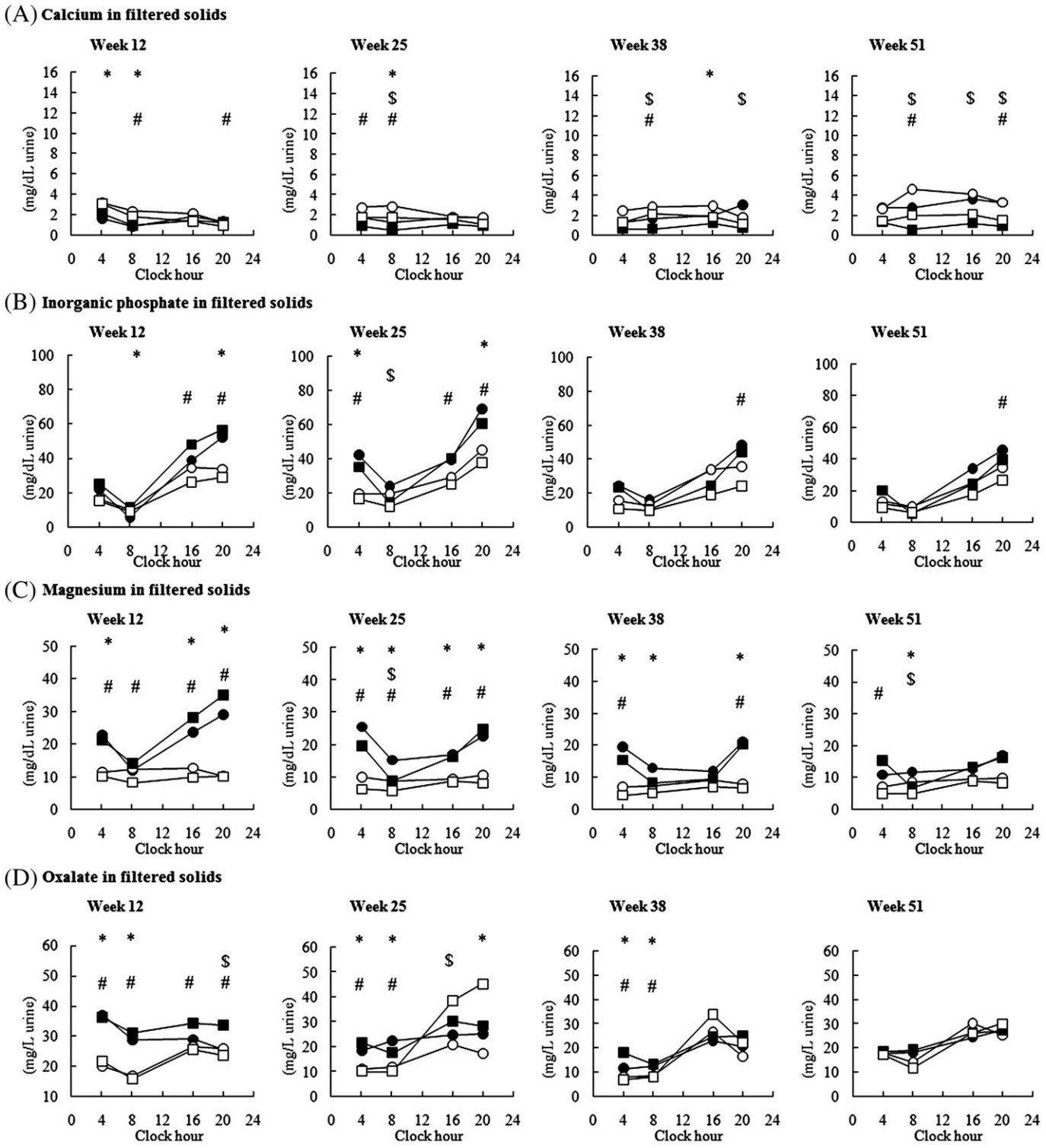


**Fig. 2.** Urinalysis for filter-trapped microcrystals in the freshly voided urine samples. Chemical analysis for electrolytes in the filter-trapped solids in the freshly voided urine samples was conducted for calcium (A), inorganic phosphate (B), magnesium (C) and oxalate (D) (10 rats/group/point). ●, placebo control + control diet; ○, placebo control + acid-forming diet; ■, pioglitazone + control diet; □, pioglitazone + acid-forming diet. *$#, Statistical significance in comparisons between ● and ○ (*), ● and ■ ($), and ■ and □ (#) ($p \leq 0.05$).

and 8 of 15 males in the CC, CA, PC and PA groups, respectively. Raised foci were noted in the ventral area in 1 of 17 males in the PC group, the dorsal area in 1 of 15 males in the PA group and both the ventral and dorsal areas in 1 of 17 males in the PC group. Among these, cell swelling and raised foci with an advanced grade were noted in 3 and 1 males in the PC group, respectively, but were not seen in any animal in the PA group. Streaks of the urothelial surface were also noted in 3 of 17 males and 4 of 15 males in the PC and PA groups, respectively.

