Experimental Study of the Anticancer Effect of Gemcitabine Combined with Sirolimus on Chemically Induced Urothelial Lesions

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Abstract. *Background: The purpose of this study was to determine the efficacy of a combination of gemcitabine and sirolimus in a mouse model of invasive bladder cancer. Materials and Methods: Gemcitabine (50 mg/kg) and sirolimus (1.5 mg/kg) were administered to animals previously exposed to N-butyl-N-4(hydroxybutyl)nitrosamine in drinking water. Tumour development was determined by histopathological evaluation. Results: Both drugs were well tolerated by animals. The incidence of lesions in mice treated with gemcitabine was lower in comparison to those not treated, however this result was not statistically significant. The incidence of invasive bladder cancer in animals treated with sirolimus was statistically lower (20%) than in animals not treated (54%) (p=0.008). The results indicate that this drug combination has no statistical significance on the development of pre-neoplastic urothelial lesions and had only a minor impact on invasive bladder cancer incidence in mice. Conclusion: The combination of gemcitabine and sirolimus had only a marginal impact on invasive bladder cancer in a mouse model.*

Bladder cancer is one of the most prevalent malignant tumours in the Western world (1). In Europe, bladder urothelial cancer is the fourth most frequent cancer among men, accounting for 7% of all cancer cases. Urothelial tumours of the bladder are usually superficial at presentation in 70 to 80% of patients and invasive in 20 to 30%. The standard treatment for patients with muscle-invasive cancer is by means of radical cystectomy. However, even using this gold standard, only around 50% of patients survive a further five years or more (2). In order to improve these disappointing results, the use of perioperative chemotherapy has been studied since the 1980s. However, only a marginal level of response has been achieved. Thus, it is crucial to develop more effective strategies and drugs for the treatment of invasive urothelial carcinoma in order to improve the prognosis of patients that are affected.

For more than two decades, gemcitabine (2'2-difluorodeoxycytidine), a pyrimidine analogue, originally investigated as an antiviral agent and later developed as an anticancer drug, has been used to treat solid tumours (pancreatic cancer, non-small cell lung cancer, breast cancer and ovarian cancer) (3-5). Gemcitabine is a prodrug that requires cellular uptake and a number of steps in order for it to be activated (4, 6). It is phosphorylated by deoxycitidine kinase into its di-and tri-phosphate metabolites and is incorporated into the DNA and RNA, causing cell growth inhibition, as well as potentially triggering apoptosis (7-9). The *in vivo* and *in vitro* effects of gemcitabine have already been investigated by several researchers in a murine model of superficial bladder cancer (10) and in human invasive urothelial cancer cell lines (11, 12).

Sirolimus, also known as rapamycin, was discovered in 1965. For years, sirolimus has been known to possess unique

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biological properties including immunosuppressive, antifungal and antiproliferative actions (13). Sirolimus is a mammalian target of rapamycin (mTOR) inhibitor (14). mTOR plays an important role in cellular signal transduction mediated by phosphatidylinositol 3 kinase (15). It is expressed in virtually all mammalian cells but is thought to play a particularly important role in cancer cells (16). The activation of mTOR results in the control of catabolism, anabolism, proliferation, growth, angiogenesis and apoptosis (16, 17). *In vivo*, the effects of sirolimus in a mouse model of invasive bladder cancer were investigated by our team (18) and by Seager *et al*. (19). Both studies concluded that sirolimus effectively prevents the progression of urothelial lesions into invasive bladder cancer. Sirolimus also reduced the number and volume of chemically induced papillary tumours in rats (20). *In vitro*, the cytotoxic effects of sirolimus were investigated by Pinto-Leite *et al*. (21) and Hansel *et al*. (22). When the results of these studies are combined, it reveals that sirolimus inhibits cellular proliferation in several lines of human invasive bladder carcinoma.

Rational polytherapy presupposes that anticancer drugs with different mechanisms of action when combined may have superior antineoplastic effect than when these agents are used individually. The *in vitro* combination of gemcitabine and sirolimus was shown to be effective against human cholangiocarcinoma cell lines (23), human pancreatic cancer cell lines (24) and breast cancer cells (25). Our literature research has revealed that only one *in vivo* research study has been carried out looking into the combined effect of these drugs in an orthotopic pancreatic cancer model (24). Taking into consideration our previous results on the effect of sirolimus alone on an invasive bladder cancer mouse model and the *in vitro* effects of gemcitabine, the present study was carried out in order to evaluate the efficacy of gemcitabine and sirolimus combination in urothelial lesions induced in mice by N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN).

