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Rosiglitazone, a PPAR gamma agonist: Potent promoter of hydroxybutyl(butyl)nitrosamine-induced urinary bladder cancers

Ronald A. Lubet[1]*, Susan M. Fischer[2], Vernon E. Steele[1], M. Margaret Juliana[3], Renee' Desmond[4] and Clinton J. Grubbs[5]

[1]*Division of Cancer Prevention, National Cancer Institute, Bethesda, MD*
[2]*Science Park-Research Division, University of Texas M.D. Anderson Cancer Center, Smithville, TX*
[3]*Department of Genetics, University of Alabama at Birmingham, Birmingham, AL*
[4]*Department of Biostatistics, University of Alabama at Birmingham, Birmingham, AL*
[5]*Department of Surgery, University of Alabama at Birmingham, Birmingham, AL*

In an initial study to determine if rosiglitazone had chemopreventive activity, Fischer-344 female rats were administered twice weekly doses of hydroxybutyl(butyl)nitrosamine (OH-BBN), a urinary bladder specific carcinogen, for 8 weeks. Two weeks following the last dose of OH-BBN, rats were administered rosiglitazone (50 mg/kg BW) daily by gavage for the remainder of the study (7 months). Only 57% of OH-BBN-treated animals developed palpable urinary bladder cancers during the course of the study, while all of the OH-BBN plus rosiglitazone treated rats developed large cancers ($p < 0.01$). Surprisingly, examination for PPAR gamma by immunohistochemistry in the urinary bladders of rats showed that while untreated bladder urothelium and preneoplastic lesions clearly expressed PPAR gamma, frank carcinomas exhibited significantly lower levels. This was confirmed by employing microarray studies of the same samples. In additional studies, lower doses of rosiglitazone (10, 2 and 0.4 mg/kg BW/day) were administered. The 10 mg/kg BW/day dose greatly enhanced bladder cancer incidence ($p < 0.01$). The dose of 2 mg/kg BW/day, which is roughly equivalent to a standard human dose, also significantly increased bladder cancer incidence (controls, 48%; rosiglitazone-treated, 84%). The lowest dose did not significantly increase tumor incidence (rosiglitazone at 0.4 mg/kg BW/day, 64%) or tumor weight in the rats, although there was a trend in that direction. Rosiglitazone alone (10 mg/kg BW/day) given in the absence of OH-BBN did not result in bladder cancer formation when given for 10 months. In summary, rosiglitazone over a wide dose range enhanced urinary bladder carcinogenesis in the OH-BBN model in rats.

Key words: rosiglitazone; urinary bladder; cancer; PPAR gamma agonist

The organ-specific carcinogen 4-hydroxybutyl(butyl)nitrosamine (OH-BBN) was observed over 30 years ago to induce urinary bladder cancers in rodents.[1] The tumors have a mixed histology with mostly transitional cell cancers and a smaller percent of squamous cell or mixed carcinomas.[2] Studies have shown that these tumors have a variety of gene expression changes routinely associated with bladder cancer in humans. These include increased levels of survivin and decreased levels of FHIT as determined by immunohistochemistry,[3,4] and various S100 proteins, cell cycle-related genes and increased expression of genes associated with the EGFr and VEGF pathways as determined by gene microarrays.[5] The rodent cancer models have been used for several decades to evaluate potential chemopreventive agents against urinary bladder cancers.[1,2] The class of agents that has undergone the most complete examination are the COX inhibitors.[6–8] A wide variety of NSAIDS (piroxicam, indomethacin), as well as the selective COX-2 inhibitor celecoxib, have proven to be highly effective in inhibiting the formation of OH-BBN induced urinary bladder cancer. Additionally, our laboratories have also found that tea polyphenols[9] and certain EGFr inhibitors[10] are also quite effective in this model.