*Analysis of mRNA from urinary bladder mucosal cells*

The relative expressions of mRNAs, which were normalized against GAPDH, are shown in Table 8. None of the relative gene expressions showed statistically significant changes on comparison between the CC and CA groups. PPARα and PPARγ were decreased and AFABP and Cyclin B were increased by PIO compared between the CC and PC groups. A tendency to decrease was also seen in CDK1.

**Table 4**
Characterization of increased microcrystals by scanning electron microscopy on Weeks 12–90.

| Week | Clock hour | Major type of microcrystals in the PC group |
|---|---|---|
| 12 | 4 PM | Scale-like |
| | 8 PM | 3-Dimensional radial aggregation[a] |
| 38 | 4 PM | Small, large, 3-dimensional radial aggregation[a] |
| 64 | 4 PM | Rod-like |
| 77 | 4 PM | Usual, small |
| | 8 PM | 3-Dimensional radial aggregation[a] |
| 90 | 4 AM | Usual |
| | 4 PM | Small |
| | 8 PM | Usual |

Urine samples for 10 males were obtained at each sampling point (4 AM, 8 AM, 4 PM and 8 PM), whereas the numbers of collectable samples were reduced to 4–6 in the PC group (pioglitazone + control diet) at Week 90 due to the increased mortality or moribundity. The types of microcrystals are listed in this table when the findings were recorded in half or more of animals in the PC group and the incidences were more than twice that in the CC group (placebo control + control diet) at the same sampling point.
[a] A distinctive aggregation of microcrystals showing a 3-dimensional radial pattern.

When the expressions in rats in the PA group were compared with those in the PC group, the decreased expression of CDK1 in the PC group was not seen in the PA group and Cyclin B was further increased with statistical significance, but statistically significant changes were not noted for the other genes. PPARs and AFABP were not affected by $NH_4Cl$ in comparisons between the CC and CA groups and between the PC and PA groups.

### *Toxicokinetics*

The Cmax and $AUC_{0-24\ h}$ values of PIO were higher than those of its metabolites, M-II, M-III and M-IV, and the acid-forming diet did not significantly affect these TK parameters (see Table 9 for typical AUC values on Weeks 25 and 77). No clear differences were observed in their TK parameters with repeated dosing. The TK parameters in this study were almost the same as those at the corresponding 16 mg/kg group in the original carcinogenicity study.

**Table 5**
Citrate and oxalate in urine collected for 6 h after dosing on Weeks 13–91.

| Group | | CC | CA | PC | PA |
|---|---|---|---|---|---|
| Dose (mg/kg) | | 0 | 0 | 16 | 16 |
| Diet | | Control | Acidified | Control | Acidified |
| No. of rats | | 10 | 10 | 10 | 10 |
| *Citrate/creatinine (g/g)* | | | | | |
| Week | 13 | 1.60 ± 0.50 | 0.27 ± 0.10 | 1.17 ± 0.64 | 0.20 ± 0.07 |
| | 26 | 1.45 ± 0.54 | 0.23 ± 0.09 * | 0.79 ± 0.64 $ | 0.12 ± 0.04 # |
| | 39 | 1.10 ± 0.56 | 0.15 ± 0.11 * | 0.51 ± 0.34 $ | 0.13 ± 0.10 # |
| | 52 | 1.53 ± 0.57 | 0.29 ± 0.14 * | 0.50 ± 0.52 $ | 0.23 ± 0.07 |
| | 65 | 1.96 ± 1.84 | 0.33 ± 0.16 | 0.72 ± 0.61 | 0.23 ± 0.06 |
| | 78 | 1.85 ± 1.64 | 0.28 ± 0.20 * | 0.33 ± 0.17 $ | 0.29 ± 0.16 |
| | 91 | 2.14 ± 1.49 | 0.38 ± 0.15 * | 0.56 ± 0.14 $ | 0.80 ± 1.13 |
| *Oxalate/creatinine (g/g)* | | | | | |
| Week | 13 | 48.85 ± 18.01 | 51.91 ± 11.41 | 59.48 ± 26.02 | 62.51 ± 8.54 |
| | 26 | 50.99 ± 11.90 | 39.96 ± 5.90 * | 47.47 ± 10.72 | 49.73 ± 5.56 |
| | 39 | 41.65 ± 8.24 | 39.11 ± 4.76 | 43.56 ± 7.79 | 43.73 ± 6.11 |
| | 52 | 47.08 ± 6.08 | 39.96 ± 6.02 | 39.55 ± 8.84 | 39.13 ± 5.01 |
| | 65 | 36.12 ± 11.40 | 34.44 ± 4.11 | 38.39 ± 8.62 | 35.74 ± 8.04 |
| | 78 | 43.06 ± 12.70 | 38.13 ± 6.30 | 36.92 ± 4.47 | 37.54 ± 7.44 |
| | 91 | 44.88 ± 16.75 | 38.76 ± 4.76 | 35.99 ± 6.22 | 35.06 ± 6.61 |

