

Inhibition by Aspirin of *N*-[4-(5-Nitro-2-furyl)-2-thiazolyl]formamide Initiation and Sodium Saccharin Promotion of Urinary Bladder Carcinogenesis in Male F344 Rats¹

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ABSTRACT

N-[4-(5-Nitro-2-furyl)-2-thiazolyl]formamide (FANFT) is metabolically activated by several enzyme systems, including prostaglandin H synthase. Aspirin is an inhibitor of prostaglandin H synthase and has been shown to inhibit FANFT-induced bladder carcinogenesis when coadministered in the diet. To further evaluate the effects of aspirin on bladder carcinogenesis in the rat, we have coadministered aspirin with FANFT during the initiation phase and with sodium saccharin during the promotion phase of carcinogenesis. FANFT was administered in the diet at a level of 0.2% for 6 weeks as the initiator and sodium saccharin was administered in the diet at a level of 5% for 61 weeks as promoting stimulus. Aspirin was administered at a level of 0.5% with FANFT or with sodium saccharin, and appropriate control groups were included. Weanling male Fischer 344 rats were utilized and the chemicals were added to Agway Prolab 3000 rat chow. A 1-week interval was included between the FANFT and sodium saccharin administration during which the rats received either aspirin containing diet or control chow, depending on the treatment regimen of the group. Thirty rats were included in each group at the beginning of the experiment, except for the control group which contained 40. Rats given FANFT followed by saccharin had a bladder carcinoma incidence of 83%. Rats given aspirin with FANFT but not with saccharin had a carcinoma incidence of 20% and the rats fed aspirin with the saccharin but not with the FANFT had an incidence of 28%. FANFT followed by control diet resulted in a bladder carcinoma incidence of 10%, as was true for the rats given FANFT plus aspirin followed by control diet. However, the hyperplastic effects induced in the bladder epithelium by saccharin without prior FANFT administration were inhibited by coadministration with aspirin. These results indicate that aspirin inhibits both FANFT initiation and sodium saccharin promotion of bladder carcinogenesis, but the mechanisms involved would most probably be different for each.

INTRODUCTION

Two-stage carcinogenesis, similar to the initiation-promotion model originally described for mouse skin, has been demonstrated for the rat urinary bladder by several investigations (1-3). FANFT⁴ is a potent urinary bladder carcinogen in rats and other species (4, 5), and it can act as an initiator of carcinogenesis when fed in the diet for 4-6 weeks (2). Sodium saccharin is a promotor of urinary bladder carcinogenesis when fed after initiation by FANFT or other bladder carcinogens (1-3).

FANFT is metabolized by a variety of enzyme systems including microsomal and nonmicrosomal nitroreductases (6), xanthine oxidase (6), and PHS (4, 7, 8). Aspirin inhibits fatty

acid cyclooxygenase, one of the PHS enzymatic activities, by acetylation of a serine moiety, significantly reducing prostaglandin E₂ synthesis (4). Rat urinary bladder epithelium contains substantial prostaglandin-synthetic capacity (4, 9), and aspirin fed in the diet inhibits prostaglandin synthesis in the rat bladder epithelium (4). It has also been shown that aspirin inhibits PHS-catalyzed metabolism of FANFT and ANFT, a deformylated metabolite of FANFT (4, 7-9). Previously, we demonstrated that aspirin inhibited the early hyperplastic (4) and the later carcinogenic (10) effects of FANFT when coadministered in the diet. Others have shown in the mouse skin model that inhibition of PHS results in inhibition of the promotion stage of carcinogenesis (11). The present study was designed to evaluate the effect of aspirin on each stage of carcinogenesis in the rat urinary bladder model, utilizing FANFT initiation and saccharin promotion.

