

## RESEARCH COMMUNICATION

## No Inhibition of Urinary Bladder Carcinogenesis in Rats with Intravesical Instillation of $\alpha$ -Galactosylceramide

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### Abstract

The immunostimulatory  $\alpha$ -galactosylceramide, KRN 7000 ((2S,3S,4R)-1-O-( $\alpha$ -D-galactopyranosyl)-2-(N-hexacosnoylamino)-1,3,4-octadecatrienol), may be anticipated to have antitumor activity *in vivo* apart from any direct toxicity to cancer cells. In this experiment, inhibition of rat bladder carcinogenesis by intravesically instilled KRN7000 was investigated. Male Fischer 344 rats, 6-weeks-old at the start, were divided into 4 groups, all first receiving the carcinogen, 0.05% N-butyl-N-(4-hydroxybutyl)nitrosamine, in their drinking water for 12 weeks. Then groups 1 and 2 respectively were administered 500 and 50  $\mu$ g/kg of KRN7000 intravesically once weekly for 17 weeks. Group 3 similarly received only 0.3 ml of saline (vehicle control). Group 4 did not undergo bladder catheterization. On macroscopic examination at 30 weeks, multiplicities and sizes of bladder tumors in the KRN 7000 high and low-dose groups were not significantly different from those of the vehicle control group. Histologic examination confirmed no significant variation in incidences of carcinomas or preneoplastic lesions in the bladder among groups 1 to 4. Thus the results indicate that intravesical instillation of KRN7000 does not inhibit bladder carcinogenesis in rats.

**Key Words:**  $\alpha$ -galactosylceramide (KRN7000) - rat -intravesical instillation therapy - bladder carcinogenesis

**Abbreviations:** BBN: N-butyl-N-(4-hydroxybutyl)nitrosamine, DC: dendritic cell, NK: natural killer, NKT: natural killerT, BCG: bacillus Calmette-Guerin, CIS: carcinoma *in situ*

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### Introduction

Patients with urinary bladder cancer have a high risk of recurrence after transurethral resection (Wallace et al., 2001). Further, this is often associated with development of biologically more aggressive lesions (Wallace et al., 2001). Although mechanisms underlying this high risk of recurrence have not been well explained, two hypotheses have been proposed. One is that cancer cells might be transplanted into urothelium at another site through the urinary stream. Urothelial trauma during endoscopy may provide opportune sites for such tumor cells to implant and grow. Laboratory studies in animals and circumstantial evidence in patients have provided evidence supporting this mechanism in some

cases (Wallace et al., 2001; Weldon et al., 2001). Soloway et al. (1983) previously reported high susceptibility of cauterized urinary bladder to tumor cell implantation in a mouse model, although Nakamura et al. (2002) suggested the presence of a system in intact urothelium, especially involving umbrella cells, that works to prevent implantation. The other hypothesis is one of urothelial multifocal carcinogenesis. Some reports describing histologic mapping of bladders resected for invasive cancer have documented additional findings of multifocal carcinomas or carcinoma *in situ* (CIS) in approximately 80 % of specimens (Wallace et al., 2001; Farrow et al., 1976; Coloby et al., 1994). Other reports based on biopsy specimens demonstrated atypia, CIS, or cancer from normal-appearing mucosa at distant sites from

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the original primary at incidences ranging between 20% and 80% (Wallace et al., 2001).

Various anticancer agents have been instilled into the urinary bladder to treat high-risk epithelium. Intravesical therapy with bacillus Calmette-Guerin (BCG) has been used for more than 20 years to treat multifocal CIS and also as adjuvant therapy after transurethral resection of Ta (non-invasive papillary carcinoma and T1 (subepithelial connective tissue invasion tumor) papillary bladder cancer. This is now considered the most successful immunotherapy for bladder tumors. Sometimes, however, the therapeutic protocol cannot be completed because of serious side effects, such as, pyrexia, generalized fatigue, and bladder pain. It is hoped that recently developed agents with less potential for side effects may be effective for intravesical immunotherapy.

The immunostimulatory  $\alpha$ -galactosylceramide, KRN 7000 ((2S,3S,4R)-1-O-( $\alpha$ -D-galactopyranosyl)-2-(N-hexacosoylamino)-1,3,4-octadecatrienol, C<sub>50</sub>H<sub>99</sub>NO<sub>9</sub>; MW. 858.33; Fig. 1) may be anticipated to have antitumor activity in vivo apart from any direct toxicity to cancer cells (Yamaguchi et al.,1996). We have already studied the effect on mouse bladder carcinogenesis by intravesical instillation using C57BL/6 mice, but could not observe any significant inhibition (Mitsuhashi et al.,2002). In the presented study, we investigated the influence on bladder carcinogenesis of intravesical instillation therapy of KRN 7000 in F344 rats.

