

BBN as an Urothelial Carcinogen

C. VASCONCELOS-NÓBREGA¹, A. COLAÇO², C. LOPES³ and P. A. OLIVEIRA²

¹Agrarian School of Viseu, Polytechnic Institute of Viseu, Viseu, Portugal;

²Department of Veterinary Sciences, CECAV, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal;

³Pathology and Molecular Immunology, Abel Salazar Institute of Biomedical Sciences –
Oporto University, Oporto, Portugal

Abstract. *Experimental urinary bladder tumours have been proposed as a useful model for the study of urinary bladder carcinogenesis, as well as for evaluating new therapeutic strategies. Consequently, the administration of chemical carcinogens is one of the most commonly used methods for inducing urinary bladder tumours. N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) is, undoubtedly, the most-used urothelial chemical carcinogen. BBN belongs to the nitrosamine family, a wide group of alkylating agents that are able to induce bladder tumours in laboratory animals. Depending on the animal species, the spectrum of urothelial lesions induced by BBN varies, but is similar to those observed in humans. BBN has a high propensity to induce mutations affecting the expression of genes such as p53, RAS and H19 among others. The aim of this study was to review BBN as a urothelial tumour inducer, taking into consideration its chemical characteristics, properties and spectrum of lesions induced, as well as its possible applications.*

Bladder cancer is among the most common malignancies worldwide, and it assumes greater prominence in developed countries. The most recent data, published in 2011, showed an estimated 386,300 new cases and 150,200 deaths in 2008. Bladder cancer is the second-most common tumour of the genitourinary tract and the second-most common cause of death in patients with genitourinary tract malignancies (1). In humans, the most common cell type of bladder cancer is transitional cell carcinoma, although adenocarcinomas, squamous cell carcinomas and sarcomas can also occur (2). The majority of patients with bladder cancer, 75-85%, are

diagnosed with superficial tumours, while the remaining 15% to 25% have invasive ones (2, 3). The superficial ones are low-grade, well-differentiated papillary tumours that do not invade or metastasize. Histological evolution in this case starts as hyperplasia, evolving to papilloma, papillary urothelial neoplasms of low malignant potential and papillary carcinoma (low- and high-grade) (4). The invasive tumours originate as flat lesions such as hyperplasia, dysplasia and carcinoma *in situ* that then evolve into invasive carcinomas (5). These are high-grade tumours that frequently have areas of squamous cells or glandular differentiation or areas of undifferentiated tumour, including small-cell or spindle-cell variations. Invasive tumours are devastating, since over 50% of the patients will die from metastatic disease (2).

Experimental Models of Bladder Cancer

The need for experimental models of tumours that are as similar as possible to those in humans has prompted researchers to attempt various approaches. Animals such as dogs, rabbits, guinea pigs and hamsters have all been used in the past to study urinary bladder carcinogenesis (6). However, for ethical reasons and due to the conditions in animal facilities, dogs cannot be used in experimental models of carcinogenesis. Animal facilities in order to maintain guinea pigs and rabbits also require special equipment, making models difficult to apply. For these reasons, small rodents, such as mice and rats, are the most common species used. These animals have a lower urinary tract that is comparable to that of humans, and neoplasms in their bladders are morphologically very alike (7). On the other hand, in most strains of rodents, bladder cancer is not commonly found unless a chemical or other treatment is applied (8). Another advantage is associated with the fact that these are small animals and there is an abundance of information concerning the biological characteristics of neoplastic development in these species (6). Mouse strains such as BALBC, C57BL and

Correspondence to: P.A. Oliveira, Agrarian School of Viseu, Polytechnic Institute of Viseu, Viseu, Portugal. E-mail: pamo@utad.pt

Key Words: Urothelial, carcinogenesis, N-butyl-N-(4-hydroxybutyl) nitrosamine, tumours, review.

Table I. Genetically altered animals used in carcinogenesis studies.

