

The Effects of Sirolimus on Urothelial Lesions Chemically Induced in ICR Mice by BBN

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Abstract. *Background:* Sirolimus was originally used as an immunosuppressant drug but recent reports have indicated that it may have other potential biological effects as an anticancer drug. *The chemopreventive efficacy of sirolimus was evaluated in an experimental model of invasive urinary bladder cancer. Materials and Methods:* ICR mice received N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) in drinking water for a period of twelve weeks. Sirolimus was administered 5 days a week. Animals were sacrificed either one or four weeks after their final treatment. Ki-67 was immunohistochemically analysed in paraffin-embedded tissue. *Results:* No evidence of host toxicity was found. The incidence of BBN-induced invasive urothelial carcinoma was significantly reduced in mice treated with sirolimus. Preneoplastic and neoplastic lesions exhibited a significant decrease in cellular proliferation. *Conclusion:* Histopathological and immunohistochemical studies showed that sirolimus reduced tumour incidence and proliferation. Sirolimus should be considered for further *in vitro* and *in vivo* studies in order to provide evidence of effectiveness.

Bladder cancer is one of the most prevalent malignant tumours in the Western world (1). In Europe, urothelial cancer of the bladder is the fourth most frequent cancer among men, accounting for 7% of all cancer. Urothelial tumours of the bladder are usually superficial at presentation

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in 70 to 80% of patients, and invasive in 20 to 30%. The standard treatment for patients with muscle-invasive cancer is radical cystectomy. However, this gold standard only provides 5-year survival in about 50% of patients (2). In order to improve these unsatisfactory results, the use of perioperative chemotherapy has been explored since the 1980s, however, only marginal response was achieved. Thus, it is essential to develop more effective strategies and drugs for the treatment of urothelial invasive carcinoma, in order to improve the prognosis of these patients.

Animal models offer useful tools for studying the effect of potential therapies for many types of cancer including urothelial carcinoma (3). Sirolimus is a macrocyclic lactone. It was originally used as an immunosuppressant, but recent reports have indicated that it may have other potential biological effects as an anticancer drug (4, 5). This compound possesses antiproliferative and antitumour activity in solid tumours and demonstrates synergistic activity in conventional chemotherapy (6). Sirolimus forms a complex with the immunophilin prolyl isomerase FK binding protein complex (FKBP-12) that binds with high affinity to mammalian target of rapamycin (mTOR) (7, 8). This interaction inhibits mTOR kinase activity and subsequently decreases the phosphorylation of 4E binding protein-1 and inhibition of the 40S ribosomal protein p70 S6 kinase (9, 10). The antineoplastic effects of sirolimus have been ascribed to its ability to inhibit translation machinery involved in the regulation of the G₁- to S-phase transition in the cell cycle (11). To date, several studies have revealed that sirolimus can potently arrest the growth of cells derived from a broad spectrum of tumour types including rhabdomyosarcoma, neuroblastoma, glioblastoma, small cell lung cancer, osteosarcoma, gallbladder, pancreatic, breast and prostate

cancer, murine melanoma, leukaemia, and B-cell lymphoma (12-16). In addition, sirolimus and its derivatives suppressed the growth of several human and murine tumours *in vivo* (small cell lung cancer, pancreatic, renal, colon, gastric, gallbladder, and breast cancer) (17-22). It was also shown that sirolimus has an antiproliferative effect on the T24 human invasive bladder cancer cell line (23).

The goal of this investigation was to evaluate the therapeutic effect of sirolimus against urothelial tumorigenesis. We aimed to determine the capacity of sirolimus capacity to treat urothelial tumours that had been chemically induced by *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN). We also determined the labelling index in urothelial lesions induced by BBN and those induced by BBN followed by treatment with sirolimus.

Materials and Methods

Animals and chemicals. Four-week-old male ICR mice (weight 25 g) were obtained from Harlan (Barcelona, Spain). All mice used in the experiment were acclimatized for one week under routine laboratory conditions before starting the experiments. They were housed randomly in groups of 4-5 in a plastic cages, with hard wood chips for bedding. The animals were maintained in a room with a controlled temperature of $23\pm 2^{\circ}\text{C}$, a 12-hour light/dark cycle and $55\pm 5\%$ humidity. All procedures were performed in accordance with the European Communities Council Directive 86/609/ECC. During the course of this study, the animals were fed *ad libitum* with standardized food (Tecklad Global Diet, Harlan, Spain). Drinking water was supplied *via* a nipple watering system. BBN and sirolimus were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan) and Wyeth, respectively.

