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Chemopreventive Effects of Nimesulide, a Selective Cyclooxygenase-2 Inhibitor, on the Development of Rat Urinary Bladder Carcinomas Initiated by *N*-Butyl-*N*-(4-hydroxybutyl)nitrosamine[1]

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ABSTRACT

The chemopreventive potential of a selective cyclooxygenase-2 inhibitor, nimesulide (NIM), against the development of rat superficial urinary bladder carcinomas after initiation with *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) was examined. Six-week-old Fischer 344 male rats were given 0.05% BBN in their drinking water for 8 weeks, followed by diets supplemented with 0, 100, 200, or 400 ppm NIM for 12 weeks, and they were then sacrificed. NIM decreased, in a dose-dependent manner, the incidence of transitional cell carcinoma (TCC) to 12 of 20 (60.0%), 8 of 16 (50.0%), and 5 of 19 (26.3%) and the multiplicity of TCCs to 0.75 ± 0.79, 0.56 ± 0.63, and 0.37 ± 0.78 per rat at 100, 200, and 400 ppm, respectively, as compared with the BBN alone group values of 18 of 20 (90.0%) and 2.35 ± 1.23. NIM did not significantly affect the cell differentiation or invasiveness of TCCs. These results indicate clear chemopreventive potential of a selective cyclooxygenase-2 inhibitor against postinitiation development of superficial rat urinary bladder carcinomas.

INTRODUCTION

Epidemiological and experimental studies have revealed that NSAIDs,[3] particularly aspirin, sulindac, and PIRO, are promising candidates for chemopreventive agents active against development of cancers in the colon and, possibly, the urinary bladder, mammary gland, skin, and liver (1–4). The preventive mechanisms remain to be elucidated in detail but have been postulated to involve their inhibition of COX and, thus, production of eicosanoids like PGs, which influence tumor growth either by directly participating in the signal cascade for cell proliferation or by disturbing immunological surveillance (2, 5, 6). Increased levels of PGs in various tumor tissues are, in fact, well known (6). However, the relative importance of COX-1 and COX-2, the two COX isozymes, is a question that needs resolution. COX-2, in contrast to the constitutively expressed COX-1, which contributes to physiological functions in most tissues, is inducible and involved in inflammation and cell proliferation (7). Reportedly, COX-2 is highly expressed in colon, stomach, skin, and mammary tumors (8–12), and its overexpression in rat intestinal epithelial cells renders them resistant to apoptosis (13). Furthermore, double knockout of the COX-2 gene suppresses intestinal polyposis in *APC* gene knockout mice (14). Thus, selective COX-2 inhibitors have attracted a great deal of attention as more effective and safer cancer chemopreventive agents than classical NSAIDs, which preferentially inhibit COX-1, causing adverse effects mainly in gastrointestinal tract, but also affect COX-2, mostly at higher doses (15, 16). Thus far, three selective COX-2 inhibitors, SC-58635, MF tricyclic, and NIM, have been reported to possess chemopreventive potential against colon cancers in rodents (14, 17–19). At present, only NIM is clinically used as a sulfonanilide class NSAID, being less ulcerogenic but having more or less the same anti-inflammatory, analgesic, and antipyretic properties as those of classical NSAIDs (20–24). Nevertheless, their chemopreventive potential against development of cancers in other organs, including urinary bladders, are as yet unknown.

Superficial type human urinary bladder cancers, which are usually low-grade TCCs and easily resectable by transurethral intervention, are found at higher incidences than invasive lesions and with a high frequency of recurrence (25). Recurrent tumors manifest themselves as more aggressive malignancies (25). Although postoperative intravesical instillation of chemotherapeutic agents, such as Adriamycin or the immunotherapeutic agent *Bacillus Calmette-Guerine*, has been performed for prophylaxis (25), development of safer and more effective means to prevent recurrence is warranted.