Multiple nuclear receptors with unknown ligands were identified approximately 15 years ago and were designated orphan receptors. Among the orphan receptors were receptors-designated PPAR (peroxisome proliferator activated receptors) that were activated by a series of agents which induce the proliferation of peroxisomes in liver tissue.[11] The PPARs actually form heterodimers with the RXR family of receptors. The resulting heterodimers when combined with their cognate ligands, for both the PPARs and RXRs, act as transcriptional activators of a wide variety of different genes. Interestingly, RXR agonists have been shown to be profoundly active chemopreventive agents both in ER^+ and ER^- models of breast cancer in rodents,[12,13] and a PPAR gamma agonist GW7845 was effective in an ER^+ model of breast cancer.[14] While screening for the potential chemopreventive properties of PPAR gamma agonists in this bladder model, we reported 4 years ago that a relatively high dose of the PPAR agonist rosiglitazone (Avandia) promoted bladder cancer formation in the OH-BBN induced rat bladder tumor model.[15] At that time, the FDA was reporting that a number of recently synthesized PPAR gamma and PPAR gamma/alpha agonists were themselves inducing bladder tumors in rats or mice or in both species (www.fda.gov/cder/present/DIA/2004/Elhage.ppt). The present study expands our initial data and demonstrates that much lower doses of rosiglitazone also have significant tumor promoting activity in this model. The receptor for this agent (PPAR gamma), although highly expressed in normal bladder urothelium and pre-neoplastic lesions, was expressed at low levels in urinary bladder cancers.

Material and methods

For all studies, female Fischer-344 rats were obtained from Harlan Sprague–Dawley, Inc. (Indianapolis, IN) at 4 weeks of age and were housed in polycarbonate cages (5 per cage). The animals were kept in a specially designed containment facility for studies using chemical carcinogens. The rooms were lighted 12 hours/day and maintained at 22 ± 2°C. Teklad diet (Harlan Teklad, Madison, WI) and tap water were provided *ad libitum*. OH-BBN was purchased from TCI America (Portland, OR) and was administered by gavage using an ethanol: water (25:75, v/v) vehicle. Each gavage was 150 mg OH-BBN in 1.0 ml. Rosiglitazone was supplied by the National Cancer Institute/Division of Cancer Prevention Repository. The vehicle for gavaging rosiglitazone was 0.5% carboxymethylcellulose: polyethylene glycol 400 (50:50, v/v) and the volume of each gavage was 0.5 ml.

During the studies, all rats were weighed 1×/week and palpated for urinary bladder tumors 2×/week. Rats were sacrificed when

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***Correspondence to:** National Cancer Institute, Executive Plaza North, Suite 2110, 6130 Executive Blvd., Bethesda, MD 20852.
Fax: 301-402-0553. E-mail: lubetr@mail.nih.gov
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TABLE I – EFFECT OF ROSIGLITAZONE (HIGH DOSE) ON OH-BBN INDUCED URINARY BLADDER CANCERS (EXPERIMENT I)

Group	No. of rats	Carcinogen[1]	Treatment[2]	Incidence of urinary bladder cancers[3]
1	34	OH-BBN	Rosiglitazone, 50 mg/kg BW/day	100[4]
2	35	OH-BBN	None	57

[1]OH-BBN was administered by gavage (2×/week for 8 weeks) to female Fischer-344 rats beginning at 56 days of age.–[2]Rosiglitazone administration was initiated two weeks after the final carcinogen treatment.–[3]Study was terminated 9 months after the initial OH-BBN treatment. Urinary bladder cancers were defined as those >5 mm in diameter and that filled >50% of the urinary bladder lumen.–[4]Statistically different from Group 2; $p < 0.01$.

they developed a large palpable bladder mass or were observed to have bloody urine. At necropsy, urinary bladders were excised, weighed and inflated with 10% buffered formalin and were observed under a high intensity light for gross lesions. Each lesion was dissected and processed for pathologic classification. Final urinary bladder cancer incidence was analyzed by the Fischer's exact test, and weights were analyzed using chi square as previously described.[6,9] Time of appearance of urinary bladder cancers were evaluated using Kaplan–Meier analysis.

Experiment I

OH-BBN was administered by gavage (2×/week) for 8 weeks beginning when the rats were 56 days of age. Two weeks after the last OH-BBN treatment (rats were 125 days of age), administration of rosiglitazone was initiated and continued until the end of the study. Rosiglitazone was given by gavage at a dose of 50 mg/kg BW/day, 7 days/week.