Shown as group mean values ± S.D. of creatinine ratio (g/g). CC, placebo control + control diet; CA, placebo control + acid-forming diet; PC, pioglitazone + control diet; PA, pioglitazone + acid-forming diet. *$#, Statistical significance in comparisons between CC and CA (*), CC and PC ($), and PC and PA (#) ($p \leq 0.05$).

## Discussion

Urinary acidification by $NH_4Cl$ has been a useful methodology to verify the mode of action through crystalluria in bladder tumorigenesis induced by sodium ascorbate (Fukushima et al., 1986), sodium o-phenylphenate (Fujii et al., 1987), monosodium glutamate (de Groot et al., 1988), carbonic anhydrase inhibitors (Durand-Cavagna et al., 1992), sodium saccharin (Cohen et al., 1995a), and muraglitazar (Dominick et al., 2006). In our study, the incidence of PIO-treated males bearing at least one of the advanced proliferative lesions was significantly reduced from 11 of 82 males fed the control diet to 2 of 80 males fed the acid-forming diet. The urinary microcrystals were increased by PIO during the first 6 months and were markedly suppressed by the acid-forming diet. The mechanism of the suppression by $NH_4Cl$ against urinary bladder tumors was considered to be specific to tumorigenesis in the urinary bladder, because the incidence, multiplicity, chronology and malignancy of any spontaneous tumor in any of the organs examined were not suppressed or enhanced in the 30-month feeding of the acid-forming diet containing 1% and 2.1% $NH_4Cl$ to rats, and non-specific and unconditional protection by mild chronic acidosis against any type of spontaneous tumorigenesis was not observed (Lina and Kuijpers, 2004). Therefore, it is most likely that the specific mechanism of the suppression by $NH_4Cl$ is related to urinary acidification leading to a reduced number of urinary microcrystals.

In the study with naveglitazar (Long et al., 2008), changes in urinary solids were not detected, and one of the possible reasons for the discrepancy could be differences in the experimental conditions. For example, the increase in microcrystals by PIO was most marked at 4 AM during the dark phase in our study, whereas urine was not collected during the dark phase in the study with naveglitazar (Long et al., 2008). The increased microcrystals during dark phase are not surprising when the circadian rhythms of the physiological activities of eating, drinking and urination in nocturnal rats are taken into consideration (Cohen et al., 2007). Lack of quantification of the microcrystals using an objective method in the previous studies might be another reason. The microcrystal numbers were quantitatively counted under microscopy, and the tendency to increase was also supported by the data obtained objectively with a laser diffraction particle size analyzer in our study; however, any quantitative numbers of microcrystals were not disclosed in the previous studies.

Oxalate can bind with many metal ions such as calcium to form insoluble precipitates in urine. In mechanistic studies with muraglitazar (Dominick et al., 2006; Van Vleet et al., 2007), an increased urinary oxalate was reported as a possible PPARα-related effect on hepatic metabolism of glyoxalate, the primary source of urinary oxalate, but oxalate in filter-trapped solids or supernatants was not increased by PIO, indicating possible differences in PPARα-agonist activity between these 2 compounds. Increases in calcium-containing crystals and calculi were reported in a 4-week experiment in rats given PIO (Suzuki et al., 2010), while typical changes in our study were increased number of magnesium ammonium phosphate crystals and appearance of their 3-dimensional radial aggregation (Table 4). We did not collect urine at Week 4 and features of urinary solids may be variable depending on sampling points.

In the urinalysis using supernatants from urine accumulated over a 6-hour period, decreases in citrate, an organic ion with anti-lithogenic effects, were the most marked and constant effect of PIO. In rat studies with muraglitazar same phenomena are suggested to be due to PPARγ-related induction of aconitase in the citric acid cycle (Dominick et al., 2006; Van Vleet et al., 2007). In humans, however, urinary citrate has been reported to be high in type 2 diabetes mellitus patients with glycosuria (Messana et al., 1998; van Doorn et al., 2006), and rosiglitazone increased the plasma levels of citrate but had no marked effects on urinary citrate (van Doorn et al., 2006). Therefore, the human relevance of the decreased urinary citrate in nondiabetic