Chemopreventive potentials have been reported for NSAIDs, including PIRO, sulindac, ketoprofen, and aspirin, against urinary bladder carcinogenesis in rodents (3, 26–28), but the evidence is somewhat equivocal, depending upon experimental protocols and models used (27, 29–31). Urinary bladders are known to possess relatively high COX activity, which is partly involved in distention-induced muscle contraction upon micturition (32), and can contribute to metabolic activation of carcinogens such as *N*-[4-(5-nitro-2-furyl)-2-thiazoyl]formamide (33). Application of tumor promoters elevates PGE_2 levels in the rat bladder (34), whereas NSAIDs block their tumor promotion potential (33, 35). Moreover, COX activity in urothelial cells in culture can be elevated by 12-*O*-tetradecanoylphorbol-13-acetate (36). Although immunoblotting analysis has revealed COX-1 rather than COX-2 protein expression in normal rat urinary bladders (37), the roles of the different COX isozymes in physiological processes and their relevance to carcinogenesis in the urinary bladders are largely unknown. This study was, therefore, performed to assess the preventive potential of a COX-2 inhibitor, NIM, against postinitiation development of superficial bladder cancers in the rat.

MATERIALS AND METHODS

Chemicals. BBN was obtained from Tokyo Kasei Kogyo (Tokyo, Japan), NIM was from Helsinn Healthcare SA (Pambio Noranco, Switzerland), and PIRO was from Sigma Chemical Co. (St. Louis, MO).

Animals, Diet, and Drinking Water. A total of 106 Fischer 344 male rats (Japan SLC Inc., Hamamatsu, Japan), 6 weeks old at the commencement of the experiments, were used. The animals were housed four to a plastic cage, with hardwood chips for bedding, in an air-conditioned room with a 12-h light/12-h dark cycle. Diets containing NIM and PIRO were prepared once a week by

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[3] The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; PIRO, piroxicam; COX, cyclooxygenase; PG, prostaglandin; NIM, nimesulide; TCC, transitional cell carcinoma; BBN, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine; SH, simple hyperplasia; NPH, nodulo-papillary hyperplasia.

Table 1 *Experimental details for rats given BBN for 8 weeks followed by NIM or PIRO for 12 weeks*[a]

Group no.	Treatment	No. of rats (final)	Body weight (g) Initial	Week 9	Week 11	Week 13	Week 15	Week 17	Week 20 (final)	Liver weight (g) (ratio to body weight × 10^{-3})		Average intake Diet (g/day/kg body weight)	Average intake Chemical (mg/day/kg body weight)
1	BBN alone	20	117 ± 18	304 ± 19	314 ± 19	330 ± 20	339 ± 21	357 ± 21	365 ± 23	10.8 ± 0.66	(30.4 ± 1.0)	44.3 ± 3.3	0
2	BBN + 75 ppm PIRO	19	120 ± 6	306 ± 12	317 ± 12	335 ± 12	345 ± 14	368 ± 15	366 ± 15	11.8 ± 0.66[b]	(32.1 ± 1.4)[c]	44.3 ± 7.3	3.32
3	BBN + 100 ppm NIM	20	119 ± 6	303 ± 15	310 ± 17	330 ± 18	340 ± 20	361 ± 20	369 ± 24	12.1 ± 0.97[c]	(32.9 ± 2.1)	43.2 ± 3.4	4.32
4	BBN + 200 ppm NIM	16	118 ± 7	305 ± 13	310 ± 14	326 ± 16	338 ± 16	361 ± 17	366 ± 14	12.1 ± 1.10[c]	(33.2 ± 3.4)[c]	44.9 ± 3.5	8.98
5	BBN + 400 ppm NIM	19	118 ± 5	302 ± 14	308 ± 14	325 ± 15	337 ± 14	356 ± 15	365 ± 15	12.5 ± 0.75[c]	(34.2 ± 1.6)[c]	45.3 ± 3.2	18.12
6	75 ppm PIRO	4	118 ± 2	310 ± 7	322 ± 9	344 ± 9	353 ± 7	377 ± 11	377 ± 8	11.4 ± 0.43	(30.2 ± 0.5)	44.9 ± 3.5	3.37
7	400 ppm NIM	4	121 ± 5	315 ± 18	319 ± 18	339 ± 18	345 ± 20	362 ± 23	364 ± 17	11.7 ± 0.67	(32.1 ± 2.5)	45.3 ± 3.2	18.12

[a] Values are means ± SD. Four rats in group 4 died by an accidental overflow of tap water during the weekend on week 16.
[b] Significantly different from group 1 ($P < 0.01$).
[c] Significantly different from group 1 ($P < 0.05$).

mixing the compounds with CE-2 powdered basal diet (Japan Clea Co., Ltd., Tokyo, Japan), and the mixture was administered using stainless steel containers. Drinking water containing BBN was prepared twice a week by dissolving the carcinogen in distilled water with the aid of Tween 80 (300 μl/10 liters), and was given in light-opaque bottles. The diet and water were available *ad libitum*, and body weights and food consumption were measured weekly.