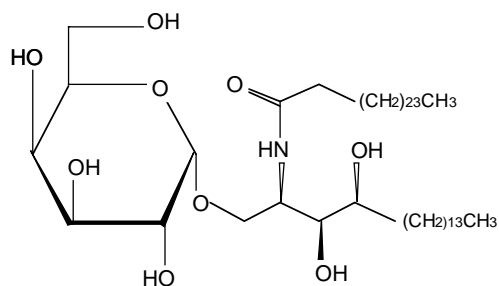
## Materials and Methods

### Experimental Animals

Female Fischer 344 rats, 5 weeks old, were purchased from Charles River Japan (Kanagawa) and housed in plastic cages with a bedding of paper chips, three rats per cage, in an environmentally controlled room maintained at a temperature of 22±2°C, a relative humidity of 55±10%, and a 12 h /12 h light-dark cycle. Standard diet (Oriental MF; Oriental Yeast, Tokyo) and water were available *ad libitum* throughout the experiment.

### Chemicals

N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) was obtained from Tokyo Kasei (Tokyo). KRN 7000 was kindly provided by KIRIN Brewery (Tokyo).



KRN7000 ( $\alpha$ -galactosylceramide; C<sub>50</sub>H<sub>99</sub>NO<sub>9</sub>, MW = 858.34)

**Figure 1. Chemical Structure of KRN 7000**

### Experimental Design

At 6 weeks of age, rats were divided into four groups as specified below (Fig. 2) and all given 0.05% BBN in the drinking water for 8 weeks to initiate bladder carcinogenesis. One week after completion of the initiation period, groups 1 and 2 (15 rats each) were administered 500 and 50 $\mu$ g/ml, respectively, of KRN 7000 in 0.3 ml of saline once weekly for 17 weeks by intravesical instillation under anesthesia with intraperitoneal pentobarbital (Nembutal, ABBOTT Laboratories, Illinois, USA). As the catheter for intravesical instillation, we used the sheath of an intravenous injection catheter, inserting it through the urethra into the urinary bladder, after expulsion of the urine in the bladder, KRN 7000 was instilled using a 1 ml syringe pump. About 2 h after the injection, the agent was allowed to drain from the bladder through the catheter. Group 3 (15 rats) received 0.3 ml of saline intravesically as a control. Group 4 (9 rats) underwent no catheterization.

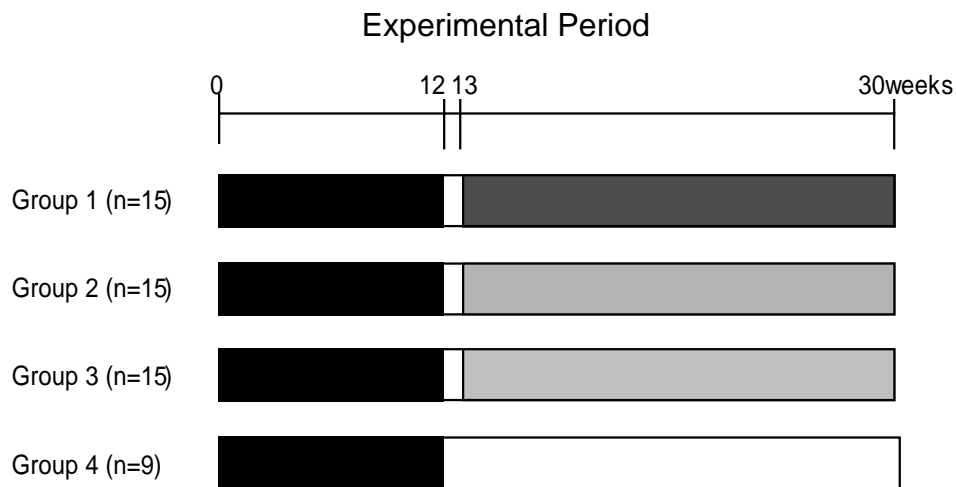
During the experiment, health was checked daily. Rats were weighed every 2 weeks during the initiation period and then before each intravesical instillation. Food consumption and water intake were recorded every 4 weeks over a period of 2 days. The experiment was terminated at 30 weeks after the commencement, on the day after the last instillation. Surviving animals were killed under light ether anesthesia. Each urinary bladder was inflated with 10% buffered formalin as a fixative and then removed. First, the numbers of bladder tumor were counted, and their sizes measured, macroscopically. The bladder was then weighed and cut longitudinally into multiple strips according to the number and size of macroscopic tumors. These tissues were embedded in paraffin and examined histopathologically. Sections were cut and stained with hematoxylin and eosin (H and E) for microscopic examination. The liver, kidneys and spleen also were also removed, weighed, examined macroscopically, and processed for staining with H and E for histopathologic assessment. Rats dying or killed because of a moribund state at an earlier time underwent similar autopsy examinations.