Transgenic animal	Genetic alteration	Animal	Spectrum of lesions
UPII-SV40T	SV40T	Mouse	Non-invasive bladder cancer; CIS (low copy number); invasive carcinomas (high copy number) (12, 16-18)
CK19-TAg	SV40T	mouse	CIS; invasive carcinomas (19)
Nrf2 ^{-/-}	null mutation in both <i>Nrf2</i> alleles	mouse	Higher sensitivity to BBN than the wild type (20-22)
H-Ras	Activated <i>Ha-ras</i> oncogene harboring a point mutation at codon 61 of the second exon converting CAG to CTG	mouse	Urothelial hyperplasia followed by superficial papillary tumors of the bladder (12, 23, 24)
H-Ras	<i>H-Ras</i> ^{Q61L} mutation	mouse	Develop early urothelial lesions (25)
p53 ^{+/-}	Null mutation in one <i>p53</i> allele	mouse	Higher sensitivity to BBN than the wild type (26, 27)
p53 ^{flox/flox} ; Pten ^{flox/flox}	Conditional alleles for both <i>p53</i> and <i>Pten</i>	mouse	CIS; high-grade invasive carcinoma with areas of transitional cell, squamous, sarcomatoid carcinoma (28)
p27 ^{kip1}	null mutation in both <i>p27</i> alleles	mouse	Higher sensibility to BBN than the wild type; early onset of tumour (29)
Hras 128	3 Copies of the human c-Ha-Ras proto-oncogene	rat	Higher sensitivity to BBN than the wild-type (30, 31)

CIS: Carcinoma *in situ*; BBN: *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine.

ICR, and rat strains such as Wistar, Sprague-Dawley and Fisher, are among the animals most used in bladder cancer research. The Brown Norway and the DA/Han rat are two rat strains with a high incidence of spontaneously occurring urothelial tumours of the bladder, and for this reason these models can be used in experimental protocols without the application of chemical carcinogens (9). Besides these, there are also genetically altered animals (that lack genes, express genes or have mutated genes) (10, 11) that exhibit increased susceptibility to developing urothelial lesions and even cancer (6). Examples of these animals are presented in Table I. These genetic alterations provide an ideal system for understanding the roles of molecular events which take place in bladder carcinogenesis (11, 12, 29, 30), allowing it to be determined if genetic changes, including oncogene overexpression, mutation, or tumour-suppressor gene loss, can increase the risk of neoplastic progression (29, 31).

These models are useful for extrapolating the results from animal carcinogenicity studies into human risk assessment (31) and also provide *in vivo* platforms for testing strategies for preventing bladder cancer (30, 32). There are several disadvantages associated with the use of transgenic models in cancer research, one being that mutation induction in a given tissue does not always lead to tumour development in that tissue. Moreover, these models also require a significant investment of time and resources towards tumour establishment (31, 33, 34).

Over the years, different methods have been developed to induce tumours in the mouse and rat urinary bladder:

Foreign bodies: In 1951, Jull successfully induced the development of urinary bladder tumours *via* surgical implantation of pellets containing cholesterol, paraffin and

several chemicals (35, 36). Later, in 1966, Bryan and Springberg intravesically implanted other compounds such as arachic acid, hexaethylbenzene, hexamethylbenzene, palmitic acid and stearamide, individually compressed as pellets, into the mouse bladder and verified that all these compounds were associated with the development of bladder tumours. Foreign bodies within the lumen of the urinary bladder play a role in promoting carcinogenesis, since they can irritate or traumatize the urothelium, stimulating mitotic activity and thereby causing the development of nodular and papillary hyperplasia (35-37).

Urinary calculi: Urinary tract calculi represent foreign bodies, similar to pellets, but do not require surgical implantation into the bladder lumen (3, 8, 38). They may act as a mitotic stimulus that makes calculus-bearing rodent bladders more susceptible to tumorigenesis (39). Numerous chemicals such as uric acid, calcium oxalate, uracil, melamine, among others, have been associated with the development of urinary calculi (8, 40-43). The calculi can arise from the administered chemical itself or from an endogenous metabolic product that is caused by the administration of the chemical. Lalich first reported the induction of stone formation in the urinary bladder of rats *via* oral administration of uracil (41). The administration of 3% uracil in the rat diet for a period of two weeks induced urolithiasis and mechanical irritation of the rat bladder urothelium, which at first responded by the development of papillomatosis and papillary/nodular hyperplasia. These lesions gradually disappeared after cessation of dietary uracil, through an enhanced apoptotic process (40). Nevertheless, uracil administered to mice and rats at low dosages does not produce calculi or tumours, which shows that a threshold effect for secondary carcinogenesis is

necessary. This high-dose (threshold) phenomenon appears to occur more readily in rodents than in humans. In fact, urolithiasis is not a cause of bladder cancer in humans (44). Urothelial carcinogenesis related to calculus formation is also influenced by factors such as urine pH, volume, osmolality, cationic and anionic concentration of minerals and quantitative and qualitative differences in the presence of urinary protein (8, 45). An increased urinary pH and an elevated sodium concentration increase cell proliferation in the urothelium and promote tumorigenesis (39, 45-47).