Materials and Methods

Animals. Four-week-old male ICR mice were obtained from Harlan Interfauna Inc (Barcelona, Spain), and housed in plastic cages using wood chips for bedding. All the mice were placed in ventilated chambers, in groups of 4-5 mice in plastic cages, in which the temperature (23±2˚C) and humidity (50±10%) were controlled, while they were also subject to a 12/12 hour light/dark cycle. All animals had access to a standard laboratory diet and bottled water *ad libitum*. After allowing a one-week acclimatisation period, the animals were then used in this study. The animals were treated in accordance with the European Community's Council Directive 86/609/ECC.

Chemicals. BBN was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Gemcitabine (Gemzar®) and Sirolimus (Rapamune®) were purchased from Lilly (Queijas, Portugal) and Wyeth (Alges, Portugal), respectively.

Animal experiments. For the invasive urothelial bladder cancer model, a total of 91 ICR male mice were randomly divided into 7 groups (Group I: n=10; Group II: n=12; Group III: n=10; Group IV: n=14; Group V=15; Group VI=15; Group VII=15) (Figure 1). Groups II, IV, V, VI and VII received BBN (0.05%) in drinking water, *ad libitum*, over the course of twelve weeks. Groups I and III were used as negative controls and were not exposed to BBN.

One week after BBN exposure was brought to an end, the animals in Groups I and II were euthanised, in order to classify the urothelial lesions induced by BBN. The other groups were given normal tap water until the end of the experimental procedure. One week after ending BBN treatment began. Animals in Group V were intraperitoneally administered gemcitabine (50 mg/kg) twice a week for six weeks. Animals in Group VI were intraperitoneally administered sirolimus (1.5 mg/kg) alone, 5 days a week for 6 weeks. Sirolimus was administered simultaneously with gemcitabine to the animals of Group VII, using the same methodology (dosage, administration route and periodicity). The mice in group IV were the control group. After ending their 12-week exposure to BBN, these animals were given normal tap water until the end of the experimental study (at 20 weeks).

The animals' drinking solutions were changed once a week or earlier if necessary, and the volume drunk was recorded. Weekly food intake was also noted. All mice were monitored throughout the experiment for signs of distress and loss of body weight. Animals' body weights were initially measured once a week. After the beginning of treatment, body weights of animals' from Group V were measured twice a week and for animals belonging to Groups VI and VII, this measurement was made daily, allowing the adjustment of drug dosages to individual weight variations.

Evaluation of treatment. All the surviving animals were euthanized by means of pentobarbital overdose anaesthesia one week after the end of treatment (week 20). After macroscopic evaluation, their organs (lungs, heart, spleen, kidneys and liver) were sampled and their weights recorded. Urinary bladders were fixed *in situ* with 100 μl of phosphate-buffered formaldehyde and then immersed, just as the other organs, in the same solution for 12 hours. After fixation, the bladders were cut longitudinally and their mucosal surface was carefully examined for the existence of macroscopic lesions. The organs were then cut and they were embedded in paraffin and sections of 2 μm were routinely stained with haematoxylin and eosin. This was carried out in order to evaluate the morphological changes induced by BBN exposure and to determine the effects of treatments using gemcitabine and sirolimus.

Histology. Histological slides were observed under a light microscope by two different investigators in a blind fashion. Histological lesions found in the various groups were classified and staged according to the World Health Organization/International Society of Urological Pathology's consensus classification of urothelial (transitional cell) neoplasms of the urinary bladder (26). Urothelial lesions were categorized as 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 or epidermoid metaplasia.

Statistics. A descriptive analysis was performed for all variables included in the study. Data was statistically analysed using SPSS 12