MATERIALS AND METHODS

FANFT (Lot 811009) was obtained from Saber Laboratories (Morton Grove, IL), and aspirin (Lots 53F-0520 and 99C-0226) and sodium saccharin (Lot 053F-0635) were from Sigma Chemical Co. (St. Louis, MO). FANFT, aspirin, and sodium saccharin were mixed into powdered Agway Prolab 3000 rat chow (Agway, Inc., St. Mary's, OH) at doses of 0.2, 0.5, and 5%, respectively, by weight into the same diet or separately, and the diets were pelleted. Male F344 rats (Charles River Breeding Laboratories, Inc., Kingston, NY), 5 weeks old at the beginning of the experiment, were housed in plastic cages with corn cob bedding in an air-conditioned room at 24°C and 50% humidity on a 12-h light-dark cycle. The rats were randomly divided into 9 groups and treated according to regimens illustrated in Fig. 1. In the first stage of the experiment (Weeks 0-7), the groups fed aspirin plus FANFT (Groups 1 and 4) were fed the diet containing aspirin without FANFT for 2 days prior to and for 7 days after FANFT administration to be sure the enzyme system was inhibited once FANFT was fed and remained inhibited during the time necessary for clearance of the FANFT from the body. Similarly, in the second stage of the experiment (Weeks 7-68), the rats fed aspirin plus sodium saccharin (Groups 2 and 6) were fed the diet containing aspirin without sodium saccharin for 2 days prior to sodium saccharin administration. During periods of the experiment when rats were fed diet without added test chemicals, rats were fed pelleted basal chow (Agway, Inc.). The food and water were available *ad libitum*. The rats were weighed and food consumption periodically determined. Water consumption was measured at Week 63, and urinary pH was measured at Week 50 on freshly voided urine using a microelectrode (M1-410; Microcombination pH probe; Micro-electrodes, Inc., Londonderry, NH). All surviving rats were killed at Week 68 of the experiment. At autopsy, skin, s.c. tissues, and organs in thoracic and abdominal cavities were macroscopically examined. The urinary bladder and the stomach were inflated *in situ* with 10% phosphate-buffered formalin, pH 7.4. The liver was weighed and portions of the liver, kidney, and other tissues with grossly observed pathological alterations were fixed in 10% phosphate-buffered formalin. The urinary bladder was cut into 6-8 longitudinal strips for further processing. All bladder strips and representative sections of other tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Statistical comparisons were by Fisher's exact test with correction for continuity and Student's *t* test (12).

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⁴ The abbreviations used are: FANFT, *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide; ANFT, 2-amino-4-(5-nitro-2-furyl)thiazole; PHS, prostaglandin H synthase.

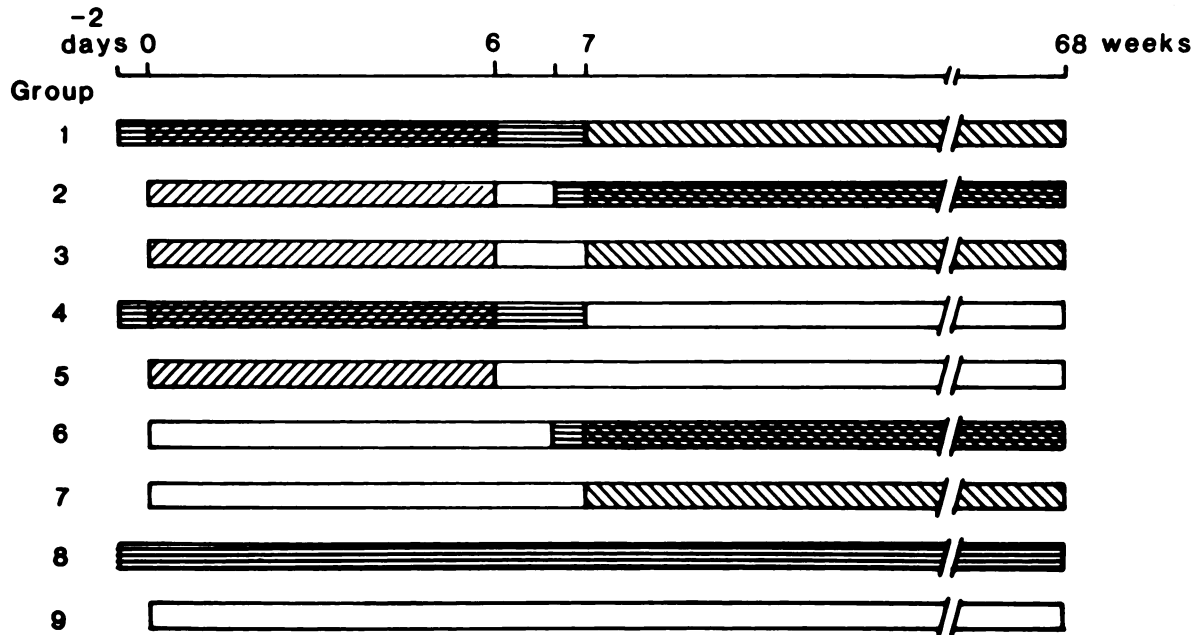


Fig. 1. Effect of aspirin on two-stage urinary bladder carcinogenesis. ▨, 0.2% FANFT; ▩, 5% sodium saccharin; ■, 0.5% aspirin; □, control diet; ▤, combinations of aspirin with FANFT or saccharin.