### Statistical Analysis

Statistical analyses were completed with Stat-View software for Macintosh microcomputers. The Fisher's exact probability test was used to analyze the incidences of bladder tumors, and the Student's t test for testing differences in body weights and relative organ weights between groups. A value of p less than 0.05 was considered to indicate significance.

## Results

By the middle of the experiment, a total of 3 rats (from group 1 at week 27, group 2 at week 19 and group 3 at week 20) had died of overdoses of pentobarbital anesthesia as a complication of instillation treatment. A further 3 rats (2 from group 2, at weeks 27 and 30, and 1 rat from group 3 at week 29) died with hydronephrosis, possibly due to urinary



**Figure 2. Experimental Protocol for Our Study, 6-Week-old Female F344 Rats.**

Black, 0.05% BBN in drinking water for first 12 weeks. Dark gray, 500 µg/ml of KRN7000 by intravesical instillation once weekly. Light gray, 50 µg/ml of KRN7000 by intravesical instillation once weekly. Crosshatched; only saline by intravesical instillation once weekly.

tract obstruction by bladder tumor growth. The bladder samples from these 3 rats were also taken into account for the assessment.

#### *Body Weight, Food Consumption, Water Intake and Organ Weights*

Body weights were almost similar in all groups during the initiation period, but after instillation treatments started, growth gain was less in groups 1 to 3 than in group 4 (Table 1). Final body weights were elevated in proportion to the dose of KRN7000 at week 28, but this was not significant.

In the latter half of the study, rats in group 3 (vehicle control group) consumed more food and drank more water than the other groups, while rats in group 4 (no KRN 7000 treatment group) consumed less water than the other groups but a similar amount of food to group 3.

Relative liver and spleen weights were similar in groups 1 to 3, while those in group 4 were significantly lower than in groups 1 or 2 (Table 2). Mean kidney weights were similar between groups 1 and 2, but significantly greater than in groups 3 and 4. Mean urinary bladder weights in group 1 (KRN 7000 high dose group) were significantly higher than in groups 2 to 4.

#### *Macroscopic Findings*

Multiplicities of bladder tumors were calculated from macroscopic counts, because there were too many lesions to be counted precisely from microscopic preparations. Values were somewhat larger in the KRN 7000 treated group without significance and that in group 3 (vehicle control group) was less than in group 4 (non catheterized group) (Table 3).

No abnormal gross findings were evident for the liver, spleen and kidney.

#### *Histopathological Findings*

Histopathological changes of urinary bladder epithelium were classified into 3 categories, PN hyperplasia, papilloma and carcinoma. Histopathological examination revealed that almost all of the macroscopically visible lesions were papillary or nodular (PN) hyperplasias, which is a preneoplastic change (Ito et al.,1975; Fukushima et al.,1982;Cohen et al.,1976), papillomas and carcinomas. Other bladder neoplasms were not observed in our samples. The data are summarized in Table 4. The incidences of PN hyperplasia, papilloma and total carcinoma were similar in all three bladder instillation groups.

**Table 1. Body Weights, Water Intake and Food Consumption**

Group	Treatment		w.2 (n)	Body weight (g)			Water intake	Food consumption
	0.05% BBN	Intravesical Instillation		w.12 (n)	w.16 (n)	w.28 (n)		
1	+	10 µg/kg b.w. KRN7000	110.1±3.7 (15)	174.2±4.2 (15)	170.9±5.1 (15)	183.2±6.1 (14)	21.1	7.5
2	+	0.1µg/kg b.w. KRN7000	111.19±3.3 (15)	171.94±7.8 (15)	169.1±6.5 (15)	181.8±7.4 (12)	21.3	8.0
3	+	0.9% saline	111.33±4.0 (15)	171.54±7.4 (15)	169.5±7.9 (15)	178.1±15.4 (13)	23.5	8.4
4	+	no treatment	110.46±3.2 (9)	173.42±5.3 (9)	176.6±6.4 (9)	189.7±6.2 (9)	18.7	8.3

Data are mean ± SD values.