**Table 6**
Light microscopic findings of the proliferative lesions in the urinary bladder up to the terminal sacrifice.

| Study | Mechanistic study | | | | Original study (e) | |
|---|---|---|---|---|---|---|
| Group | CC | CA | PC | PA | CC | PC |
| Dose (mg/kg) | 0 | 0 | 16 | 16 | 0 | 16 |
| Diet | Control | Acidified | Control | Acidified | Control | Control |
| No. of rats examined | 78 | 78 | 82 | 80 | 60 | 60 |
| Carcinoma | 0 | 0 | 1 | 0 | 0 | 5 |
| (Ventral) | (0) | (0) | (1) | (0) | (0) | (5) |
| (Dorsal) | (0) | (0) | (0) | (0) | (0) | (0) |
| Papilloma | 0 | 0 | 7$ | 1# | 0 | 4 |
| (Ventral) | (0) | (0) | (4)$ | (0)# | (0) | (4) |
| (Dorsal) | (0) | (0) | (3) | (1) | (0) | (0) |
| Any type of tumor | 0 | 0 | 7$ | 1# | 0 | 7 |
| (Ventral) | (0) | (0) | (4)$ | (0)# | (0) | (7) |
| (Dorsal) | (0) | (0) | (3) | (1) | (0) | (0) |
| Nodular hyperplasia | 0 | 0 | 4$ | 1 | 0 | 2 |
| (Ventral) | (0) | (0) | (4)$ | (1) | (0) | (2) |
| (Dorsal) | (0) | (0) | (1) | (0) | (0) | (1) |
| Papillary hyperplasia | 0 | 0 | 5$ | 0# | 0 | 1 |
| (Ventral) | (0) | (0) | (5)$ | (0)# | (0) | (1) |
| (Dorsal) | (0) | (0) | (0) | (0) | (0) | (1) |
| Proliferative lesions more severe than simple hyperplasia (a) | 0 | 0 | 11$ | 2# | 0 | 9 |
| (Ventral) | (0) | (0) | (8)$ | (1)# | (0) | (9) |
| (Dorsal) | (0) | (0) | (4)$ | (1) | (0) | (2) |
| Simple hyperplasia (b) | 12 | 13 | 28$ | 40 | 3 | 10 |
| (Ventral) | (9) | (12) | (27)$ | (31) | (3) | (8) |
| (Dorsal) | (12) | (8) | (24)$ | (34) | (3) | (5) |
| Simple hyperplasia alone (c) | 12 | 13 | 20 | 39 | 3 | 10 |
| Simple hyperplasia and more severe proliferative lesions (d) | 0 | 0 | 8 | 1 | 0 | 0 |

CC, placebo control + control diet; CA, placebo control + acid-forming diet; PC, pioglitazone + control diet; PA, pioglitazone + acid-forming diet. Results of statistical analysis are shown for the mechanistic study: statistically significant comparisons between CC and PC groups ($), and PC and PA groups (#) (Peto/Peto exact test, $p \leq 0.05$). (a), animals bearing at least one of papillary hyperplasia, nodular hyperplasia, papilloma and carcinoma; (b), animals bearing simple hyperplasia alone or both simple hyperplasia and more severe proliferative lesions; (c), animals bearing simple hyperplasia alone (statistical analysis was not performed); (d), animals bearing both simple hyperplasia and more severe proliferative lesions (statistical analysis was not performed); (e), the original 2-year carcinogenicity study consisted of 7 groups.

rats in our study is unclear. Urinary citrate ranged in the order of CC > PC > PA = CA, and the reduction by PIO alone can be one of the enhancing factors in bladder tumorigenesis in the PC group. However, the suppressive effects of dietary $NH_4Cl$ against bladder tumorigenesis were not related to the decreased level of urinary citrate in the PA group where the urinary solids were already reduced by acidification itself, regardless of the level of urinary citrate.

The developmental sequence of the various proliferative lesions in the PC group can be divided into 2 major classes: simple hyperplasia and more severe proliferative lesions ('advanced' lesions). The behavior of these 2 classes was significantly different in terms of 3 factors: (1) spontaneous occurrence in the controls; (2) responsiveness to $NH_4Cl$; and (3) intravesical localization (Table 6).

Advanced lesions consisting of papillary hyperplasia, nodular hyperplasia, papilloma and carcinoma were not seen in any animal in the CC and CA groups, while those were seen in 11 of 82 animals in the PC group (13%) and were noted only in 2 of 80 animals in the PA group (2.5%); one had nodular hyperplasia only at a single location and another had papilloma alone, suggesting that these single occurrences might be spontaneous. The advanced lesions, especially carcinoma and papillary/nodular hyperplasia, were predominantly located in the ventral sections in the PC group, which indicate that the deposition of solids and subsequent proliferative lesions are apt to occur on the ventral epithelium due to the effects of gravity in the bladder of quadrupeds. The BrdU index, a reflection of mitotic activity in the bladder mucosal cells, was increased in the animals treated with PIO at the interim necropsy, supporting the histopathological evidence of proliferation in the urinary bladder mucosal cells, and concurrent acidification of the urine by $NH_4Cl$ tended to suppress the BrdU index (Table 7). By scanning electron microscopy of the urinary bladder, an advanced grade of cell swelling and raised foci were noted in the PC group but not in the PA group, which also indicated the