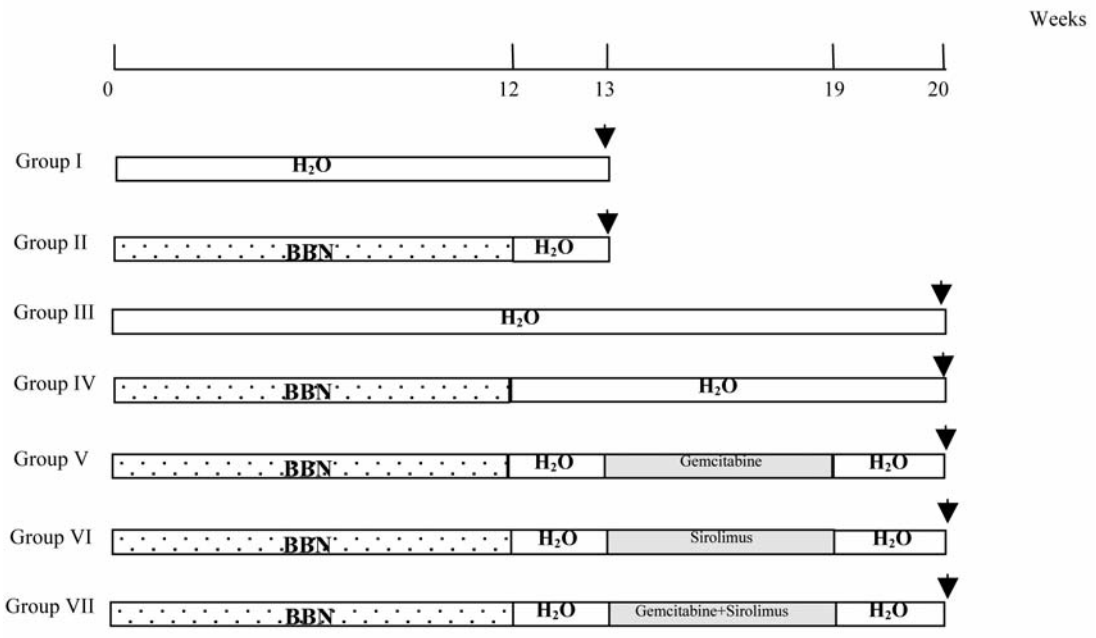

Figure 1. *Experimental design (black arrows indicate euthanasia).*

(SPSS Inc. USA). The differences between mean values of body, liver, bladder, kidney weights, as well as relative bladder, liver and kidney weights, were assessed among those animals treated with gemcitabine, sirolimus, gemcitabine plus sirolimus and the controls. Relative organ weights (liver, kidney, spleen, lungs, heart and bladder) were calculated as the ratio of the organ weight to the body weight. Parametric data were analysed via analysis of variance (ANOVA) with the Bonferroni correction multiple-comparison method; non-parametric data were computed using the χ^2 test or Fisher's exact test with the Bonferroni correction. A *p*-value of <0.05 was considered to be statistically significant.

Results

General findings. Five animals were found dead during the course of the experiment: one mouse from Group IV (BBN); one mouse from Group VI (BBN+sirolimus) and three from Group VII (BBN+gemcitabine+sirolimus). Given the fact that these animals were not found until 24 hours after their death, a complete necropsy was not performed due to the advanced *post-mortem* changes that had taken place.

Only those animals surviving all the experimental procedures were included in the effective numbers of mice subject to histopathological examination. The treatments were well tolerated. During the experimental procedure, all animals exhibited normal cage activity. The mean food and water intake was constant and similar across the different groups throughout the whole treatment period (data not shown).

When comparing the initial and final weight of the mice that had been subjected to different treatments, we concluded that only the data for Group VII (BBN+gemcitabine+sirolimus) are statistically different from those of Group III and IV (not exposed and not treated, respectively) (Table I).

Table I shows the mean values of liver, kidneys and bladder weights, as well as relative liver, kidneys and bladder weights. The relative weights of organs (liver, kidneys and bladder) were calculated as the ratio of the mouse's organ weight by the mouse's (final) weight. The initial and final weights of mice from different groups are also presented.

Effects of BBN on urothelial tumorigenesis. We conducted detailed histopathological examinations in order to determine the degree of urothelial lesions induced by BBN and determine their response to treatment. Table II summarizes the incidence and classification of histologically diagnosed urothelial bladder lesions. When comparing Groups I and II, we observed that 100% of those who did not receive BBN in drinking water (animals from Group I) exhibited a normal urothelium. Animals from Group II, which drank *ad libitum* water with BBN, developed pre-neoplastic and/or neoplastic lesions, namely simple and nodular hyperplasia, dysplasia, CIS, invasive carcinoma and epidermoid metaplasia.

Effects of gemcitabine, sirolimus and their association on urothelial tumorigenesis in mice. To determine the efficacy of drugs to treat chemically induced urinary lesions in mice, all the treated groups (V, VI, and VII) were compared to the group which was exposed to BBN but not treated (IV).

