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Validity of Short-term Examination for Antipromoters of Bladder Carcinogenesis

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Various compounds were screened for antipromoter activity in bladder carcinogenesis in rats with a view to using them clinically to inhibit postoperative intravesical ectopic tumor growth of superficial papillary bladder cancer. Their inhibitions of the effect of sodium saccharin in maintaining increased agglutinability of bladder cells by concanavalin A were examined in 4-week tests. The compounds found to inhibit the effect of saccharin were α-tocopherol, ascorbic acid, aspirin, all-*trans* aromatic retinoid, α-difluoromethylornithine, sodium cyanate and *p,p'*-diaminodiphenylmethane. Considering the toxicities of some of these chemicals, ascorbic acid and α-difluoromethylornithine were concluded to be the most promising for future clinical trials.

Key words: Rat bladder cancer — Antipromoter — Ascorbic acid — α-Difluoromethylornithine — Concanavalin A

About 70 percent of all human bladder cancers are of the papillary superficial type, which is characterized by frequent multiple intravesical ectopic tumor growth after transurethral resection.[1,2] The incidence of such growth is as high as 50% within 1 year after resection.[3] This phenomenon, called "recurrence," is explained in two ways[4]: one is that carcinoma *in situ* (CIS) and dysplasia are present but difficult to recognize in the nontumorous mucosa of the bladder[5] and that they develop into visible tumors after a certain period. In this process, tumor promoters are probably important in new tumor growth. The other explanation is that cancer cells are intravesically spread during repeated endoscopic manipulations. This work was based on the first possibility, because we observed a close relation between visible bladder cancer and CIS and dysplasia in the nontumorous mucosa of the bladder.[6,7]

Previously we developed a short-term assay for detection of tumor promoters of bladder cancer,[8] and using this assay system, we identified the tumor-promoting effects of L-isoleucine and L-leucine.[9] In the present work, by modifying this system, we examined the antipromoter effects of various compounds on bladder carcinogenesis in rats.

Materials and Methods

Animals Male F344 rats (5 weeks old) were purchased from Charles River Japan Co., Kanagawa. They were kept 4 to a wire cage in an animal room at 23° and 50% humidity with a 12-hr light-dark cycle and used at 6 weeks old. Animals were given free access to tap water and CE-2 pellet diet (CLEA Japan, Tokyo) or CE-2 powder diet containing test chemicals.

Chemicals The following chemicals were used: N-butyl-N-(4-hydroxybutyl)nitrosamine (BHBN, Tokyo Kasei Co., Tokyo); concanavalin A (Con A), α-methylmannoside and aspirin (Sigma Chemical Co., Ann Arbor, Mich.); sodium saccharin (Aisan Chemical Co., Aichi); L-ascorbic acid, indomethacin, selenium powder, sodium cyanate and *p,p'*-diaminodiphenylmethane (Wako Pure Chemical Ind., Osaka); α-tocopherol and butylated hydroxytoluene (Tokyo Kasei Co.). Thienyl-2-thioacetic acid, α-difluoromethylornithine (DFMO) and all-*trans* aromatic retinoid were kindly provided by Nippon Synthetic Chemical Ind., Tokyo, Merrel Dow Research Institute, Cincinnati, Ohio, and Roche Japan Co., Tokyo, respectively.

Treatment of Animals Three groups of 4 animals each were given 0.01% BHBN in the drinking water for 1 week. As we have repeatedly observed previously, after this treatment the agglutinability of their isolated bladder cells by Con A was increased. In group 1, which was given tap water and control CE-2 diet after the period of BHBN treat-

ment, this increased agglutinability disappeared within 3 weeks.[8] In group 2, given sodium saccharin, a known promoter of bladder carcinogenesis, at a concentration of 5.0% in the diet, the increased agglutinability persisted for at least 3 weeks. Animals in other groups were given a test chemical together with sodium saccharin. When a chemical inhibited the maintenance of increased agglutinability by sodium saccharin, it was considered to be a possible antipromoter of bladder carcinogenesis. The chemicals tested and the doses examined are listed inTable I. Final body weights at week 4 (mean±SD) for each group are also included in Table I. The choice of these chemicals was based on reports of their inhibition of carcinogenesis, irrespective of the target organ. The doses of chemicals used were based on the LD50 values or the doses reported to inhibit carcinogenesis.

Agglutination Assay Agglutination was assayed as described previously.[10] The significance of differences between agglutination values was examined by using Student's *t*-test.

Results and Discussion

The first two rows in Table I show agglutination values at the end of the third week after cessation of BHBN administration with or without sodium saccharin treatment. These values were obtained by calculating mean± standard deviation based on the data collected from each experiment, in which these groups were always included as controls. The agglutination value with BHBN alone was 3±1, but when 5.0% sodium saccharin was given after BHBN treatment, a high value of 22±3 was maintained. Test chemicals were given at the indicated doses with saccharin, and the resulting agglutination values are listed in the last column.

First we tested antioxidants. α-Tocopherol at a concentration of 2.0% inhibited the maintenance of agglutination by saccharin but it also significantly suppressed the increase in body weight in 3 weeks. Ascorbic acid at a concentration of 0.25% inhibited the effect of sodium saccharin. Ascorbic acid was previously tested in humans as an inhibitor of bladder carcinogenesis because it prevents oxidation of 3-hydroxyanthralinic acid in the urine.[11] However, detailed results have not been reported. Butylated hydroxytoluene, which was reported to promote bladder carcinogenesis at high concentration,[12] was examined at a lower concentration in this experiment because it was found to inhibit carcinogenesis in the skin[13] and stomach.[14] However, at the concentrations tested, butylated hydroxytoluene did not influence the effect of sodium saccharin. Thienyl-2-thioacetic acid, which is an oxygen radical scavenger,[15] also did not have any inhibitory effect.