RESULTS

During the initial period of the experiment (6 weeks), the rats fed either FANFT only or FANFT plus aspirin had reduced body weight gain compared to the other groups. These rats attained weights comparable to the control groups unless followed by a diet containing saccharin with or without aspirin (Table 1). All rats treated with saccharin, regardless of initial treatment, showed a decreased weight gain compared to control rats. Also, rats fed aspirin for the entire course of the experiment (Group 8) showed decreased weight gain. Rats fed long term saccharin plus aspirin (Groups 2 and 6) showed the greatest difference in weight compared to the control group, and their final body weights were significantly less than those of rats fed either chemical alone.

The daily food consumption was similar between groups, and the estimated average cumulative dose of chemical was proportional to the period of the treatment except in Group 2, which had a slightly higher (not significantly increased) intake of sodium saccharin compared to other groups (Table 1). Water intake (Table 1), measured at Week 63, was increased by sodium saccharin or long term aspirin feeding and increased even more

when both were fed together. The urinary pH of the rats fed sodium saccharin alone, measured at Week 50, was slightly higher than in control rats, but the pH of the rats fed aspirin with saccharin was comparable to the controls (Table 1). At the end of the experiment, the liver weight (Table 1) was significantly lower in the rats fed saccharin or long term aspirin, and it was lowest in the rats fed sodium saccharin and aspirin (significantly lower than with either chemical alone).

The first urinary bladder tumor was identified in a rat which died during the 46th week of the experiment. Rats dying or killed because of marked weight loss and/or hematuria after this time were included in the effective number of rats for determining incidences. By the time of the terminal sacrifice at 68 weeks, the highest mortality rate in any group was 37% (11 of 30) in rats fed sodium saccharin after FANFT (Group 3); 10 of these rats had died because of bladder tumors. The bladder lesions were histologically classified as simple hyperplasia, nodular or papillary hyperplasia, papilloma, and carcinoma as described previously (13). The bladder malignancies were all transitional cell carcinoma. One rat had a poorly differentiated carcinoma with invasion into the prostate. The incidences of bladder lesions are summarized in Table 2. Each rat is tabulated

Table 1 Final weights, water intake, urinary pH, and chemical consumption in rats

Group	Final av. wt (g)		Water intake at Wk 63 (ml/day)	Urinary pH at Wk 50	Av. cumulative dose (g/rat)		
	Body	Liver			FANFT	Saccharin	Aspirin
1	413 ± 32 ^{a,b,c}	12.5 ± 1.2 ^{a,b,c} (27) ^d	39.7	7.11 ± 0.35 ^{a,b,c} (29) ^e	1.82	374	5.3
2	375 ± 20 ^b	11.1 ± 0.9 ^b (29)	53.5	6.55 ± 0.27 (29)	1.78	404	40.6
3	415 ± 24 ^{b,c}	12.4 ± 1.0 ^{b,c} (19)	44.6	7.11 ± 0.22 ^{b,c} (29)	1.65	385	0
4	465 ± 29	14.5 ± 1.3 (28)	25.3	6.99 ± 0.30 ^b (30)	1.79	0	5.2
5	459 ± 33	14.9 ± 1.7 (29)	26.5	6.47 ± 0.30 (28)	1.70	0	0
6	378 ± 19 ^b	10.8 ± 0.8 ^b (29)	52.6	6.40 ± 0.28 (28)	0	381	38.3
7	434 ± 24 ^{b,c}	13.0 ± 1.0 ^{b,c} (28)	38.6	6.67 ± 0.21 ^f (30)	0	389	0
8	429 ± 34 ^{b,c}	12.8 ± 1.1 ^{b,c} (29)	38.8	6.52 ± 0.40 (29)	0	0	48.8
9	462 ± 23	14.6 ± 1.3 (36)	24.6	6.45 ± 0.29 (30)	0	0	0

^a Mean ± SD.

^b Significantly different from control (Group 9) at *P* < 0.001 by Student's *t* test.

^c Significantly different from Groups 2 and 6 at *P* < 0.001 by Student's *t* test.

^d Numbers in parentheses, number of rats killed at the end of the experiment for which liver weights were determined.

^e Numbers in parentheses, number of rats examined.