Irradiation: Radiotherapy not only results in beneficial effects regarding tumour control, but is also associated with adverse effects in normal tissues. For this reason, irradiation is one of the most widely researched carcinogens (48). The urinary bladder, due to its location, is an organ that is frequently affected by pelvic radiotherapy (49-52). In man, bladder cancer tends to occur 5 to 10 years after radiation and is characteristically of high grade and locally advanced at diagnosis. The relative risk of secondary bladder malignancy ranges from 1.5- to 4-fold and is likely to be proportional to the dose of radiation given (49). In experimental studies with female mice of the inbred C3H/Neu strain, a single dose of irradiation with x-rays (20 Gy) induced bladder dysfunction, with early effects, observed between 0 and 31 days afterwards, and later effects, seen between 120 and 360 days (50). Another study, using female F344 Fischer rats, showed the development of preneoplastic urothelial hyperplasia and urothelial carcinoma, when rats were observed 20 months post-irradiation (53). Several experimental studies showed that the rate of bladder tumours achieved with irradiation depends on the dosage, the period of exposure and the follow-up time (49-55). Due to this low incidence and the long delay before a tumour appears, which are related to the slow cell-turnover rate of the urothelium, this is not considered a productive model. Moreover, an x-ray source and a authorized facility where it can be used are required (56).

Administration of chemical substances: This remains the most important method used to induce tumours in rodents (40, 56, 57). The discovery that human bladder cancer might be related to chemical exposure dates back to the 19th century (58). In 1895, Dr. Ludwig Rehn reported bladder cancer in German dye workers who manufactured aniline dyes. Later, this was shown to be related to the presence of 2-naphthylamine in the dyes. Since that time, many other chemicals and environmental agents have been associated with bladder cancer development (59-61). The administration of chemicals to induce tumours in mouse and rat is characterized by a long delay in tumour development and a high incidence of tumours. However, chemical substances should be carefully chosen, in order to avoid non-organ specificity of the carcinogenic/mutagenic effect. In recent decades, several chemical compounds have been proven to be particularly effective in induction of bladder cancer. When

administered *via* an appropriate route, at the appropriate doses and in the appropriate strain of animal, all produce 100% incidence of bladder tumours.

Bladder Tumour Cell Lines

Bladder tumour cell lines are also valuable as *in vitro* models that enable the study of the underlying biology and the identification of potential therapeutic targets, from which promising agents would ideally be advanced into translational studies. Various *in vitro* models have been suggested, such as the three-dimensional spheroid models, which attempt to simulate the behaviour of cancer towards therapeutic agents (62). Bladder tumour cell lines, although not suitable for studying the evolution of a tumour, unlike animal models, are frequently used to establish orthotopic tumour implantation. Similar to animal models, bladder cancer cell lines can also be used to test the therapeutic and preventive actions of experimental drugs (63, 64). There are approximately 50 human bladder cancer cell lines established to date (64). Mouse cell lines availability for the study of bladder cancer is considerably lower. An example is BTT-T739, a transitional cell carcinoma cell line derived from bladder tumours that are chemically induced by BBN in T-739 mice (65, 66). However, some authors question the relevance of rodent cell lines to normal and malignant human urothelial cells (67).

Nitrosamines

Nitrosamines were first reported by Geuther in 1863; however, they remained a group of compounds considered to be of no great importance for almost a century. This was until 1954, when Barnes and Magee reported nitrosodimethylamine liver toxicity in rats and humans, and later in 1956, when the same investigators reported hepatic cancer in rats fed with the same compound (68). The description of the carcinogenic activity of nitrosamine compounds, and their use in inducing tumours in the urinary bladder, in 1964 by Duckrey and collaborators provided experimental urinary tract pathology with a useful research instrument. Since then, *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) has become established as one of the most, if not the most, suitable carcinogen for induction of urinary bladder carcinoma (68, 69).

BBN

BBN is a clear yellow to reddish-yellow colour liquid. BBN is also a carcinogen that is metabolically derived from an *N*-nitroso compound found in cigarette smoke (19). BBN is a metabolite of dibutyl nitrosamine (DBN). Both, DBN and BBN induce tumours of the urinary bladder, but DBN can also induce liver and oesophageal tumours (69-71).