BBN; N-butyl-N-(4-hydroxybutyl) nitrosamine

**Table 2. Relative Organ Weights of Animals.**

Group	Treatment		No. of effective animals	Bladder weight (%)	Liver weight (%)	Spleen weight (%)	Kidney weight (%)
	0.05% BBN	Intravesical Instillation					
1	+	500 mg/kg b.w. KRN7000	14	0.55±0.33 <sup>a</sup>	2.8±0.16 <sup>b</sup>	0.25±0.010 <sup>b</sup>	0.72±0.033 <sup>bc</sup>
2	+	50 mg/kg b.w. KRN7000	13	0.34±0.19	2.8±0.27	0.25±0.017 <sup>b</sup>	0.71±0.043 <sup>bc</sup>
3	+	0.9% saline	14	0.35±0.43	2.7±0.27	0.24±0.022	0.67±0.040
4	+	no treatment	9	0.34±0.16	1.2±0.10	0.11±0.011	0.65±0.017

Data are mean ± SD values. BBN; N-butyl-N-(4-hydroxybutyl) nitrosamine;

a Significantly different from KRN7000 low dose group (group 2), no KRN7000 treated group (group 3) and no intravesical instillation group (group 4), P<0.05.

b Significantly different from no intravesical instillation group (group 4), P<0.05.

c Significantly different from no KRN7000 treated group (group 3), P<0.05.P<0.05.

## Discussion

Kobayashi et al. (1995) reported that KRN 7000, isolated from extracts of the sponge, *Agelas mauritanus* (Morita et al.,1995), can markedly augment natural killer (NK) activity of spleen cells *in vivo*, while showing significant antitumor activity in mice with lung metastasis following transplantation of B16 melanoma cells. Nakagawa et al. similarly described strong antitumor activity in mice with Colon 26 hepatic metastases, accompanied by marked activation of NK cells in the liver (Nakagawa et al.,1998). Administration of KRN 7000 resulted in a high percentage of cures in mice, which thus acquired tumor-specific immunity (Ohteki et al.1998). Moreover the antitumor effect was stronger than those of known chemotherapeutic agents such as mitomycin C, 5-fluorouracil, or adriamycin; it was equal to that of interleukin-12. A sequential mechanism of antitumor activity can be proposed. First, molecules of KRN 7000 are taken up by a dendritic cells (DCs) and presented on the DC membranes together with a CD1 molecule. The CD1/KRN7000 complex is recognized by natural killer T (NKT) cells with V $\alpha$ 14/V $\beta$  8, (Nakagawa et al.,1998), attacking tumor cells (Lu et al.,1994) and releasing various cytokines such as interleukin-4 and interferon-  $\gamma$ (Nakagawa et al.,1998). In turn, NK cells and macrophages are activated and injure tumor cells non-specifically (Kawano et al.,1997). Further, CD8<sup>+</sup> cytotoxic T lymphocytes with tumor-specific effects are induced. This combination of antitumor mechanisms for KRN7000 is considered unique, differing in important ways from those of other antitumor agents. In

recent studies, activation of DCs by KRN 7000 *in vitro* was associated with a significant antitumor effect when they were injected into cancer- bearing mice (Yamaguchi et al.,1996; Kawano et al.,1998). Such treatment of various cancers using KRN 7000 has been considered promising.

As specialized leukocytes, DCs in different organs show various degrees of maturity and sometimes have been given organ-specific names (Toura et al.,1999), such as Langhans cells in skin. DCs also circulate in the blood circulation, accounting for 0.14% of peripheral blood leukocytes. Although the mechanism of DC activation has not been fully elucidated, DCs apparently infiltrate cancerous tissue, take up tumor antigens, and migrate to the periphery of the cancer where they present the antigen to T lymphocytes. In tumors, DCs often have been observed in association with T cells. According to another report, cancer cells expressing CD1d molecules become targets of NKT cells activated by KRN 7000 (Koezuka et al.,2000). Thus detection of CD1d expression by cancer cells might be an indication of a potential anticancer effect (Spada et al.,1998). Little has been reported about localization of DCs in the urinary bladder or its cancers, but Hart et al. (1981) found MHC class II<sup>+</sup> DCs beneath the epithelial layer, as has been seen for other epithelial surfaces (Maric et al.,1996). Inoue et al. reported that S100 protein-positive DCs, suspected to be a prognostic factor in various cancers, also are present in carcinomas of the urinary bladder (Inoue et al.,1993).