Table I. *Mean (±SD) of initial and final body weights, and relative weights of liver, kidney and bladder at the end of the study.*

Group	I	II	III	IV	V	VI	VII
	H_2O	BBN	H_2O	BBN+H_2O	BBN+ Gemcitabine	BBN+ Sirolimus	BBN+ Gemcitabine+ Sirolimus
Mean initial body weight (g)	30.05±2.04	28.12±1.51	27.81±1.62	28.38±1.29	28.33±2.17	29.42±2.01	28.4±1.68
Mean final body weight (g)	43.50±3.23	38.66±3.08	41.68±2.76[a]	42.06±2.73[b]	40.14±3.61	41.26±3.95	39.92±2.86
Mean weight (g)							
Bladder	0.280±0.114	0.267±0.115	0.16±0.52	0.38±0.29[d]	0.19±0.08	0.197±0.08	0.15±0.08
Right kidney	0.327±0.018	0.347±0.041	0.35±0.07	0.38±0.06	0.35±0.05	0.37±0.08	0.32±0.039
Left kidney	0.341±0.042	0.343±0.051	0.34±0.07	0.41±0.166	0.36±0.05	0.35±0.09	0.33±0.12
Liver	2.32±0.41[c]	1.95±0.28	2.3±0.25	2.08±0.25	2.29±0.35	2.27±0.23	2.08±0.24
Mean relative weight							
Bladder	0.0063±0.0022	0.0069±0.003	0.0038±0.0013	0.0095±0.0077[e]	0.0045±0.0018	0.0049±0.0022	0.004±0.0019
Right kidney	0.0075±0.00054	0.009±0.001	0.0084±0.0015	0.009±0.0014	0.008±0.0009	0.009±0.0022	0.0083±0.0011
Left kidney	0.0078±0.001	0.0089±0.001	0.0081±0.0013	0.0098±0.0042	0.0085±0.0008	0.0086±0.0023	0.0087±0.0029
Liver	0.053±0.0091	0.050±0.0051	0.055±0.0049	0.05±0.0048	0.054±0.0064	0.057±0.0038	0.054±0.005

[a]Significantly from Group VII (p=0.002); [b]significantly from Group VII (p<0.001); [c]significantly from Group II (p<0.005); [d]significantly from Groups III, V, VI, VII (p<0.005); [e]significantly from Groups III, V, VII (p<0.005).

Table II. *Incidence of histological lesions*.

Group	I	II	III	IV	V	VI	VII
Histological lesion	H_2O	BBN	H_2O	BBN+H_2O	BBN+ Gemcitabine	BBN+ Sirolimus	BBN+ Gemcitabine+ Sirolimus
Normal urothelium	10/10 (100%)	0 (0%)	10/10 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Simple hyperplasia	0 (0%)	8/12 (67%)	0 (0%)	10/13 (76.9%)[a]	10/15 (66.67%)	10/14 (71.43%)	10/12 (83.3%)
Nodular hyperplasia	0 (0%)	7/12 (58.3%)	0 (0%)	9/13 (69.23%)	7/15 (46.67%)	4/14 (28.6%)	5/12 (41.7%)
Dysplasia	0 (0%)	12/12 (100%)	0 (0%)	13/13 (100%)	15/15 (100%)	12/14 (85.71%)	9/12 (91.7%)
CIS	0 (0%)	4/12 (33%)	0 (0%)	3/13(15%)	0 (0%)	0 (0%)	0 (0%)
Papilloma	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1/14 (7.14%)	0 (0%)
Papillary neoplasm of low malignant potential	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1/15 (6.67%)	0 (0%)	0 (0%)
Low-level papillary carcinoma	0 (0%)	0 (0%)	0 (0%)	1/13(7.7%)	1/15 (6.67%)	0 (0%)	0 (0%)
High-level papillary carcinoma	0 (0%)	0 (0%)	0 (0%)	1/13(7.7%)	0 (0%)	0 (0%)	0 (0%)
Invasive carcinoma	0 (0%)	4/12 (33%)	0 (0%)	7/13(54%)[b]	3/15 (20%)	1/14 (7.14%)[c]	5/12 (41.7%)
Epidermoid metaplasia	0 (0%)	8/12 (67%)	0 (0%)	10/13 (76.92%)[d,e]	6/15 (40%)	5/14 (35.71%)	6/12 (50%)
Time of euthanasia (weeks of age)	13				20		

[a]Statistically different from group VI (p=0.035); [b]statistically different from group VI (p=0.008); [c]statistically different from group VII (p=0.037); [d]statistically different from group VI (p=0.031); [e]statistically different from group V (p=0.049); CIS: carcinoma *in situ*.