BBN has been used as a representative carcinogen that efficiently induces bladder cancer in laboratory animals. BBN is commonly administered *via* the oral route, in drinking water or by gavage, at a dose that ranges from 0.01-0.05% (72-74). However, it can also be administered subcutaneously and introduced directly into the urinary bladder by intravesical instillation (6, 70, 75-78). When BBN is administered in drinking water, the use of opaque bottles is necessary, since this is a photosensitive compound (79). Gavage administration, although highly effective, is not frequently used because aspiration pneumonia can occasionally occur as a consequence of tracheal intubation. It is also possible that oesophageal injury and gastric rupture may occur, as well as restraint-associated distress, particularly with repeated use. All of these undesirable effects can increase rodents' morbidity and mortality (80, 81). Subcutaneous administration performed in infant mice resulted not in bladder tumours but mainly in lung and liver tumours (76). Intravesical instillation of BBN with successful induction of papillomas and carcinomas in female ACI/N rats was reported by some authors (75). Others using the same route of administration of BBN studied the damage and repair of DNA in the urinary bladder epithelium of Wistar rats (77). For intravesical instillation, anaesthesia is necessary to prevent distress, to carry out the technique safely and to avoid animal pain. Females are used rather than males, given the anatomical differences that render this technique impossible in males. This route of administration also has some advantages, namely the fact that the compound is only contained within the environment of the urothelium, therefore protecting the animals from systemic exposure (82). Regardless of its disadvantages, oral administration remains the most common route for BBN administration.

BBN is an indirect carcinogen: after ingestion it is metabolized mainly in the liver, but also in the bladder, into several metabolites. The alcoholic group of BBN is oxidized into a carboxylic group by the enzymatic system alcohol/aldehyde dehydrogenase, resulting in *N*-butyl-*N*-(3-carboxypropyl)nitrosamine (BCPN) (Figure 1) (83-85). BBN is also converted to BBN-glucuronide by uridine diphosphate-glucuronosyltransferase-catalyzed conjugation, but this metabolite does not possess carcinogenic properties. BCPN, unlike BBN-glucuronide, is a bladder carcinogen. These metabolites reach the urinary bladder through urine and come into contact with the urothelium, binding covalently to cellular macromolecules and initiating the carcinogenic process (78, 85-88). Several other minor metabolites, resulting from beta-oxidation according to the Knoop mechanism, can also be detected, such as *N*-butyl-*N*-(2-hydroxy-3-carboxypropyl)nitrosamine, *N*-butyl-*N*-(carboxymethyl)nitrosamine and *N*-butyl-*N*-(2-oxopropyl)nitrosamine (86, 87, 89). Due to its mechanism of action, BBN is considered to be a genotoxic or DNA-reactive carcinogen (88); it causes DNA damage in the bladder epithelium and selectively induces urinary bladder

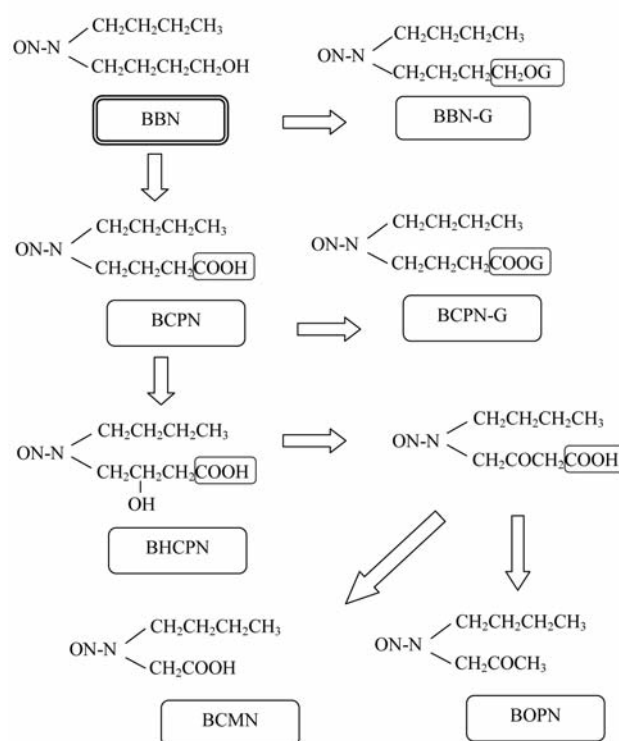


Figure 1. Metabolism of *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN). (BBN-G: BBN-glucuronide; BCPN: *N*-butyl-*N*-(3-carboxypropyl)nitrosamine; BCPNG: BCPN-glucuronide; BHCPN: *N*-butyl-*N*-(2-hydroxy-3-carboxypropyl)nitrosamine; BCMN: *N*-butyl-*N*-(carboxymethyl)nitrosamine; BOPN: *N*-butyl-*N*-(2-oxopropyl)nitrosamine (64, 81).