Antitumor effects of intravesical BCG therapy are well known, and involve activation of the immunologic response (Braudeau et al.,2001). However, BCG often shows serious

**Table 3. Tumor Multiplicities and Sizes of Bladder Tumor in Macroscopic Findings**

Group	Treatment		No. of effective animals	Tumor numbers per rat					Total
	0.05% BBN	intravesical instillation		diameter (d; mm)	d $\leq$ 1	1<d $\leq$ 2	2<d $\leq$ 5	5<d $\leq$ 10	
1	+	500 $\mu$ g/ml KRN7000	14	3.57	3.07	2.86	0.43	0.21	10.1±3.4
2	+	50 $\mu$ g/ml KRN7000	13	4.55	2.09	2.18	0.27	0	9.09±4.7
3	+	0.9% saline	14	3.08	1.31	1.92	0.15	0.23	6.69±4.9
4	+	no treatment	9	3.11	3.11	0.78	0	0	7.00±2.0

Data are mean SD ± values. BBN; N-butyl-N (4-hydroxybutyl) nitrosamine

**Table 4. Data for Urinary Bladder Histopathological Findings**

Group	Treatment	intravesical instillation	No. of effective animals	Simple hyperplasia	PN hyperplasia	Papilloma	TCC	SCC	AC
1	+	500µg/ml KRN7000	14	10 (71.4)	14 (100)	4 (28.6)	14 (100)	14 (100)	0
2	+	50µg/ml KRN7000	13	4 (33.3)	12 (100)	6 (50)	12 (100)	7 (58.3)	0
3	+	0.9% saline	14	7 (53.8)	13 (100)	4 (30.8)	13 (100)	6 (46.2)	1 (7.7)
4	+	no treatment	9	6 (54.5)	9 (100)	2 (15.4)	9 (100)	4 (36.4)	0

Data are incidence (%). BBN; N-butyl-N-(4-hydroxybutyl) nitrosamine, PN shows papillary or nodular, TCC shows transitional cell carcinoma, SCC shows squamous cell carcinoma, and AC shows adenocarcinoma.

side effects and, therefore new immunotherapeutic agents have been sought for bladder tumors. We expected that intravesical instillation of KRN 7000 would be effective against bladder cancer, as well as being essentially free of serious side effects. In mice, piloerection soon after intravenous administration and liver toxicity from KRN 7000 have been reported (Kobayashi et al.,1995), but we did not find any abnormalities in our samples of liver, spleen, or kidney, and observed no piloerection upon treatment.

In the present experiments, we planned to use an intravesical concentration of KRN 7000 that corresponded to the most effective dose in *in vitro* studies (100 ng/ml). We therefore used a similar dose for the high-dose group and one tenth of this for the low-dose group. The ideal duration of drug retention in the bladder and the volume to be instilled effective are uncertain. Agents for intravesical instillation therapy in humans (pirarubicin hydrochloride or cytarabine) usually are retained for only 30 min, and are instilled in a solution volume of 20 ml. We based duration and relative amounts on these protocols, in order to avoid missing a therapeutic effect of KRN 7000. The frequency of instillation corresponded to chemotherapy instillation protocols for the human bladder, once weekly. Intraperitoneal injection every 4 days has been reported to show a clear anticancer effect in mice, while overly frequent administration of the agent was avoided so as not to decrease cytokine induction (unpublished data).

In the present experiment, long cumulative intervals of respiratory insufficiency related to anesthesia may have caused lower body weights in catheterized rats. Multiplicity and tumor size of the bladder became larger in population to the dose of KRN 7000 without significance. Some studies of intravesical instillation therapy have suggested that catheterization itself promotes bladder carcinogenesis (Hart et al.,1981; Ohtani et al.,1984). However since the tumor multiplicity in group 3 (vehicle control group) was less than that in group 4 (non catheterized group), the explanation that the increase of multiplicity of bladder tumor in the KRN 7000 high dose group reflect tumor-stimulating effects of the catheterization itself appear inappropriate. In past, there have been reports that intravesically instilled Adriamycin (doxorubicin) and mitomycin C, which are widely used to treat human bladder cancer today, rather promote bladder carcinogenesis in BBN treated rodents (Ohtani et al.,

1984;Ikegami et al., 1967), and its reason has not been clarified, yet. In our present study, no significant differences in incidence of bladder carcinoma and putative preneoplastic lesions of urinary bladder was noted between groups, so it can be said that instillation treatment with KRN 7000 neither inhibited nor facilitated bladder carcinogenesis in these rats. Further investigation is necessary, however, to avoid premature conclusions from this specific negative result.

In conclusion, we could not observe any inhibitory effect of intravesical instillation of KRN 7000 in Fischer 344 rats exposed to a bladder carcinogen.

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