**Table 7**
BrdU labeling index in urinary bladder mucosal cells at Week 78.

| Group | CC | CA | PC | PA |
|---|---|---|---|---|
| Dose (mg/kg) | 0 | 0 | 16 | 16 |
| Diet | Control | Acidified | Control | Acidified |
| No. of rats | 22 | 19 | 16 | 15 |
| Ventral | 0.052 ± 0.082 | 0.103 ± 0.195 | 0.381 ± 0.572 $ | 0.152 ± 0.151 |
| Dorsal | 0.082 ± 0.099 | 0.064 ± 0.108 | 0.224 ± 0.248 $ | 0.179 ± 0.243 |

Shown as group mean values ± S.D. (%). CC, placebo control + control diet; CA, placebo control + acid-forming diet; PC, pioglitazone + control diet; PA, pioglitazone + acid-forming diet. $, Statistical significance in comparisons between CC and PC ($p \leq 0.05$); no significance in comparisons between CC and CA, and PC and PA.

**Table 8**
Relative mRNA expression in urinary bladder mucosal cells at Week 78.

| Group | CC | CA | PC | PA |
|---|---|---|---|---|
| Dose (mg/kg) | 0 | 0 | 16 | 16 |
| Diet | Control | Acidified | Control | Acidified |
| No. of rats | 11 | 11 | 7 | 9 |
| CDK1 | 100 ± 44 | 94 ± 31 | 72 ± 23 | 101 ± 22 # |
| Cyclin B | 100 ± 22 | 111 ± 40 | 133 ± 37 $ | 191 ± 58 # |
| PPARα | 100 ± 23 | 100 ± 25 | 53 ± 15 $ | 58 ± 12 |
| PPARγ | 100 ± 24 | 94 ± 24 | 47 ± 12 $ | 53 ± 8 |
| PPARδ | 100 ± 20 | 104 ± 20 | 86 ± 19 | 89 ± 13 |
| RXRα | 100 ± 16 | 100 ± 16 | 86 ± 32 | 84 ± 10 |
| AFABP | 100 ± 30 | 106 ± 37 | 572 ± 186 $ | 419 ± 104 |

Shown as group mean values ± S.D. (%); normalized against GAPDH. CC, placebo control + control diet; CA, placebo control + acid-forming diet; PC, pioglitazone + control diet; PA, pioglitazone + acid-forming diet. $#, Statistical significance in comparisons between CC and PC ($), and PC and PA (#) ($p \leq 0.05$); no significance in comparisons between CC and CA.

**Table 9**
Toxicokinetic parameters ($AUC_{0-24\ h}$) of pioglitazone and its metabolites.

| Study | Mechanistic study | | | | Original study | |
|---|---|---|---|---|---|---|
| Group | PC | | PA | | PC | |
| Dose (mg/kg) | 16 | | 16 | | 16 | |
| Diet | Control | | Acidified | | Control | |
| Week | 25 | 77 | 25 | 77 | 26 | 79 |
| $AUC_{0-24\ h}$ (μg·h/mL) | | | | | | |
| Pioglitazone | 191.1 | 132.8 | 186.8 | 205.3 | 173.8 | 155.2 |
| M-II | 56.2 | 43.6 | 35.3 | 40.8 | 69.6 | 71.7 |
| M-III | 18.4 | 14.1 | 25.6 | 24.5 | 14.2 | 17.4 |
| M-IV | 15.2 | 13.1 | 21.1 | 29.4 | 15.4 | 15.9 |

Shown as group mean values (3 rats/point/group); PC, pioglitazone + control diet; PA, pioglitazone + acid-forming diet; M-II, M-III and M-IV, metabolites of pioglitazone.

suppressive effects of $NH_4Cl$. These data strongly suggest that the PIO-induced occurrence of the advanced lesions in the PC group and their suppression by $NH_4Cl$ in the PA group were consistent with the crystalluria hypothesis, which is the most plausible mode of action for bladder tumorigenesis by PIO in male rats.