tumours in mice and rats (78, 90). In BBN-induced bladder tumours, the clonal mutations detected were predominantly G-A or C-T transitions (15/27, 56%) and substitutions of T (10/27, 37%) (90).

Urothelial Lesions Induced by BBN

BBN-induced urothelial lesions in rodents resemble human urothelial lesions in their morphological characteristics. However, susceptibility to BBN-induced bladder tumours differs significantly among mouse and rat strains. From our experience, we can say that the mouse and rat spectrum of urothelial lesions depends on the sex, strain, age and period of exposure to BBN. Macroscopic lesions observed in mice and rats are dependent on the length of exposure to the compound. Bladder cancer in mice develops relatively early after BBN exposure and macroscopic lesions are observed after 12 weeks of exposure to BBN (0.05%). In rats, the exposure period to BBN (0.05%) must be at least 20 weeks, to enable the development of macroscopic lesions. In Figure 2, we can observe the macroscopic characteristics of BBN-induced lesions in mice and rats. Macroscopically, mouse

Table II. Spectrum of urothelial lesions induced by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN).

Animal specie	Age	BBN	Time of exposure	Experimental groups	Spectrum of lesions										Reference(s)		
					NU	SH	NH	D	PH	CIS	PAP	PTLMP	LGPC	HGPC	IC	SqM	
Rat Fisher 344 (♀)	5 weeks	0.05% (drinking water)	20 weeks	20 W BBN + 1 W H ₂ O (n=12)	0 (0%)	2/12 (16.6%)	10/12 (83%)	10/12 (83%)	0 (0%)	0 (0%)	4/12 (33.3%)	4/12 (33.3%)	10/12 (83%)	2/12 (16.6%)	2/12 (16.6%)	6/12 (50%)	85
			-	CTRL (n=10)	10/10 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
Rat	5 weeks	0.05% (drinking water)	20 weeks	20 W BBN + 1W H ₂ O (n=10)	0 (0%)	8/10 (80%)	9/10 (90%)	9/10 (90%)	0 (0%)	6/10 (60%)	5/10 (50%)	5/10 (50%)	5/10 (50%)	1/10 (10%)	3/10 (30%)	1/10 (10%)	Data not published
Wistar (♀)	-	-	-	CTRL (n=10)	10/10 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Rat	5 weeks	0.05% (drinking water)	20 weeks	20 W BBN + 8 W H ₂ O (n=13)	0 (0%)	11/13 (85%)	13/13 (100%)	9/13 (70%)	10/13 (76.9%)	0 (0%)	3/13 (23%)	8/13 (61.5%)	11/13 (84.6%)	10/13 (76.9%)	5/13 (38.5%)	9/13 (70%)	Data not published
Wistar (♀)	-	-	-	CTRL (n=10)	10/10 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Mice ICR (♂)	4 weeks	0.05% (drinking water)	12 weeks	12 W BBN + 1 W H ₂ O (n=12)	0 (0%)	8/12 (67%)	7/12 (58.3%)	12/12 (100%)	0 (0%)	4/12 (33%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	4/12 (33%)	8/12 (67%)	86
-	-	-	-	CTRL (n=10)	10/10 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
Mice ICR (♂)	4 weeks	0.05% (drinking water)	12 weeks	12 W BBN + 1 W H ₂ O (n=10)	0 (0%)	2/10 (20%)	5/10 (50%)	10/10 (100%)	0 (0%)	6/10 (60%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	3/10 (30%)	7/10 (70%)	87
-	-	-	-	CTRL (n=10)	10/10 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
Mice ICR (♂)	5 weeks	7.25 mg/mice (gavage)	10 weeks	10 W BBN + 10W H ₂ O (n=6)	0 (0%)	3/6 (50%)	0 (0%)	5/6 (83.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	Data not published
Mice ICR (♀)	5 weeks	7.25 mg/mice (gavage)	10 weeks	10 W BBN + 10W H ₂ O (n=6)	0 (0%)	4/6 (66.7%)	0 (0%)	4/6 (66.7%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2/6 (33.3%)	