Immunohistochemistry for PPAR gamma

Bladders from untreated, OH-BBN treated and tumor-bearing rats were fixed in formalin overnight and transferred to 70% ethanol prior to embedding in paraffin. Sections were cut and deparaffinized and endogenous peroxidase activity was blocked with 3% H_2O_2. Antigen retrieval was achieved by microwaving in 10 mM citrate buffer for 10 minutes. Nonspecific antibody binding was blocked with 10% rabbit serum in PBS. Following washing, the sections were incubated with PPAR gamma primary antibody (Santa Cruz sc-7273) at 1:200 dilution for 1 hour at room temperature. Washed slides were then incubated with a 1:250 dilution of biotinylated rabbit anti-mouse antibody (Accurate Chemical) for 15 minutes. Staining was achieved with streptavidin horseradish peroxidase and 3,3′-diaminobenzidine (Dako) treatment. No staining was observed when the primary antibody was omitted.

PPAR gamma expression RNA studies

The RNA data comparing PPAR gamma expression in normal bladder epithelium vs bladder cancers (Fig. 3) is based on our prior published paper.[5] In brief, bladder epithelium was physically separated by scraping the epithelium from the underlying muscle and stroma. Total RNA from normal bladder epithelium and bladder tumors were isolated by Trizol cDNA for each sample. CDNA for each sample was synthesized using a Superscript cDNA Synthesis Kit (Invitrogen) and a T7-(dT)24 primer: Then, the biotin-labeled cRNA was transcribed *in vitro* and repurified. The labeled cRNA was applied to the Affymetrix Rat 230 2.0 GeneChips (Affymetrix, Santa Clara, CA); every gene or EST is represented by a probe set consisting of approximately 16 probe pairs (oligonucleotides) of 25-mer oligonucleotides. Array normalization and gene expression estimates were obtained using Affymetrix Microarray Suite 5.0 software (MAS5). The array mean intensities were scaled to 1,500. These estimates formed the basis for statistical testing. Differential expression was determined using the combined basis of *t*-test.

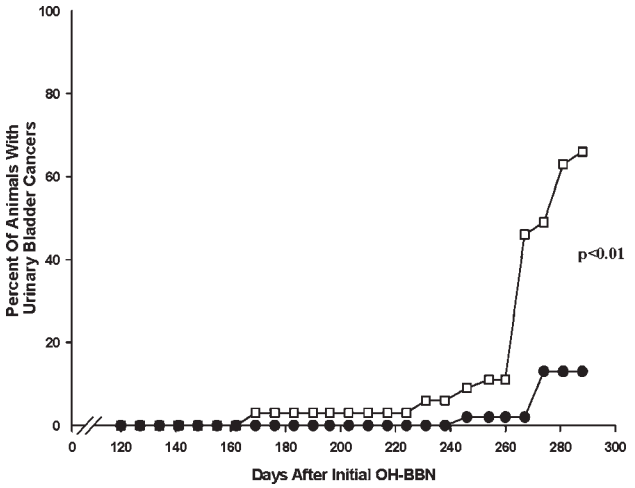

FIGURE 1 – Percent of animals that developed a palpable urinary bladder cancer during the evaluation of rosiglitazone at a high dose (Experiment I). The initial dose of OH-BBN was given to Fischer-344 rats at 56 days of age and rosiglitazone was started 2 weeks after the last dose of OH-BBN (or at 125 days of age). The groups were: □, rosiglitazone (50 mg/kg BW/day); ●, none.

Experiment II

Beginning when the rats were 56 days of age, OH-BBN was given by gavage (2×/week) for 8 weeks. Rosiglitazone treatment was started 2 weeks after the last OH-BBN treatment and continued until the end of the study. The compound was administered 10, 2 or 0.4 mg/kg BW/day, 7 days/week. The group receiving the high dose of rosiglitazone was terminated at 8 months after the initial OH-BBN treatment because most of the animals had been sacrificed with large urinary bladder tumors. A concurrent control was sacrificed simultaneously with this group. The groups receiving the 2 lower doses of rosiglitazone were not terminated until 10 months after the initial carcinogen treatment. In addition, a single group of rats were treated with rosiglitazone (10 mg/kg BW/day) for a period of 10 months without any carcinogen treatment. These animals were sacrificed at the same time as the animals receiving the 2 lower doses of rosiglitazone in OH-BBN treated rats.