Experimental Protocol. Animals were divided into seven groups, with 19–20 rats each for groups 1–5 and 4 rats each for groups 6 and 7. Animals in groups 1–5 were given 0.05% BBN in drinking water for the first 8 weeks, and then group 1 was fed the basal diet, group 2 was fed the diet with 75 ppm PIRO, and groups 3–5 were fed the diet with 100, 200, and 400 ppm NIM, respectively, for 12 weeks. The animals in groups 6 and 7 served as controls and were given tap water for the first 8 weeks, followed by 75 ppm PIRO and 400 ppm NIM, respectively. All of the animals were sacrificed under ether anesthesia 20 weeks after the commencement of the experiment. Their urinary bladders were fixed in 10% phosphate-buffered formalin after inflation by intraluminal injection of the fixative. The livers from 8 rats in groups 1–5 and from all 4 rats in groups 6 and 7 were weighed and fixed in the fixative.

Histopathological Examination. The fixed urinary bladders were longitudinally bisected and further transversely cut into six to eight slices. Macroscopically detectable lesions were recorded as a guide for the histological examination. The bladders and livers were routinely embedded in paraffin and sectioned for H&E staining. The bladder lesions were histologically diagnosed basically according to the criteria described by Oyasu *et al.* (38, 39) as SH, NPH, or TCC. TCCs were further classified into three grades in terms of cell differentiation and into Ta, T1, T2, and T3, depending upon the depth of the invasion (28, 38, 39).

Statistical Analysis. Data on body and liver weights, diet intakes, and tumor multiplicities were analyzed for significance of differences between control and NIM diet groups using one-way ANOVA with multiple comparison post hoc tests by Dunnett and between control and PIRO diet groups using Student's *t* test. Data on tumor incidence and classification for invasion and differentiation were analyzed by Fisher's exact test or χ^2 test.

RESULTS

Body and Organ Weights and Food Intake. Experimental details are summarized in Table 1. Mortalities during the experimental period were limited to four rats in group 4, which died accidentally during week 16. Body weights, determined only at critical points, initially, finally, at week 9 when the PIRO and NIM dietary regimens were begun, and every 2 weeks afterward, are shown in Table 1. These values demonstrated no significant intergroup differences. Liver weights in rats given 200 and 400 ppm NIM and 75 ppm PIRO following BBN were significantly increased in terms of absolute values and ratios to body weights as compared with group 1 (BBN alone) values. Average, as well as weekly (data not shown), food intake during the experimental period from week 9 to week 20 did not show significant intergroup differences.

Incidences and Numbers of Urinary Bladder Lesions. Macroscopically, multiple whitish protuberant nodular lesions were observed in the urinary bladders of group 1 (BBN alone). Their numbers and sizes were clearly diminished in all of the groups treated with NIM and PIRO, particularly with the latter and the 400 ppm dose of NIM. Data for incidences and numbers of histologically diagnosed urinary bladder lesions are summarized in Table 2. No significant intergroup differences were observed in the incidences of SH and NPH (Fig. 1*a*). However, NIM decreased, in a dose-dependent manner, both the incidence and multiplicity of TCCs in almost all cases. Seventy-five ppm PIRO also significantly decreased the incidence and multiplicity of TCCs, confirming our previous finding (28). TCCs that developed in this study, were all either Ta (Fig. 1*b*) or T1 (Fig. 1*c*), in terms of depth of invasion, or grade 1 (Fig. 1*b*) or grade 2 (Fig. 1*c*), in terms of differentiation. Neither NIM nor PIRO exerted any sig-

Table 2 *Effects of NIM and PIRO on the development of urinary bladder lesions in rats initiated by BBN*