The inhibitors of prostaglandin synthesis, indomethacin and aspirin, were examined because both chemicals have been reported to inhibit mammary,[16] esophageal[17] and bladder[18] carcinogenesis. Indomethacin given in the drinking water at 40 and 20 mg/liter induced thickening of the bladder wall and ulcer formation in the bladder without inhibiting agglutination. Even at a concentration of 0.5%, aspirin inhibited the effect of sodium saccharin, but caused hemorrhage of the gastric mucosa in 3 weeks.

All-*trans* aromatic retinoid is reported to be a potent inhibitor of bladder carcinogenesis in rats,[19] and we found that even at 25 ppm it inhibited the effect of sodium saccharin. At higher concentrations of 50 and 100 ppm, however, it had deleterious effects such as causing hair loss, nail bed bleeding and loss of body weight even when administered for only 3 weeks. A concentration of 25 ppm did not have any apparent deleterious effects on the animals, but we have found that this concentration cannot be given to patients with bladder cancer to inhibit recurrence, because it has side effects on the skin and mucosa (unpublished findings).

α-Difluoromethylornithine (DFMO), an inhibitor of ornithine decarboxylase in polyamine biosynthesis,[20] inhibited agglutination at a level of 0.10% without having any side effects. DFMO has been found to exert inhibitory effects on carcinogenesis in several organs such as the skin,[21] colon,[22] mammary gland[23] and urinary bladder.[24]

Sodium cyanate, which is an inhibitor of protein synthesis, inhibited agglutination at its maximum concentration tested, but this may be toxic, because the animals ate very little of the diet containing sodium saccharin and sodium cyanate. The same was probably true for *p,p'*-diaminodiphenylmethane. *p,p'*-Diaminodiphenylmethane was reported to inhibit the promoting effect of sodium saccharin in rat bladder carcinogenesis,[25] but since it is

Table I. Inhibition of Effect of Sodium Saccharin on Con A-Agglutination of Isolated Bladder Cells

Chemicals	Dose	Agglutination[a]	Body weight (g)
BHBN alone		3±1	208±9
BHBN+sodium saccharin		22±3	210±10
BHBN+sodium saccharin			
+α-tocopherol	2.0%	9±2*	178±2**
	1.0	10±1	188±4**
	0.5	18±3	198±5
	0.1	24±3	200±4
Ascorbic acid	0.50%	8±1*	212±10
	0.25	9±3*	208±3
	0.10	22±2	214±2
Butylated hydroxytoluene	0.50%	19±2	208±7
	0.10	20±3	200±9
	0.01	19±3	215±4
Thienyl-2-thioacetic acid	0.150%	15±2	190±8**
	0.075	17±1	213±8
	0.025	23±3	215±9
Indomethacin	40 mg/liter	17±1	189±8**
	20	17±2	196±5
Aspirin	1.0%	9±2*	165±6**
	0.5	6±1*	209±9
	0.1	14±2	216±3
All-*trans* aromatic retinoid	100 ppm	3±1*	179±8**
	50	5±2*	200±4
	25	8±2*	207±6
	10	15±2	210±10
α-Difluoromethylornithine	0.50%	7±1*	204±6
	0.25	7±1*	205±5
	0.10	8±2*	208±8
	0.05	16±2	206±5
Sodium cyanate	2.6×10^{-2} mmol/g	8±1*	180±10**
	2.6×10^{-3}	15±1	200±6
	2.6×10^{-4}	20±2	208±5
p,p'-Diaminodiphenylmethane	0.10%	3±1*	166±10**
	0.01	21±3	200±4
Selenium	2.0 ppm	20±3	207±4
	0.1	24±3	213±5

a) Mean±SD. *$P<0.01$, **$P<0.05$.

strongly toxic, it seems inappropriate for clinical use. Selenium at concentrations of 2.0 ppm and 0.1 ppm did not have any effect on the agglutination.

Although sodium saccharin is an inert compound, we have not examined the possibility of chemical interaction between the test compounds and sodium saccharin. When we extend this work, we need to examine this possibility together with testing the effect of a change of urinary pH.

Considering the toxicities of some of the chemicals examined in this study, ascorbic acid and DFMO seem to be the most promising for clinical use. Administration of 5.0% sodium saccharin increased the urinary pH to 7.4, while on coadministration of 0.5% ascorbic acid with sodium saccharin, the urinary pH decreased to 6.8. Increase of urinary pH and urinary excretion of sodium ion are proposed to be major mechanisms of tumor promotion in rat bladder carcinogenesis.[26] Thus,

the inhibition by ascorbic acid of the maintenance of agglutination by sodium saccharin may be due to its effect in lowering the urinary pH. However, there is strong evidence that DFMO exerts its inhibitory effect on carcinogenesis in various organs by suppressing polyamine synthesis.[21-24] We have studied the inhibitory effect of oral DFMO on BHBN-induced bladder carcinogenesis in rats and also examinated the chronic toxicity of DFMO (Y. Honma *et al.*, unpublished). In our experiment, DFMO at levels of 0.2% and 0.5% clearly inhibited bladder carcinogenesis in rats without having any serious toxic effects. We are continuing experiments to accumulate more information on the possibility of clinical use of DFMO and ascorbic acid in treatment of bladder cancer.

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