Results

In Experiment I, rats were administered a high dose of rosiglitazone (50 mg/kg BW/day) beginning 2 weeks after the last OH-BBN treatment. Of the rats treated only with the carcinogen, 7/35 developed palpable masses during the course of the study, while 20/35 had large urinary bladder masses at the end of the study. These palpable masses were subsequently diagnosed as transitional cell carcinomas (Table I). However, in the group that also received rosiglitazone, 23/34 developed palpable tumors during the course of the study, while all 34 rats in this group had developed large urinary bladder cancers by the end of the study (Fig. 1). The majority of the rats in the rosiglitazone-treated group were sacrificed prior to the scheduled end of the study. Although all lesions in the urinary bladder were histologically examined, the actual number of tumors in the bladder of rats in each group could not be determined because the large cancers made it difficult to score additional lesions.[9]

Expression of the PPAR gamma receptor in normal urothelium and in OH-BBN-induced bladder lesions was examined both by immunohistochemistry (Fig. 2) and microarray analysis (Fig. 3). Both revealed higher levels of expression in the normal epithelium as contrasted with bladder cancers. It also appeared that PPAR gamma staining was higher in bladder tissues from rats receiving rosiglitazone when compared to that from controls (Fig. 2).

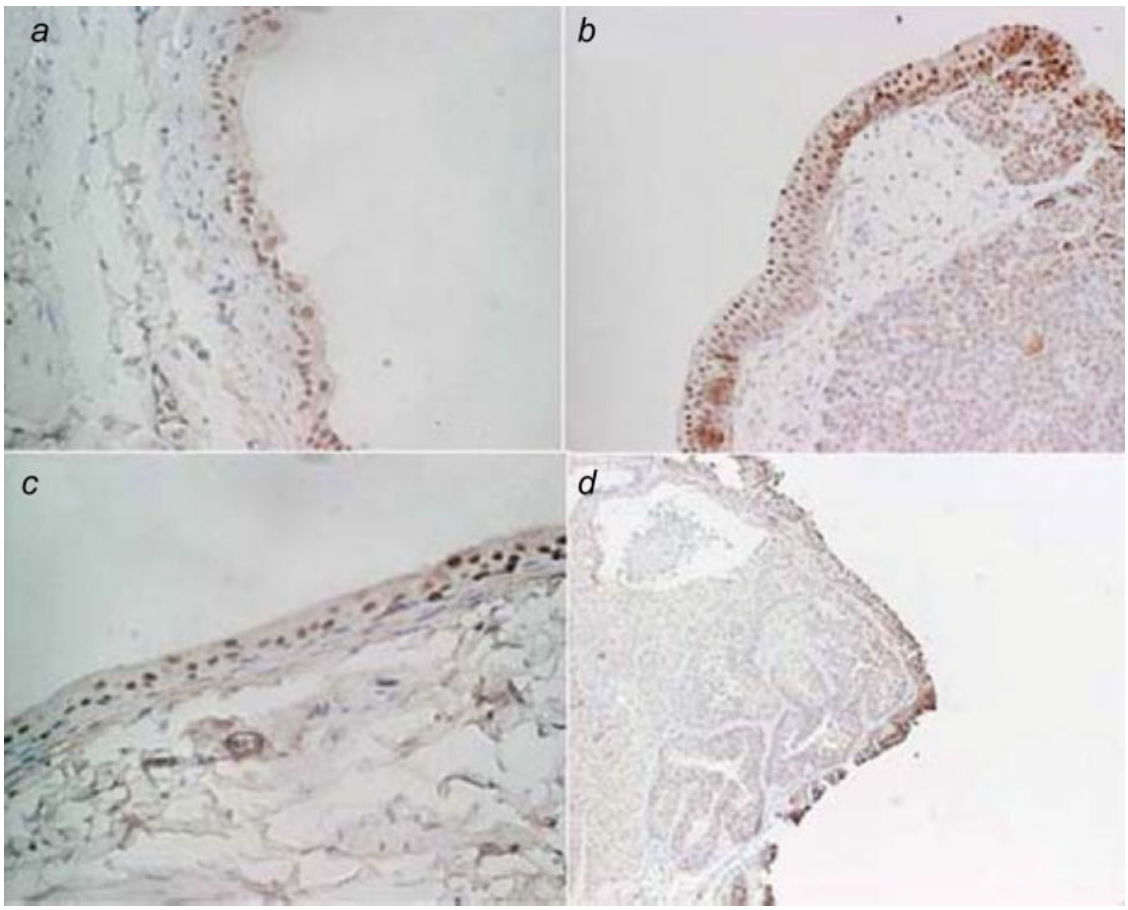

FIGURE 2 – PPAR gamma expression in rat urinary bladder tissues. Formalin-fixed paraffin sections of normal rat bladders or bladder cancers were immunostained for PPAR gamma. (*a*) Normal bladder epithelium (40×). Note strong nuclear staining. (*b*) Bladder epithelium from rosiglitazone treated rat (40×). (*c*) OH-BBN induced bladder cancer from rosiglitazone treated rat (40×). (*d*) Bladder cancer from carcinogen-only treated rat (20×). Cancer expression of PPAR gamma is greatly reduced relative to overlying epithelium.

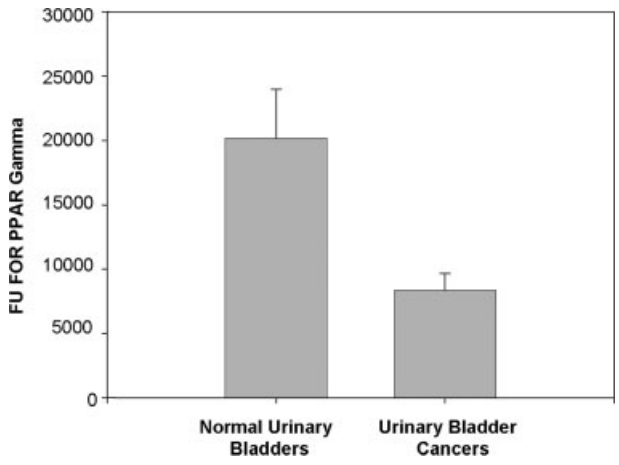