Group no.	Treatment	Effective no. of rats[a]	Incidence (%)[b] SH	NPH	TCC	No. of TCCs (%) Multiplicity (no. per rat)	Total no. counted	Classified by invasion[c] Ta	T1	Classified by differentiation[d] Grade 1	Grade 2
1	BBN alone	20	20 (100)	20 (100)	18 (90.0)	2.35 ± 1.23	47	6 (12.8)	41 (87.2)	17 (36.2)	30 (63.8)
2	BBN + 75 ppm PIRO	19	19 (100)	18 (94.7)	3 (15.8)[e]	0.16 ± 0.37[e]	3	0 (0)	3 (100)	0 (0)	3 (100)
3	BBN + 100 ppm NIM	20	19 (95.0)	19 (95.0)	12 (60.0)	0.75 ± 0.79[f]	15	0 (0)	15 (100)	5 (33.3)	10 (66.7)
4	BBN + 200 ppm NIM	16	16 (100)	14 (87.5)	8 (50.0)[g]	0.56 ± 0.63[f]	9	0 (0)	9 (100)	3 (33.3)	6 (66.7)
5	BBN + 400 ppm NIM	19	19 (100)	16 (84.2)	5 (26.3)[h]	0.37 ± 0.68[f]	7	1 (14.3)	6 (85.7)	2 (28.6)	5 (71.4)

[a] Based on histological examination.
[b] SH, simple hyperplasia; NPH, nodulopapillary hyperplasia; TCC, transitional cell carcinoma.
[c] Ta, no invasion; T1, invasion to the lamina propria.
[d] Grade 1, either papillary or nodular, showing a minimal cytological atypia and infrequent mitoses; grade 2, larger and more pleomorphic than grade 1 carcinomas, and nucleoli were prominent. Mitoses were readily detectable.
[e] Significantly different from group 1 ($P < 0.0001$).
[f] Significantly different from group 1 ($P < 0.01$).
[g] Significantly different from group 1 ($P < 0.05$).
[h] Significantly different from group 1 ($P < 0.0002$).

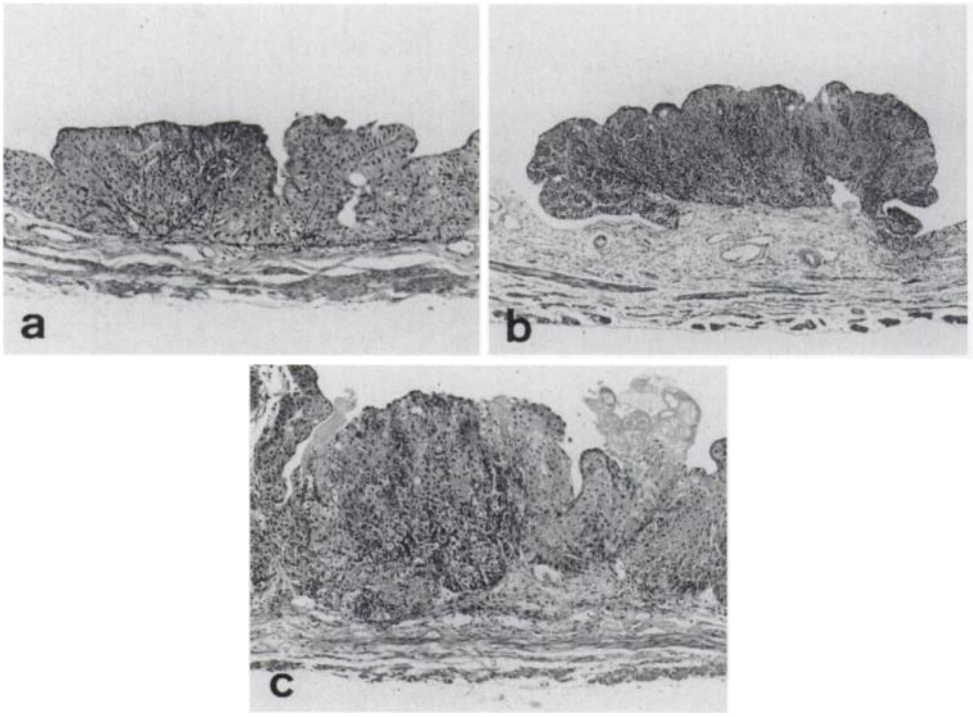

Fig. 1. Representative histological lesions seen in the urinary bladders of rats given BBN in drinking water for 8 weeks, followed by a basal diet or the diets supplemented with PIRO or NIM for 12 weeks. NPH (*a*) and TCC (*b*), classified as grade 1 and Ta, seen in rats given BBN followed by 400 ppm NIM. *c*, TCC, classified as grade 2 and T1 seen in a rat given BBN followed by a basal diet. H&E staining. Magnifications, ×40 (*a*), ×20 (*b*), and ×33 (*c*).

nificant effect on tumor grade or invasion. No urinary bladder lesions were observed without BBN treatment.