Animal experiments. A total of 63 mice were randomly divided into six groups of 10 mice per group, and one group of three mice as shown in Figure 1. Mice in group 1 were given 0.05% BBN solution as their drinking water for a period of 12 weeks. Mice in group 2 were the carcinogenic control group. To evaluate the histological changes induced in the urothelium by BBN treatment alone, the mice from groups 1 and 2 were killed thirteen weeks after the exposure had begun. After being treated with BBN solution for twelve weeks, groups 4 and 6 were then maintained with normal tap water and after this week had elapsed, sirolimus (1.5 mg/kg) was administered intraperitoneally five days a week for six consecutively weeks, *i.e.* until week 19 of the experiment. Mice were then untreated until their sacrifice at 20 and 23 weeks, respectively. After being treated with BBN solution, groups 4 and 6 were not subject to any treatment for one week. Mice in group 3 were the sirolimus control group, these animals drank tap water and were treated with sirolimus at the same dose. Sirolimus dosage was chosen based on previous experiments that used animal tumour models (22). The sirolimus solution was freshly prepared every day. The animals' drinking solution was changed once per week and the volume drunk was recorded. Weekly food and water intakes were also noted. For the duration of the study, the mice's state of health was monitored daily. Their body weights were initially measured once a week and then on a daily basis after treatment had begun.

Evaluation of treatment. All surviving animals were sacrificed either one or four weeks after their last treatment. Their urinary bladders were inflated *in situ* and collected. All organs were examined macroscopically for any changes and their weights were recorded. Lungs, heart, spleen, kidneys and liver were also submerged in formalin. Twelve hours later, the bladders were longitudinally cut and their mucosal surface was carefully examined for the existence of papillary or nodular lesions. All organs were carefully observed and cut, and then were also embedded in paraffin. Sections of 2 μm were cut and stained with haematoxylin and eosin.

Immunohistochemical. Proliferating cells were detected using anti-mouse Ki-67 antibody (TEC-3; DakoCytomation, Lisbon, Portugal). Immunohistochemical technique was performed according to the method published by our research group (24). Paraffin sections from a breast tumor with known immunoreactivity to Ki-67 antigens were used as a positive control. In order to analyse Ki-67 expression, slides were examined with a light microscope coupled to a digital camera system (Nikon) at a final magnification of $\times 400$. In each case, a minimum of 500 nuclei were counted in up to 10 consecutive microscopic fields per lesion. The Ki-67 labelling index (the fraction of proliferating cells) was calculated by dividing the number of Ki-67-positive cells by the total cell count. All sections were analyzed blind, twice.

Histology. Histological slides were observed under a light microscope. All the slides were examined without prior knowledge of the treatment given to the animals whose tissue samples were under investigation. Found lesions were classified and staged according to the World Health Organization/International Society of Urological Pathology consensus classification of urothelial (transitional cell) neoplasms of the urinary bladder (25). Urothelial lesions were categorized into either: simple hyperplasia, nodular hyperplasia, dysplasia, carcinoma *in situ* (CIS), papillary neoplasms of low malignant potential, low-grade papillary tumours, high-grade papillary tumours, invasive urothelial carcinoma, spinocellular carcinoma and squamous metaplasia.

Statistical methods. A descriptive study was performed for all the variables included in the study. Statistical analysis was performed using the SPSS 12.0 statistical package for Windows (SPSS Inc. USA). The differences between mean values of body, liver, bladder and kidney weights, and relative bladder, liver and kidney weights, as well as Ki-67 expression were assessed between those animals treated with sirolimus and the controls. Parametric data were analysed *via* analysis of variance (ANOVA) with Bonferroni's multiple comparison; non-parametric data were computed using the χ^2 test or Fisher's exact test with Bonferroni's correction. A *p*-value of < 0.05 was considered to be statistically significant.