W: week(s); NU: normal urothelium; SH: simple hyperplasia; NH: nodular hyperplasia; D: dysplasia; PH: papillar hyperplasia; CIS: carcinoma *in situ*; PAP: papilloma; PTLMP: papillary tumour of low malignant potential; LGPC: low-grade papillary carcinoma; HGPC: high-grade papillary carcinoma; IC: invasive carcinoma; SqM: squamous carcinoma.

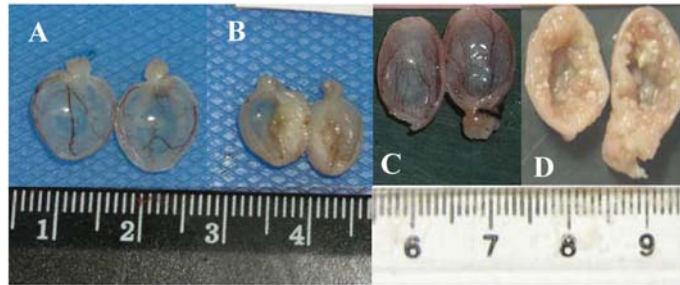


Figure 2. Macroscopic appearance of the mouse (A, B) and rat (C, D) bladder. B and D were exposed to *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine. A: Normal bladder; B: invasive carcinoma; C: normal bladder; D: papillary neoplasm.

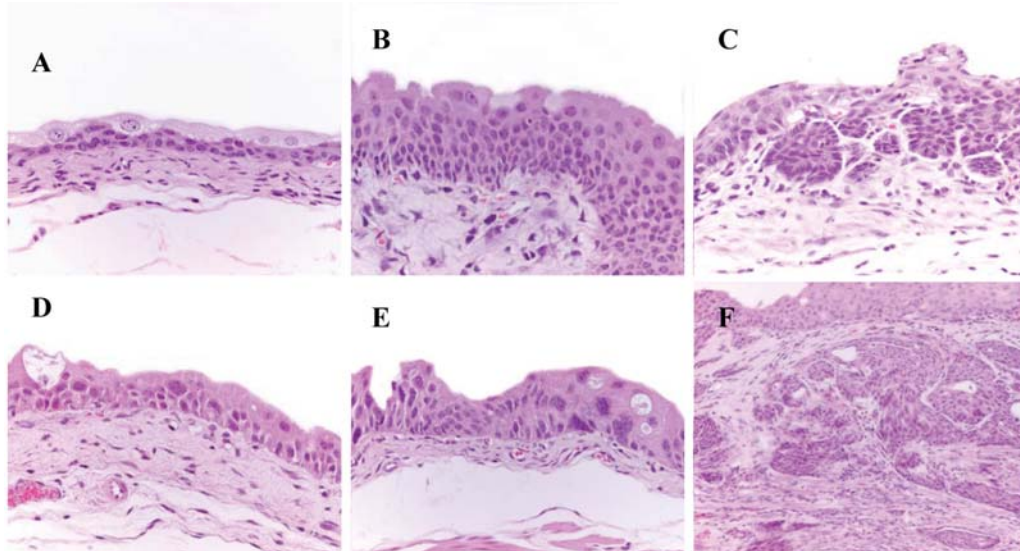


Figure 3. Histopathological evaluation of lesions identified in mouse urothelial carcinogenesis induced by BBN. A: Normal urothelium (control group, H&E, ×400). B: Simple hyperplasia (H&E, ×600). C: Nodular hyperplasia (H&E, ×400). D: Dysplasia (H&E, ×600). E: Carcinoma in situ (H&E, ×400). F: Invasive carcinoma with squamous differentiation (H&E, ×400).