FIGURE 3 – PPAR gamma expression in rat urinary bladder cancers as determined by microarray analysis. Frozen normal rat bladders or bladder cancers were processed for RNA analysis as described in Material and Methods. RNA analysis employing Affymatrix rat microarrays and the determination of fluorescence units for PPAR gamma receptor were as described by Yao et al.[5] Comparison of expression in normal urinary bladders and bladder cancers was performed using the student *t*-test. Expression was higher in normal bladders than in bladder cancers ($p < 0.01$).

Experiment II was conducted to determine if lower doses of rosiglitazone would also alter the incidence of urinary bladder cancers induced by OH-BBN. As indicated in Table II and Fig. 4*a*, the highest dose of this agent (10 mg/kg BW/day) had a highly significant effect on the growth of the urinary bladder cancers; increasing both the incidence and the weights of the resulting bladder tumors. The weight of the normal urinary bladder is approximately 90 mg. At 8 months after the initial carcinogen treatment, rats on the 10 mg/kg BW/day dose of rosiglitazone and concurrent controls were sacrificed. At that time the palpable tumor incidence was 28% in the OH-BBN controls and 93% in the rats treated with rosiglitazone, while the tumor weights (urinary bladder plus tumors) had increased to 157 mg in the carcinogen treated group and 594 mg in the carcinogen plus rosiglitazone group. The 2 mg/kg BW/day dose level of rosiglitazone, which is slightly greater than a standard human dose, also caused increases in both the incidence of urinary bladder cancers [controls, 48%; rosiglitazone (2 mg/kg BW/day), 80% ($p < 0.05$)] and their weight (Fig. 4*b*, Table II). The 0.4 mg/kg BW/day dose of rosiglitazone caused nonsignificant increases in the incidence and weight of the bladder cancers. However, there was a trend toward increases in both tumor incidence (67% treated vs 48% controls) and tumor weight (713 mg treated vs 557 mg controls) at this dose. In this study, the urinary bladders of rats not treated with the carcinogen but receiving rosiglitazone at 10 mg/kg BW/day for 10 months were histologically evaluated (Table II). The urothelium of these animals was normal and the weight of the bladders did not differ from controls.