Results

Animal growth and water and food consumption. The oral administration of BBN was well tolerated by all mice involved. There was no significant difference in body weight among the various groups over the course of the entire study period. The mean food and water intake was constant and similar in the different groups throughout the whole treatment period (data not show). Seven mice from the

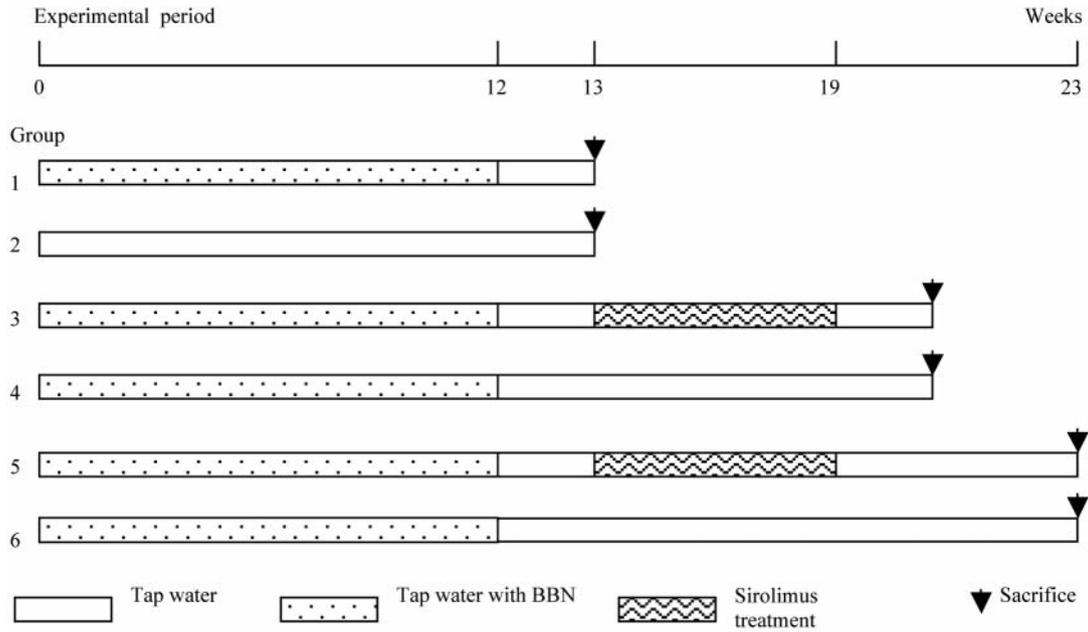


Figure 1. Experimental design to examine the effect of treatment with rapamycin in a model of urothelial carcinogenesis in male ICR mice.

various experimental groups died before the end of the study: none in groups 1, 2, 3 and 4; 1 from group 5 (10%); 3 from group 6 (30%) and 3 from group 7 (30%). As such, the mortality rate was higher in group 5 than group 4 and was similar between groups 6 and 7. Only those animals surviving the 23-week period were included in the effective numbers of mice subject to histopathological examination. During the treatment period, animals exhibited normal cage activity. The mean liver, kidney and bladder weights, as well as relative liver, kidney and bladder weights at the end of the study are indicated in Table I. Organ relative weights (liver, kidneys and bladder) were calculated as the ratio of the mouse organ weight to the mouse body weight.

Effects of sirolimus on urothelial tumorigenesis. We conducted detailed histopathological examination to determine the degree of urothelial tumour progression in relation to the therapeutic agent's effect on tumour development. No histopathological changes in urothelial cells were observed in the control groups (group 2 and 3). Table II summarizes the incidence of BBN-induced urothelial lesions in each group. There was a significant difference in invasive urothelial carcinoma between groups 4 (treated with sirolimus) and 5 (not treated with sirolimus) ($p=0.005$). Mice treated with sirolimus for 6 weeks and sacrificed 4 weeks (group 6) later had a lower incidence of invasive urothelial carcinoma (43%) than group 7 which was not treated with sirolimus. The incidence of tumours in groups of mice not

treated with sirolimus were greater than in those treated with this compound. However, squamous cell metaplasia and premalignant urothelial lesions such as simple hyperplasia, nodular hyperplasia, dysplasia and CIS were detected in mice treated and not treated with sirolimus. Low- and high-grade papillary tumours were only observed in the bladders of BBN-treated mice, in groups 5 and 7. Such neoplastic lesions were not detected in sirolimus-treated mice.

Non-urothelial lesions. No microscopic changes were seen in the liver, lungs, kidneys or gastrointestinal tract of control animals. However, we did observe two squamous cell carcinomata of the renal pelvis in some animals from groups 4 and 6. Squamous cell carcinoma of the renal pelvis are uncommon in mice; therefore these tumours were most likely induced by BBN. We also identified a hepatic metastasis of urothelial carcinoma in one animal from group 6.