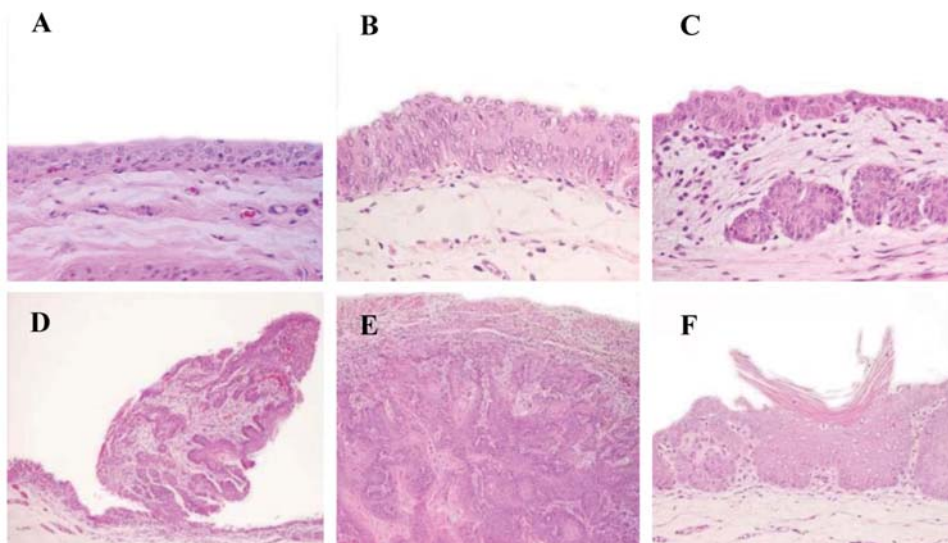


Figure 4. Histopathological evaluation of lesions identified in rat urothelial carcinogenesis induced by BBN. A: Normal urothelium (control group, H&E, ×400). B: Simple hyperplasia (H&E, ×200). C: Nodular hyperplasia (H&E, ×200). D: Papilloma (H&E, ×100). E: Invasive carcinoma (H&E, ×200). F: Squamous metaplasia (H&E, ×100).

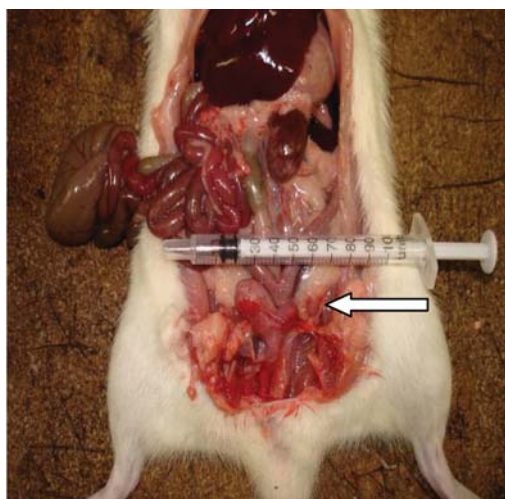


Figure 5. Macroscopic metastasis in the lymph node of a female Wistar rat exposed to *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine.

lesions have nodular characteristics, whereas rat lesions exhibit a papillary pattern. In both animals, the dimensions are variable, ranging from a few millimetres to the complete occupation of the entire bladder lumen.

Microscopic lesions are also different in mice and rats. For the past ten years, we have been using the bladder tumour model in which BBN was administered by gavage and in drinking water using mice (ICR) and rats (Wistar; Fisher 344). Further studies on the spectrum of urothelial lesions have been conducted by our team. As shown in Table II, the spectrum and incidence of urothelial lesions can be seen to increase over time.

Mice usually develop dysplasia, with or without hyperplasia, carcinoma *in situ* and invasive carcinoma (Figure 3). Microscopic lesions in rats usually start as simple hyperplasia, evolving into papillary and nodular hyperplasia, papilloma, papillary tumour of low malignant potential, low-grade papillary carcinoma, high-grade papillary carcinoma,