TABLE II – EFFECTS OF ROSIGLITAZONE (LOWER DOSES) ON OH-BBN INDUCED URINARY BLADDER CANCERS (EXPERIMENT II)

Group	No. of rats	Carcinogen[1]	Treatment[2]	Time of sacrifice[3]	Incidence of urinary bladder cancers[4]	Weight of bladder plus tumors (mg)[5]
1	30	OH-BBN	Rosiglitazone, 10 mg/kg BW/day	8 months	93[6]	594 (278%↑)[6]
2	29	OH-BBN	None	8 months	28	157
3	30	OH-BBN	Rosiglitazone, 2 mg/kg BW/day	10 months	80[7]	936 (68%↑)[7]
4	29	OH-BBN	Rosiglitazone, 0.4 mg/kg BW/day	10 months	67	713 (28%↑)
5	25	OH-BBN	None	10 months	48	557
6	10	–	Rosiglitazone, 10 mg/kg BW/day	10 months	0	91 (1%↑)
7	10	–	None	10 months	0	90

[1]OH-BBN was administered by gavage (2×/week for 8 weeks) to female Fischer-344 rats beginning at 56 days of age.–[2]Rosiglitazone administration was initiated 2 weeks after the final carcinogen treatment.–[3]The groups were sacrificed either 8 months or 10 months after the initial OH-BBN treatment.–[4]Incidence of large urinary cancers; defined as those >5 mm in diameter and that filled >50% of the bladder lumen.–[5]Average weight of urinary bladder plus tumors at time of sacrifice of the rats. Number in parenthesis is percent difference from appropriate controls.–[6]Statistically different from respective control groups; $p < 0.01$.–[7]Statistically different from respective control groups. $p < 0.05$.