Immunohistochemistry. To determine if 6 weeks of sirolimus treatment had any antitumour effects, we assessed the proliferation rate of preneoplastic and neoplastic lesions by measuring the Ki-67 labelling index. Normal urothelia, in the control group, were uniformly negative for Ki-67 expression. Ki-67 immunostaining was evident in the form of diffuse or dot-like nuclear and nucleolar staining. Invasive urothelial carcinoma from the sirolimus-treated groups, sacrificed 4 weeks after treatment was ended, had a significantly lower labelling index than did the invasive urothelial carcinomas

Table I. Liver, bladder, kidney weights of mice in each group.

Group	Treatment	Effective number of mice	Organ weight				Organ relative weight			
			Liver (g)	Right kidney (g)	Left kidney (g)	Bladder (g)	Liver (g)	Right kidney (g)	Left kidney (g)	Bladder (g)
1	BBN	10	2.28±0.24	0.39±0.05	0.37±0.03	0.16±0.04 ^a	0.056±0.0068	0.009±0.0009	0.009±0.0007	0.0039±0.0012
2	Control	10	2.29±0.29	0.34±0.07	0.35±0.05	0.13±0.02	0.055±0.0073	0.008±0.0011	0.008±0.0008	0.0032±0.0005
3	H ₂ O→SIR	3	2.41±0.18	0.34±0.02	0.34±0.01	0.12±0.01	0.065±0.0049	0.007±0.0010	0.007±0.0010	0.0035±0.0001
4	BBN→SIR	10	2.28±0.45	0.34±0.05	0.32±0.05	0.17±0.06	0.060±0.0063	0.009±0.0024	0.008±0.0022	0.004±0.0017
5	BBN→H ₂ O	9	2.37±0.61	0.36±0.05	0.33±0.04	0.24±0.08	0.059±0.0116	0.008±0.0010	0.008±0.0011	0.0059±0.0016
6	BBN→SIR	7	2.38±0.41	0.33±0.05	0.34±0.05	0.18±0.05	0.062±0.0081	0.009±0.0008	0.008±0.0008	0.0046±0.0014
7	BBN→H ₂ O	7	2.28±0.21	0.37±0.05	0.39±0.07	0.25±0.15 ^b	0.057±0.0065	0.009±0.0008	0.009±0.0017	0.0063±0.0034

Values are mean±S.D. SIR, Sirolimus; BBN, *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine; ^a*p*=0.018, statistically different from group 2, ^b*p*=0.015 statistically different from group 5. Organ relative weights (organ weight/animal weight).

Table II. Incidence of urothelial lesions in male ICR mice exposed to BBN and treated with sirolimus.

Group (n) Treatment	Number of mice (%) with histopathological classification									
	Normal urothelium	Simple hyperplasia	Nodular hyperplasia	Dysplasia	CIS	PNLMP	Low-grade papillary tumour	High-grade papillary tumour	Invasive urothelial carcinoma	Squamous cell metaplasia
1 (10) BBN	-	2 (20)	5 (50)	10 (100)	6 (60)	-	-	-	3 (30) ^a	7 (70)
2 (10) Control	10 (100)	-	-	-	-	-	-	-	-	-
3 (3) H ₂ O→SIR	3 (3)	-	-	-	-	-	-	-	-	-
4 (10) BBN→SIR	-	5 (50)	2 (20)	7 (70)	2 (20)	-	-	-	2 (20) ^{a,b}	6 (60)
5 (9) BBN→H ₂ O	-	3 (33.3)	3 (33.3)	9 (100)	2 (22.2)	1 (11.1)	1 (11.1)	2 (22.2)	8 (88.8) ^a	7 (78)
6 (7) BBN→SIR	-	6 (85.7)	-	7 (100)	4 (57.1)	-	-	-	3 (43)	3 (43)
7 (7) BBN→H ₂ O	-	-	4 (57.1)	7 (100)	-	-	-	2 (28.5)	5 (71.4) ^c	5 (71.4)

CIS, Carcinoma *in situ*; PNLMP, papillary neoplasms of low malignant potential; values are Mean±S.D; ^aOne case with concomitant squamous cell carcinoma; ^b*p*=0.005, statistically different from group 5; ^cthree cases with concomitant squamous cell carcinoma.

from the group that was not treated (mean: 22.33% versus 39.25% , *p*=0.021). The mean labelling index values for each lesion evaluated are summarized in Table III.