On the other hand, the incidence of simple hyperplasia alone showed different properties from those of the advanced proliferative lesions in terms of the 3 factors noted above. Simple hyperplasia was seen in the 2 control groups with no difference in incidence between the CC and CA groups or between ventral and dorsal sections, revealing that the spontaneous simple hyperplasia in the control groups was not always explicable by the crystalluria hypothesis. In fact, the spontaneous simple hyperplasia was not diminished by $NH_4Cl$ at dietary concentrations of 1% or 2.1% in a 30-month study in rats (Lina and Kuijpers, 2004). Limitation of the suppressive effects of $NH_4Cl$ against simple hyperplasia and no evidence of predominant intravesical localization of simple hyperplasia were also the case in the PC and PA groups when the numbers of animals with simple hyperplasia alone were compared. However, when the numbers of animals bearing both simple hyperplasia and the advanced lesions were counted, the suppressive effects of $NH_4Cl$ against proliferative lesions became obvious. That is, 8 of 28 animals with simple hyperplasia in the PC group also had at least 1 of the advanced lesions, whereas only 1 of 40 animals had both simple hyperplasia and the advanced lesion in the PA group, as shown in Table 6. This may suggest that most of the simple hyperplasia noted alone in the PC and PA groups was caused by the exaggeration of spontaneous lesions whose mechanism is different in nature from the advanced lesions caused by PIO in accordance with the crystalluria hypothesis. The incidence of simple hyperplasia in the CC group in the present study was slightly higher than that of the placebo control group in the original study (Table 6). Simple hyperplasia consisted of at least 3 layers of epithelial cells except the umbrella cells, which were occasionally exfoliated due to autolysis in some animals, and this diagnostic criterion was strictly applied not only for diffuse changes but also for tiny focal changes to increase the sensitivity of this experimental model. In fact, the majority of simple hyperplasia in any group was minimal in severity. This may explain to some extent the higher incidence of simple hyperplasia in the present study. Additionally, because stress induced by extensive handling of rats can lead to urothelial exfoliation and consequent regenerative hyperplasia (Cohen et al., 1996), frequent manipulation for collecting urine specimens, which is not routinely conducted in a standard 2-year carcinogenicity study, may increase the incidence of simple hyperplasia in the present study.

The increased mRNA expression of AFABP, one of target genes of PPARγ, in the PC group revealed that the PPARγ agonistic potency of PIO was unaffected after chronic treatment (Table 8). This increased expression is consistent with the previous report of the *in vitro* experiment in which PIO up-regulated AFABP mRNA expression in a bladder cancer cell line derived from a high-grade tumor in humans (Boiteux et al., 2009). The AFABP expression was also high in the PA group while the acid-forming diet itself did not cause any changes in PPARs and AFABP in comparisons between the CC and CA groups and between the PC and PA groups, suggesting that the suppressive effects of $NH_4Cl$ against the advanced proliferative lesions are not due to modification of the PPARγ agonistic effects of PIO in the urinary bladder mucosa as was the case in muraglitazar (Achanzar et al., 2007). The expression levels of AFABP protein and/or mRNA in humans were high in normal bladder urothelium and low-grade transitional cell carcinomas, while the levels were markedly low in neoplasms of advanced grades (Boiteux et al., 2009; Celis et al., 1996; Gromova et al., 1998). Moreover, PPARγ is not responsible for the loss of AFABP in invasive urothelial carcinomas in humans (Ohlsson et al., 2005). The increased expression of AFABP mRNA by PIO in our study suggested that the mechanisms supportive of the reported relationship between decrease or loss of AFABP and tumorigenicity in human bladder cannot be applied to the mode of action in the rat bladder tumors induced by PIO. CDK1 and Cyclin B had been selected as sensitive markers for detection of proliferative changes in the urinary bladder epithelium induced by subacute treatment with acetazolamide in our preliminary study (data not shown), but the results in the present study did not always match the histopathologic findings and the potential of these genes as cell proliferation markers could not be validated in a steady state after chronic exposure. Expressions of PPARα and PPARγ were decreased by PIO in the present study, and the same decreases were confirmed after a short treatment with acetazolamide in rats (data not shown). Thus, these PPAR genes in the urinary bladder mucosa may be suppressed in a non-specific fashion when cells are in the process of proliferation regardless of the PPAR agonistic activity of the test articles. These data support the formation of urinary solids as the mode of action for PIO-induced bladder proliferative lesions, rather than a direct effect of PIO on the PPAR receptors in the urothelium.

In conclusion, the present study provides strong evidence that the increased incidences and degree of the advanced proliferative lesions, i.e. carcinoma, papilloma and nodular and papillary hyperplasia, in male rats receiving PIO were significantly suppressed by the acid-forming diet. Based on the collective data currently available, it is most likely that the crystalluria hypothesis is applicable to the developmental sequence of the proliferative bladder lesions accelerated by PIO.

## Conflict of interest statement

The authors declare that there are no conflicts of interest which may influence interpretations of the data.

## Acknowledgments

The study was founded by Takeda Pharmaceutical Company Limited. We acknowledge Dr. Samuel M. Cohen, Department of Pathology and Microbiology, University of Nebraska Medical Center, for his advice on the experimental design, technical procedures, scientific interpretation of our data and possible mode of action in urothelial tumorigenesis. We also acknowledge Kevin R. Isaacs, an independent consultant in toxicological pathology, for his contribution as a third party reviewing pathologist.