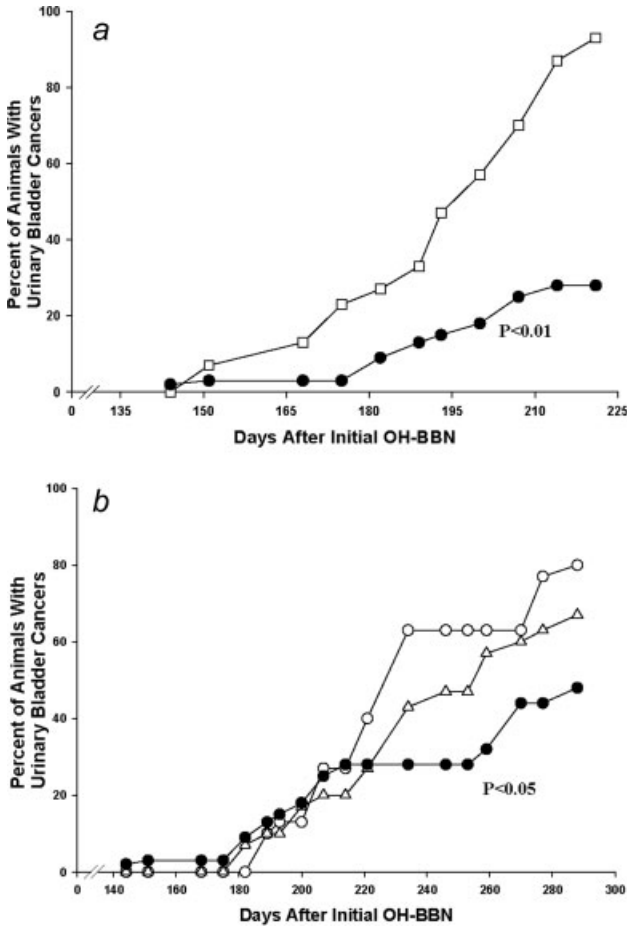

FIGURE 4 – Percent of animals that developed a palpable urinary bladder cancer during the evaluation of rosiglitazone at lower doses (Experiment II). (*a*) Rosiglitazone administered at 10 mg/kg BW/day; study terminated at 8 months after the initial OH-BBN treatment. The groups were: □, rosiglitazone (10 mg/kg BW/day); ●, None. (*b*) rosiglitazone administered at 2 and 0.4 mg/kg BW/day; study terminated at 10 months after the initial OH-BBN treatment. The groups were: O, rosiglitazone (2 mg/kg BW/day); Δ, rosiglitazone (0.4 mg/kg BW/day); and ●, none.

Discussion

Our laboratories and others have employed the OH-BBN-induced rat urinary bladder cancer model to examine the preventive efficacy of a wide variety of agents. As mentioned in the Introduction, the resulting cancers display a mixed transitional cell and squamous cell histology.[1,2] These cancers have undergone molecular characterization; for example, our laboratories have recently shown that FHIT expression[3] is decreased and that there is an increased expression of survivin.[4] These proteins are similarly altered in expression in human urinary bladder cancers. Furthermore, using affymatrix microarrays we found a wide variety of genes whose expression was increased when comparing bladder cancers and normal urothelium,[5] including a variety of genes related to the EGFr and VEGF pathways and genes involved in the cell cycle and proliferation (e.g., Ki67, cyclin DI, myc). As shown in Table I, the control rats in the initial study developed bladder cancers that were observed either during the course of the experiment (20%) or at the time of sacrifice (37%). Therefore, 57% of all control rats developed large urinary bladder cancers. These tumors were quite large (>5 mm in diameter), typically filled at least 50% of the urinary bladder lumen and weighed greater than 200 mg.

The results with rosiglitazone, the PPAR gamma agonist and antidiabetic agent,[16] were unexpected. Rosiglitazone in Experiment I caused a decrease in cancer latency (Fig. 1) and a large increase in cancer incidence (Table I). Furthermore, only 20% of control rats, but 70% of the rosiglitazone treated rats, had to be sacrificed prior to the end of the study because of development of the bladder cancers. This implies that rosiglitazone is a strong tumor promoter in this model; yielding increases in bladder cancers within 2 1/2 months of the time initially administered.

On the basis of these observations, a separate small study (10 rats/group) was performed in which rats that had been treated with OH-BBN received rosiglitazone beginning 5.5 months following the first dose of OH-BBN (data not shown). Rosiglitazone treatment was, therefore, initiated approximately 65% of the way into the experiment when rats would be expected to have microinvasive cancers but virtually no palpable tumors. Within 3 months, all of the rats treated with OH-BBN plus rosiglitazone had developed palpable bladder tumors while only 20% of rats treated with OH-BBN alone developed palpable tumors. These data imply that rosiglitazone is an effective promoting agent even when given late in the carcinogenic process. The dose of rosiglitazone employed (50 mg/kg BW/day) did not cause any deaths or cause a decrease in body weights. However, this dose did cause an increase in the size of the heart; increasing heart/body weight ratios by 15%.

Since the previous dose of rosiglitazone employed (50 mg/kg BW/day) was considerably higher than the human equivalent, lower doses were subsequently evaluated (Experiment II). The typical dose of rosiglitazone in a human is equivalent in rats to approximately 1.5 mg/kg BW/day. Thus, rosiglitazone at 10, 2 and 0.4 mg/kg BW/day were tested. These doses of rosiglitazone caused a dose dependent increase in the formation of palpable urinary bladder cancers. Because of the large increase in bladder tumors in rats treated with OH-BBN and 10 mg/kg BW/day of rosiglitazone, it was necessary to sacrifice those rats and a concurrent control group at 8 months following the first dose of

OH-BBN. At that time, 93% of rats treated with this dose of rosiglitazone but only 28% of control rats had developed large tumors. The rats treated with lower doses of rosiglitazone (2 or 0.4 mg/kg BW/day) and a concurrent control were not sacrificed until 10 months after the last dose of OH-BBN. This resulted in a urinary bladder cancer incidence of 48% in the controls by the end of the experiment, and an incidence of 67% and 80% in rats exposed to 0.4 or 2 mg/kg BW/day of rosiglitazone, respectively. Although the 0.4 mg/kg BW/day dose, much lower than the human equivalent dose, did not cause a significant increase in tumor incidence or weight, a trend toward increases was observed. In contrast, when rats were exposed to rosiglitazone (10 mg/kg BW/day) alone, in the absence of any OH-BBN, for a period of 10 months (Table II), bladder cancers or other pathological lesions were not observed.