Discussion

The results of this study showed, for the first time, that the incidence of BBN-induced invasive urothelial carcinoma can be significantly reduced in mice treated with sirolimus. No evidence of host toxicity as measured by body weight, cage activity, fur texture and organ weights (liver, bladder and kidney) were found in animals treated with sirolimus. Squamous cell metaplasia, simple hyperplasia and nodular hyperplasia were detected in mice both treated and not treated with sirolimus. Parada *et al.* studied the effect of sirolimus in rat urothelial tumours induced by BBN and concluded that sirolimus had no chemopreventive effects

(26). However, the rat model is only recommended for studying papillary tumours while the mouse model is the most appropriate for studying invasive urothelial tumours (3). In our study, low- and high-grade papillary tumours were the lesions less frequently and only observed in BBN-treated mice; these lesions were not detected in sirolimus-treated mice. However, we observed that dysplasia and CIS were present in groups treated with sirolimus (highest in group 6). Invasive urothelial carcinoma was significantly reduced in mice treated with sirolimus, however, this reduction was not statistically significant when the animals were killed four weeks after their final sirolimus injection. The efficacy of sirolimus must be balanced and its role in urothelial invasive carcinoma therapy strategy (combination with other drugs) must be tested. Squamous cell metaplasia was found in cases treated with sirolimus and not-treated groups. Nevertheless, squamous cell carcinoma of renal

Table III. Labelling index for the various urothelial lesions and experimental groups.

Group (n) Treatment	Histopathological classification						
	Normal urothelium	Simple hyperplasia	Dysplasia	CIS papillary tumour	High-grade carcinoma	Invasive urothelial metaplasia	Squamous cell
1 (10) BBN	-	19.5±4.73 ^a	23.75±4.23 ^b	29±2.82	-	38.50±17.67	29.57±3.55
2 (10) Control	-	-	-	-	-	-	-
3 (3) H ₂ O→SIR	-	-	-	-	-	-	-
3 (10) BBN→SIR	-	15.50±3.32 ^c	19.33±5.54 ^d	24±2.82	-	21.50±0.70	28.42±5.13
4 (9) BBN→H ₂ O	-	15.00±2.58 ^{e,f}	20.71±3.54	-	35±5.56	26.40±3.97	24.75±5.56
5 (7) BBN→SIR	-	15.40±4.97 ⁱ	20.66±4.72	21±8.67	-	22.33±2.51 ^j	28.33±2.08
6 (7) BBN→H ₂ O	-	26.00±1.82 ^g	27.33±9.71	-	43±11.78	39.25±4.99 ^h	24.75±4.57

Statistically different from invasive carcinoma ^a $p=0.012$, ^b $p=0.043$, ^c $p=0.003$, ^g $p=0.036$, ^j $p=0.003$ (group 7); statistically different from squamous metaplasia: ^e $p=0.005$, ^d $p=0.016$, ^f $p=0.019$, ^h $p=0.021$; ⁱ $p=0.005$, statistically different from simple hyperplasia (group 7).

pelvis and hepatic metastasis were only identified in animals not treated with sirolimus. Chemotherapy using sirolimus has already been experimentally applied to tumours such as lung, gastric, gallbladder and breast cancer (20, 22). Wang *et al.* showed that sirolimus does not absolutely abolish the occurrence/recurrence of urothelial carcinoma among renal transplant recipients and its potency as an anticancerous immunosuppressant for transplant recipients with urothelial carcinoma deserves to be further defined in larger studies (27). Pinto-Leite *et al.* and Fechner *et al.* evaluated the effect of sirolimus on several human bladder carcinoma cell lines and concluded that sirolimus delayed the proliferation of the cancer cell lines (23, 28). At the cellular level, the exact mechanisms for the effect of sirolimus on urothelial tumours of ICR mice require further investigation. However, our initial analysis suggests that sirolimus treatment induced the reduction of cellular proliferation in the cells of responsive tumours and preneoplastic and neoplastic lesions were shown to be responsive to sirolimus treatment and exhibit a significant decrease in cellular proliferation as given by Ki-67 protein, a widely known suitable and useful marker of the proliferating fraction of a given cell population (24, 29). This could have occurred through the inhibition of mTOR phosphorylation and decreased protein synthesis (9). Integrated analyses of mouse and human bladder cancer provide a rationale for investigating mTOR inhibition for treatment of patients with invasive disease (30). In conclusion, the results of this study suggest that sirolimus is active against urothelial bladder cancer and reduces invasive urothelial carcinoma incidence in this mouse model of human invasive urothelial carcinoma. Thus, sirolimus should be considered a potential drug against bladder cancer in combination with standard drugs and should be considered for further *in vitro* and *in vivo* studies.

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