## 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., Foster, W.R., Burgun, J.J., Car, B.D., Cosma, G.N., Dominick, M.A., 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.

Boiteux, G., Lascombe, I., Roche, E., Plissonnier, M.L., Clairotte, A., Bittard, H., Fauconnet, S., 2009. A-FABP, a candidate progression marker of human transitional cell carcinoma of the bladder, is differentially regulated by PPAR in urothelial cancer cells. Int. J. Cancer 124, 1820–1828.

Burin, G.J., Gibb, H.J., Hill, R.N., 1995. Human bladder cancer: evidence for a potential irritation-induced mechanism. Food Chem. Toxicol. 33, 785–795.

Celis, J.E., Østergaard, M., Basse, B., Celis, A., Lauridsen, J.B., Ratz, G.P., Andersen, I., Hein, B., Wolf, H., Orntoft, T.F., Rasmussen, H.H., 1996. Loss of adipocyte-type fatty acid binding protein and other protein biomarkers is associated with progression of human bladder transitional cell carcinomas. Cancer Res. 56, 4782–4790.

Chopra, B., Hinley, J., Oleksiewicz, M.B., Southgate, J., 2008. Trans-species comparison of PPAR and RXR expression by rat and human urothelial tissues. Toxicol. Pathol. 36, 485–495.

Cohen, S.M., 1995. Role of urinary physiology and chemistry in bladder carcinogenesis. Food Chem. Toxicol. 33, 715–730.

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., Cano, M., Garland, E.M., St, John M., Arnold, L.L., 1995a. Urinary and urothelial effects of sodium salts in male rats. Carcinogenesis 16, 343–348.

Cohen, S.M., Garland, E.M., Cano, M., St John, M.K., Khachab, M., Wehner, J.M., Arnold, L. L., 1995b. Effects of sodium ascorbate, sodium saccharin and ammonium chloride on the male rat urinary bladder. Carcinogenesis 16, 2743–2750.

Cohen, S.M., Cano, M., Anderson, T., Garland, E.M., 1996. Extensive handling of rats leads to mild urinary bladder hyperplasia. Toxicol. Pathol. 24, 251–257.

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

De Groot, A.P., Feron, V.J., Immel, H.R., 1988. Induction of hyperplasia in the bladder epithelium of rats by a dietary excess of acid or base: implications for toxicity/carcinogenicity testing. Food Chem. Toxicol. 26, 425–434.

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., Schilling, B.E., 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.

Drugs@FDA, 1999. The drug approval package for Actos (pioglitazone hydrochloride) tabletsApplication No. 021073 http://www.accessdata.fda.gov/drugsatfda_docs/nda/99/021073A_Actos.cfm1999.

Durand-Cavagna, G., Owen, R.A., Gordon, L.R., Peter, C.P., Boussiquet-Leroux, C., 1992. Urothelial hyperplasia induced by carbonic anhydrase inhibitors (CAIs) in animals and its relationship to urinary Na and pH. Fundam. Appl. Toxicol. 18, 137–143.

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

Fujii, T., Nakamura, K., Hiraga, K., 1987. Effects of pH on the carcinogenicity of o-phenylphenol and sodium o-phenylphenate in the rat urinary bladder. Food Chem. Toxicol. 25, 359–362.

Fukushima, S., Shibata, M.A., Shirai, T., Tamano, S., Ito, N., 1986. Roles of urinary sodium ion concentration and pH in promotion by ascorbic acid of urinary bladder carcinogenesis in rats. Cancer Res. 46 (4 Pt 1), 1623–1626.

Gromova, I., Gromov, P., Wolf, H., Celis, J.E., 1998. Protein abundance and mRNA levels of the adipocyte-type fatty acid binding protein correlate in non-invasive and invasive bladder transitional cell carcinomas. Int. J. Oncol. 13, 379–383.

Guan, Y., Zhang, Y., Davis, L., Breyer, M.D., 1997. Expression of peroxisome proliferator-activated receptors in urinary tract of rabbits and humans. Am. J. Physiol. 273 (6 Pt 2), F1013–F1022.

Guan, Y.F., Zhang, Y.H., Breyer, R.M., Davis, L., Breyer, M.D., 1999. Expression of peroxisome proliferator-activated receptor gamma (PPARgamma) in human transitional bladder cancer and its role in inducing cell death. Neoplasia 1, 330–339.

Hard, G.C., 1995. Species comparison of the content and composition of urinary proteins. Food Chem. Toxicol. 33, 731–746.