^f Significantly different from control (Group 9) at *P* < 0.01 by Student's *t* test.

ASPIRIN INHIBITION OF FANFT-INDUCED CARCINOGENESIS

Table 2 Lesions in the urinary bladder of rats^a

Group/treatment	No. of rats		Normal	Simple hyperplasia	Nodular or papillary hyperplasia	Papilloma	Total	Transitional cell carcinoma				
	Initial	Effective ^b						Non-invasive	Invasive			
								Stalk	Submucosa	Muscle	Serosa	
1. F ^c + A→S	30	30	5 (17) ^d	10 (33)	8 (27)	1 (3)	6 (20) ^e	5	1	0	0	0
2. F→S + A	30	29	4 (14)	7 (24)	8 (28)	2 (7)	8 (28) ^e	5	2	0	0	1
3. F→S	30	29	0	2 (7)	2 (7)	1 (3)	24 (83)	6	14	3	1	0
4. F + A→C	30	30	24 (80) ^e	2 (7)	1 (3)	0	3 (10) ^e	2	1	0	0	0
5. F→C	30	30	16 (53) ^e	5 (17)	3 (10)	3 (10)	3 (10) ^e	2	0	0	0	1 ^f
6. C→S + A	30	29	29 (100)	0	0	0	0	0	0	0	0	0
7. C→S	30	29	21 (72) ^f	8 (28) ^f	0	0	0	0	0	0	0	0
8. A→A	30	29	28 (97)	0	0	1 (3)	0	0	0	0	0	0
9. C→C	40	36	36 (100)	0	0	0	0	0	0	0	0	0

^a Each rat is tabulated in the column of the most advanced lesion present in the urinary bladder as determined by histopathological examination.

^b All rats killed after 46 weeks of the experiment are included.

^c F, 0.2% FANFT; A, 0.5% aspirin; S, 5% sodium saccharin; C, control diet.

^d Numbers in parentheses, percentage of effective number of rats in the group.

^e Significantly different from Group 3 at $P < 0.001$ by Fisher's exact test.

^f Significantly different from Group 6 at $P < 0.01$ by Fisher's exact test.

^g This tumor invaded into the prostate.

Table 3 Tumor lesions in tissues other than the urinary bladder

Group	Effective no. of rats	Testis, interstitial cell tumor	Mesothelioma	Leukemia	Preputial gland, carcinoma	Liver, hyperplastic nodule	Breast, fibroadenoma	Skin, epidermal cyst	Lipoma
1	30	9 (30) ^a	0	0	0	0	1 (3)	0	0
2	29	5 (17) ^b	2 (7)	1 (3)	0	0	0	0	0
3	29	8 (28)	5 (17) ^c	0	1 (3)	0	0	1 (3)	1 (3)
4	30	14 (47)	2 (7)	0	0	0	1 (3)	0	1 (3)
5	30	16 (53)	5 (17) ^c	1 (3)	2 (7)	0	0	0	4 (13)
6	29	5 (17) ^b	0	0	0	1 (3)	0	0	2 (7)
7	29	20 (69)	1 (3)	1 (3)	0	0	0	1 (3)	1 (3)
8	29	3 (10) ^d	0	0	0	0	0	0	1 (3)
9	36	18 (50)	0	0	0	0	0	0	1 (3)

^a Numbers in parentheses, percentages of effective number of rats in the group.

^b Significantly different from control (Group 9) at $P < 0.01$ by Fisher's exact test.

^c Significantly different from control (Group 9) at $P < 0.05$ by Fisher's exact test.

^d Significantly different from control (Group 9) at $P < 0.001$ by Fisher's exact test.

on the basis of the most advanced lesion found in its bladder.

As has been demonstrated previously, saccharin promoted bladder carcinogenesis initiated by FANFT (Group 3 compared to Group 5). The incidences of carcinoma in Groups 1, 2, 4, and 5 were significantly lower than in Group 3. These data demonstrate that aspirin has an inhibitory effect on FANFT initiation of bladder carcinogenesis and also has an inhibitory effect on saccharin promotion of bladder carcinogenesis. In addition, aspirin appears to inhibit the mild hyperplasia occasionally seen in rats fed long term saccharin (Group 6 compared to Group 7).