Table III. *Incidence of pre-neoplastic and neoplastic lesions in groups IV to VII at euthanasia*.

	Group IV	Group V	Group VI	Group VII
	BBN+H_2O	BBN+Gemcitabine	BBN+Sirolimus	BBN+Gemcitabine+Sirolimus
Pre-neoplastic lesions	32/54 (59.3%)	32/43 (74.42%)	26/33 (78.8%)	24/35 (68.6%)
Neoplastic lesions	22/54 (40.7%)	11/43 (25.58%)	7/33 (21.2%)	11/35 (31.4%)

The incidence of BBN-induced invasive urothelial carcinoma was lower in those mice treated with gemcitabine (Group V) (20%) compared to non-treated animals (54%). Dysplasia was the only lesion that was not affected by gemcitabine treatment. All other histological alterations, such as simple or nodular hyperplasia, CIS, low- and high-grade papillary tumours, also presented lower incidence in animals treated with gemcitabine than in animals not treated (Group IV). However, these results were not statistically significant.

The incidence of urothelial lesions in animals treated with sirolimus (Group IV) was lower than in animals not treated. In this group, invasive urothelial carcinoma was 7.14% compared to 54% in animals not treated (p=0.008).

Group VII, treated with a combination of gemcitabine and sirolimus, exhibited a reduced number of urothelial lesions when compared with those animals not treated. The highest reductions were observed in cases of invasive urothelial carcinoma and nodular hyperplasia.

CIS was only identified in animals exposed to BBN; this lesion was not observed in animals treated with gemcitabine, sirolimus or a combination of both drugs. Regardless of the kind of pre-neoplastic and neoplastic lesions found, we observed that the incidence of pre-neoplastic lesions was greater in animals treated, although the incidence of neoplastic lesions was greater in animals not treated (Table III).

Non-urothelial lesions. The liver, lungs, kidneys and gastrointestinal tracts of all animals used were observed and no microscopic changes were identified.

Discussion

In this report, we have pursued *in vivo* pre-clinical studies of invasive bladder cancer chemically induced by BBN in a mouse model in order to study the effects of the combined use of gemcitabine and sirolimus. Several researchers have already evaluated the effect of gemcitabine alone or in association with other drugs in syngeneic and xenograft models of cancer (27, 28). To our knowledge, there have been no previous experimental studies designed to evaluate the efficacy of gemcitabine in chemically induced murine invasive bladder cancer.

Treatments using gemcitabine and sirolimus were well tolerated, with no demonstrable side-effects. Our results indicate that treatment with gemcitabine as a single agent, or sirolimus also as a single agent, reduces the incidence of chemically induced urothelial lesions in mice. The incidence of invasive bladder cancer in animals treated with sirolimus was statistically lower than in those animals not treated (p=0.008), and these results are similar to those previously published by our team (18). Although not statistically different, sirolimus use exhibited a slightly reduced the incidence of more strongly neoplastic lesions than did gemcitabine. The drugs showed a statistically significant decrease in the bladder weight of the animals treated with gemcitabine and sirolimus compared with those that went untreated. Based on previous *in vitro* studies (25) and on the different mechanisms of action of these drugs, we were expecting additive or synergic results to come from this combination. However, despite a reduction of neoplastic lesions, the results obtained were not as good as when each drug was used individually. The discrepancy between the efficacy in combination therapy *in vitro* and *in vivo* is interesting. The reasons for this difference may be multi factorial and complex. Perhaps the chemotherapeutic effect of each drug in combination might be influenced by the accompanying drug (29) and by the expression of several enzymes involved in their mechanism of absorption, metabolization, distribution and elimination (4). Giovannetti *et al*. (30) obtained similar *in vitro* results with the combination of gemcitabine and paclitaxel. They explain their results on the basis of the cell-cycle effect of the drugs, since a shift towards the S-phase after gemcitabine and a progressive G_2/M block after paclitaxel treatment were both demonstrated. This could also be a hypothesis to explain our results.

The combined used of gemcitabine and sirolimus exhibited marginal anti-neoplastic activity when used to treat superficial bladder cancer. However, further studies are required to clarify interactions between these drugs and assess their benefits when used in combination.

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