Histopathological Findings of Other Organs. No histopathological lesions were observed in the livers of rats given NIM and PIRO with or without BBN treatment, despite significantly increased weights. Thus, no reasons for the increased liver weights were found in this study. No macroscopically abnormal findings were observed in other organs, and bleeding was not apparent in the gastrointestinal tracts of NIM- and PIRO-treated animals.

DISCUSSION

These results indicated clear chemopreventive potential of the COX-2 inhibitor, NIM, against postinitiation development of superficial urinary bladder cancers in the rat. *In vitro* COX activity assays using isolated COX-1 and COX-2 enzymes from ram seminal vesicles and sheep placenta, respectively, have confirmed that NIM preferentially inhibits COX-2 in a time-dependent fashion, with an IC_{50} of 0.07 μM at the peak time point, as compared with >100 μM or 300 μM for COX-1 without time dependence (22–24). The structural basis for the time-dependent, selective COX-2 inhibition by the sulfonanilide class of inhibitors has been postulated as partly ascribable to the binding of the sulfonanilide moiety to the large binding pocket created by the substitution of valine for isoleucine in the catalytic moiety of COX-2 but not COX-1 (40). Therefore, our results strongly suggest an important role for COX-2 in the postinitiation development of rat urinary bladder cancers induced by BBN. The involved mechanisms are, at present, unknown, but further studies into whether COX-2 is as highly expressed in bladder cancers as it is in colon cancers (8, 9) or whether COX-2 is induced by bladder tumor promoters are clearly warranted. The question of whether NIM inhibits mouse invasive type (27) as well as *N*-[4-(5-nitro-2-furyl)-2-thiazoyl]formamide-induced rat (30, 31) bladder cancers is also important. The lack of adverse effects of NIM in the liver and gastrointestinal tract in this study are consistent with a previous report (20).

PIRO, which competitively and preferentially inhibits COX-1, with an IC_{50} of 9–24 μM (the IC_{50} value against COX-2 being 70–240 μM; Refs. 15 and 16), was here confirmed to exert potent chemopreventive potential against urinary bladder cancers (28). Seventy-five ppm PIRO exhibited roughly equipotent preventive potential to that of 400 ppm NIM. Because PIRO is long-acting (plasma half-lives in rats reported to be 8.3 and 13.3 h; Refs. 41 and 42, respectively) relative to NIM (plasma half-life = 5.01 ± 0.28 h in rats)[4], a simple comparison of the preventive potency between the two agents is difficult. However, the average daily intakes with 400 ppm NIM at 18.12 and with 75 ppm PIRO at 3.32 mg/kg body weight are ~5 and 10 times the respective maximum tolerated doses in man of 200 and 20 mg per person per day, respectively (20, 42). It remains to be clarified whether COX-2 inhibition by PIRO might have been involved in its chemopreventive potential.

Because NIM reportedly scavenges hydroxyl and peroxyl radicals (43), mechanisms other than inhibition of COX-2 could be involved in its effects on bladder carcinogenesis. Furthermore, induction of apoptosis by NSAIDs, including the selective COX-2 inhibitor NS-398, has been postulated to play important roles in their cancer chemopreventive potential (44–46). Moreover, a COX-independent pathway is assumed for the influence of sulindac sulfone, which is not a COX inhibitor but induces apoptosis and is a cancer chemopreventive agent (44). It remains to be clarified whether COX-2 or PGs play any role in the apoptosis-inducing signal cascade, and in fact, it has been reported that overexpression of COX-2 renders cells resistant to apoptosis induction (13). Moreover, both PIRO and NIM in the present study affected multiplicity but not grade of TCCs, suggesting that these agents exert their effects in the early rather than late stage of cancer development.

In conclusion, these results provide the first evidence that a selective COX-2 inhibitor, NIM, with much less adverse effects on the gastrointestinal tract than PIRO or other NSAIDs (20–22) might be a promising candidate chemopreventive agent active against human superficial bladder cancers.

[4] H. Asada of Sawai Pharmaceutical Co. Ltd. and H. Yamaguchi of Hisamitsu Pharmaceutical Co. Inc., personal communication.

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