Tumors in tissues other than the urinary bladder are shown in Table 3. Numerous nonneoplastic lesions were observed in the kidney which will be described elsewhere. Few renal pelvic lesions were observed and they were all transitional cell hyperplasia, a few of which showed extensive nodularity. The stomachs showed chronic gastritis of the glandular epithelium in rats fed saccharin plus aspirin. The forestomachs had no tumors in this experiment, in contrast to our previous experiment (10). At 68 weeks of the experiment, 50% of the rats fed control diet had interstitial cell tumors in one or both testes. Groups 2, 6, and 8, fed long term aspirin, showed a significant decrease in the incidence of testicular tumors. The incidence of mesothelioma was significantly increased in Groups 3 and 5. Incidences of tumors of other tissues were not significantly higher than in control rats.

DISCUSSION

It has been demonstrated previously that coadministration of aspirin and FANFT in the diet to male Fischer rats inhibits the

bladder carcinogenicity of FANFT (10). The mechanism remains unknown, although several possibilities have been evaluated, including the potential effect of aspirin on the metabolic activation of FANFT or its urinary excreted metabolite ANFT. The present results again demonstrate that the effects of FANFT on the urinary bladder can be inhibited by aspirin. In the previous experiment (10), FANFT and aspirin were coadministered for 12 weeks. In the present experiment, they were coadministered for only a 6-week period which is sufficient for initiation of bladder carcinogenesis in the rat (2). In a relatively short term experiment, utilizing labeling index determined by autoradiography following a pulse injection of [³H]thymidine, it was demonstrated that the inhibitory effect of aspirin on FANFT is relatively transient (14). Thus, there was an inhibition of the increased labeling index of the bladder epithelium induced by FANFT through 4 weeks of administration, but by 12 weeks of coadministration the labeling index was similar between the groups of rats fed FANFT or FANFT plus aspirin. Thus, utilizing an initiation-promotion type of experiment, with the briefer period of administration of FANFT with or without aspirin, probably provides a more realistic model for evaluation of the effects of aspirin on FANFT carcinogenesis.

The possible mechanisms of aspirin inhibition of FANFT-induced bladder lesions include effects of aspirin on ANFT excretion and/or metabolism (6-9). ANFT is the urinary-excreted form of FANFT (6) and, like FANFT, has the nitro group intact. It could theoretically be metabolically activated by reduction of the nitro group to a metabolically reactive intermediate. However, an alternative metabolic route is activation by PHS (4, 7, 8). This appears to involve oxidation of the aminothiazole portion of the molecule rather than the

nitrofurans (15). The urinary excretion of ANFT has previously been shown not to be reduced by coadministration with aspirin (16). In fact, the renal excretion in urinary levels of ANFT when aspirin and FANFT are coadministered is actually slightly increased.

The results of the present experiment demonstrate that not only does aspirin inhibit the initiation of bladder carcinogenesis by FANFT but it also inhibits the promotion of bladder carcinogenesis by sodium saccharin. Again, the mechanism for this is unknown. However, the relationship to prostaglandin synthesis is certainly possible here also. In other two-stage models of carcinogenesis, particularly in the mouse skin model, inhibition of prostaglandin synthesis has been found to be related to inhibition of promotion by a variety of agents, in particular the phorbol esters (11). The exact mechanism of this inhibition is unknown.

Since sodium saccharin is not metabolized, the effect of aspirin cannot be an inhibition of the metabolic activation of the compound as is potentially possible with FANFT. However, there could be an alteration in the excretion of saccharin or changing of various urinary parameters which could affect the proliferative inducing activity of saccharin. At present, there are no data available to evaluate the effect of aspirin on the excretion of saccharin in the urine. However, effects of aspirin on various urinary parameters are certainly possible and obviously do occur based even on the preliminary findings in the present experiment. Urinary volume has been shown to be greatly increased following the administration of sodium saccharin in the diet (17). This increased urinary excretion was accentuated further by coadministration with aspirin.

It has also been shown that sodium saccharin administered in the diet can result in a slightly increased urinary pH compared to rats fed control diet (17, 18). This slight increase was again observed in the present experiment and was inhibited when coadministered with aspirin.

There has been recent evidence that urinary pH is a critical parameter in the proliferation-inducing effects of saccharin, and the effect of aspirin on urinary pH might be related to this change (19). However, the pH determination in the present experiment was at only one time point and did not adequately take into account diurnal variations in urinary pH in the rat. Clearly, additional investigation is necessary to evaluate the effects of aspirin on saccharin-induced proliferation and promotion of bladder epithelium as well as the mechanism of FANFT-induced initiation and carcinogenesis.

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