Hardisty, J.F., Anderson, D.C., Brodie, S., Cline, J.M., Hahn, F.F., Kolenda-Roberts, H., Lele, S.M., Lowenstine, L.J., 2008. Histopathology of the urinary bladders of cynomolgus monkeys treated with PPAR agonists. Toxicol. Pathol. 36, 769–776.

IARC Working Group, 1999. Consensus Report. In: Capen, C.C., Dybing, E., Rice, J.M., Wilbourn, J.D. (Eds.), Species differences in thyroid, kidney and urinary bladder carcinogenesis. : IARC Scientific Publications No. 147. International Agency for Research on Cancer, Lyon, pp. 1–14.

Kawakami, S., Arai, G., Hayashi, T., Fujii, Y., Xia, G., Kageyama, Y., Kihara, K., 2002. PPARgamma ligands suppress proliferation of human urothelial basal cells *in vitro*. J. Cell. Physiol. 191, 310–319.

Lina, B.A., Kuijpers, M.H., 2004. Toxicity and carcinogenicity of acidogenic or alkalogenic diets in rats; effects of feeding $NH_4Cl$, $KHCO_3$ or KCl. Food Chem. Toxicol. 42, 135–153.

Long, G.G., Reynolds, V.L., Lopez-Martinez, A., Ryan, T.E., White, S.L., 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., 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.

Messana, I., Forni, F., Ferrari, F., Rossi, C., Giardina, B., Zuppi, C., 1998. Proton nuclear magnetic resonance spectral profiles of urine in type II diabetic patients. Clin. Chem. 44, 1529–1534.

Nakashiro, K.I., Hayashi, Y., Kita, A., Tamatani, T., Chlenski, A., Usuda, N., Hattori, K., Reddy, J.K., Oyasu, R., 2001. Role of peroxisome proliferator-activated receptor gamma and its ligands in non-neoplastic and neoplastic human urothelial cells. Am. J. Pathol. 159, 591–597.

Ohlsson, G., Moreira, J.M., Gromov, P., Sauter, G., Celis, J.E., 2005. Loss of expression of the adipocyte-type fatty acid-binding protein (A-FABP) is associated with progression of human urothelial carcinomas. Mol. Cell. Proteomics 4, 570–581.

Oleksiewicz, M.B., Thorup, I., Nielsen, H.S., Andersen, H.V., Hegelund, A.C., Iversen, L., Guldberg, T.S., Brinck, P.R., Sjogren, I., Thinggaard, U.K., Jørgensen, L., Jensen, M.B., 2005. Generalized cellular hypertrophy is induced by a dual-acting PPAR agonist in rat urinary bladder urothelium *in vivo*. Toxicol. Pathol. 33, 552–560.

Olson, M.J., Johnson, J.T., Reidy, C.A., 1990. A comparison of male rat and human urinary proteins: implications for human resistance to hyaline droplet nephropathy. Toxicol. Appl. Pharmacol. 102, 524–536.

Oyasu, R., 1995. Epithelial tumours of the lower urinary tract in humans and rodents. Food Chem. Toxicol. 33, 747–755.

Rodent Bladder Carcinogenesis Working Group, 1995. Urinary bladder carcinogenesis: implications for risk assessment. Food Chem. Toxicol. 33, 797–802.

Suzuki, S., Arnold, L.L., Pennington, K.L., Kakiuchi-Kiyota, S., Wei, M., Wanibuchi, H., Cohen, S.M., 2010. Effects of pioglitazone, a peroxisome proliferator-activated receptor gamma agonist, on the urine and urothelium of the rat. Toxicol. Sci. 113, 349–357.

Van Doorn, M., Vogels, J., Tas, A., van Hoogdalem, E.J., Burggraaf, J., Cohen, A., van der Greef, J., 2006. Evaluation of metabolite profiles as biomarkers for the pharmacological effects of thiazolidinediones in Type 2 diabetes mellitus patients and healthy volunteers. Br. J. Clin. Pharmacol. 63, 562–574.

Van Vleet, T.R., White, M.R., Sanderson, T.P., Cohen, S.M., Cano, M., Arnold, L.L., Waites, C.R., Schilling, B.E., Mitroka, J., Dominick, M.A., 2007. Subchronic urinary bladder effects of muraglitazar in male rats. Toxicol. Sci. 96, 58–71.

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

Wall, I., Tiselius, H.G., 1990. Studies on the crystallization of magnesium ammonium phosphate in urine. Urol. Res. 18, 401–406.

Yoshimura, R., Matsuyama, M., Segawa, Y., Hase, T., Mitsuhashi, M., Tsuchida, K., Wada, S., Kawahito, Y., Sano, H., Nakatani, T., 2003. Expression of peroxisome proliferator-activated receptors (PPARs) in human urinary bladder carcinoma and growth inhibition by its agonists. Int. J. Cancer 104, 597–602.