Because of the significant effects of rosiglitazone on cancer development and growth, the expression of PPAR gamma in normal bladder epithelium, hyperplastic lesions and established cancers was examined (Figs. 2 and 3). By immunohistochemistry, PPAR gamma was clearly expressed in normal urothelium and in the hyperplastic areas of bladders from rats treated with OH-BBN. Unexpectedly, lower levels of PPAR gamma were observed in established bladder cancers. We confirmed this decrease in PPAR gamma expression between bladder tumors and normal bladder epithelium using microarray data (Fig. 3). One might expect that the most striking effects of rosiglitazone would be during the earlier stages of bladder cancer development; potentially increasing growth of microscopic lesions. Therefore, it is somewhat surprising that late intervention with rosiglitazone also greatly increased the growth of palpable tumors.

Our data showing strong promoting activity, but no apparent activity as a complete carcinogen, might imply that it is only a tumor promoter that may be highly specific for the OH-BBN urinary bladder cancer model in rats. Note that many of the carcinogenic effects of the PPAR receptor agonists (e.g., PPAR alpha in liver) are highly species specific; i.e., observed in rodents but not in humans or primates.[17,18] However, more recent data suggest that certain of the highly potent PPAR alpha and gamma agonists administered alone in chronic carcinogenicity studies induce bladder tumors in rats and/or mice (www.fda.gove/cder/present/DIA2004/Elhage.ppt). An article examining the health effects of the PPAR gamma agonist pioglitazone was recently published.[19] Treatment with pioglitazone was effective in blocking the effects of diabetes, and overall did not alter the incidence of cancers. However, it was reported that 14 bladder cancers were observed in the pioglitazone group and only 6 in the controls. This result had borderline statistical significance ($p < 0.07$). Since more than half of the tumors arose within 1 year of study, the authors attributed it to randomization errors, in which case the bladder cancers were 6 and 3 for pioglitazone and controls, respectively; an insignificant difference.[19] The present results clearly support the possibility that the PPAR gamma agonists may promote bladder cancer. However, this apparent increase must be seen contextually with its highly positive effects on diabetes. Although the cause of this promoting effect is not known, it has been shown that PPAR gamma agonists may stimulate the production of angiogenic growth factors[20] as well as increased expression of the transcription factor EGR-1 and phosphorylated c-Jun; both of which may contribute to the oncogenic process.[21] This finding is particularly of interest and warrants further studies. The abstract to the present work[15] was recently discussed in a short review article on PPAR agonists and urinary bladder cancer.[22] The possibility was presented that these effects might be due to a chemical irritant effect (as, for example, previously observed with saccharin).[23] However, our observations do not meet 2 of the hallmarks of those studies. First, we observed the effects in female rats rather than male rats; and it is male rats that are considered to be particularly sensitive to these irritant effects. Second, as mentioned above, we observed these effects quite rapidly (8 weeks) when rosiglitazone was administered late; arguing against a constant long-term irritant effect.

This study yields a number of important findings and leads to 2 interesting questions: (a) are the results unique to the specific model employed? and (b) are the results specific to rosiglitazone? However, data at a FDA Web site implied that a number of the high affinity PPAR agonists, particularly dual PPAR alpha/gamma agonist, are complete carcinogens in rats. The finding that the PPAR gamma receptor is highly expressed in normal bladder epithelium, and even preneoplastic tissue, would appear to be consistent with the promoting effects of rosiglitazone in this model. However, the limited expression of this protein in established bladder cancers seems somewhat contradictory to the ability of rosiglitazone to enhance carcinogenesis, especially when administered late in the process.

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