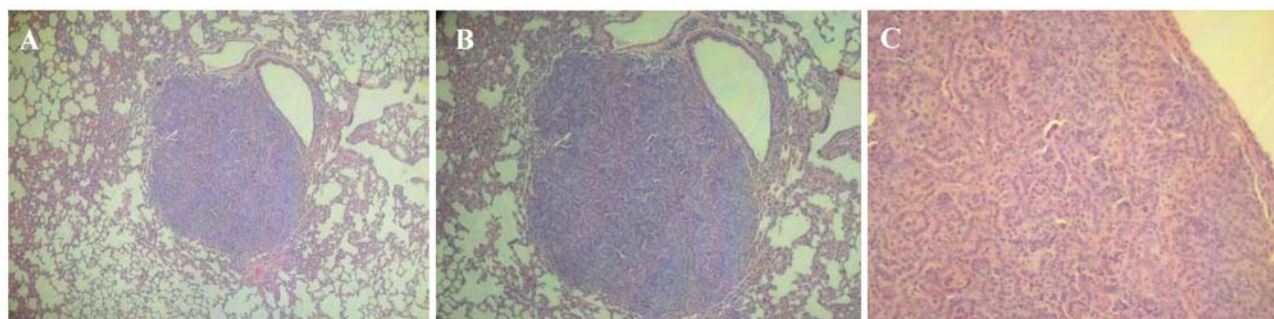


Figure 6. Lung metastasis in a male ICR mouse treated with *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine with invasive urothelial carcinoma (H&E) (A: ×100; B: ×200; C: ×400).

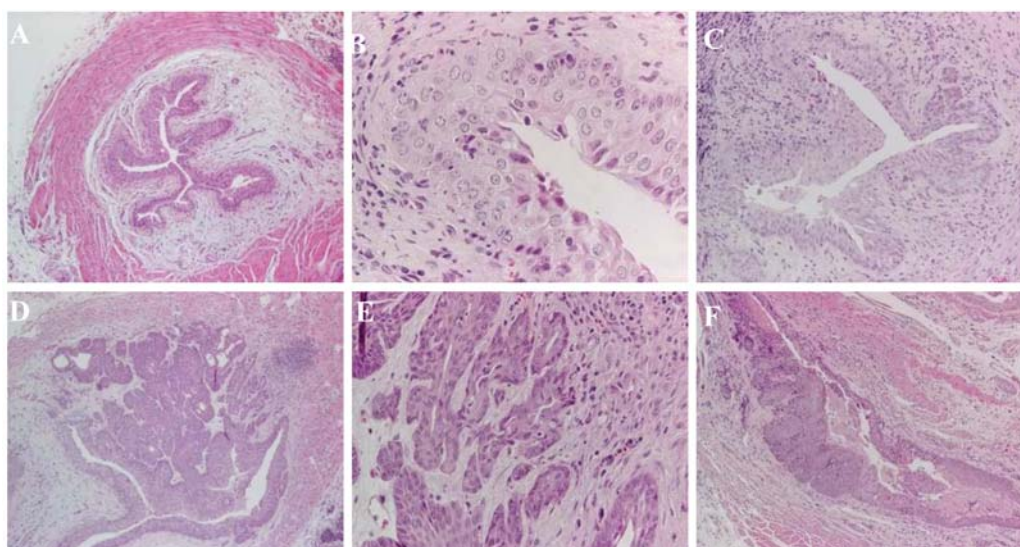


Figure 7. Histopathological evaluation of lesions identified in mouse urethra after BBN treatment. A: Normal urethra (transverse section) (H&E, ×100). B: Dysplasia (H&E, ×600). C: Nodular hyperplasia (H&E, ×200). D: Invasive carcinoma (H&E, ×100). E: Invasive carcinoma (D) with higher magnification (H&E, ×400). F: Squamous metaplasia (H&E, ×100).

and non-invasive carcinomas (Figure 4) (3, 23, 94-97). In contrast to low-grade papillary lesions in humans, in rats these lesions can eventually progress to higher grade, non-invasive carcinomas and finally to invasive neoplasms (3).

Rats are most susceptible to BBN, followed by mice, hamsters and guinea pigs. In hamsters, diffuse cell growth of the urothelium is observed in response to BBN, but no papillomatous growth is seen. Following treatment with BBN, the bladder urothelium of guinea pigs does not increase in thickness (6). In dogs, development of urinary bladder tumours induced by BBN required longer periods than needed in mice or rats, but the incidence and development of bladder tumours and morphological patterns were very similar. BBN-induced transitional cell carcinoma in dog urinary bladder was very similar to the one observed in human cases (98, 99).

Extra Urinary Bladder Tumours

In mice and rats, tumour development can lead to the emergence of considerably larger urothelial tumours, which in turn can cause obstruction of their ureters. This situation often leads to hydronephrosis and death (100), even before metastasis can arise. On the other hand, since experimental animals are euthanized early in life (at the end of the experimental period) it is not surprising that this would be too soon for metastases to develop (101). However, according to several authors, invasive carcinomas are highly malignant and frequently develop metastasis in mice, particularly to the lungs (3, 102) but also to regional lymph nodes and occasionally other organs (3). Throughout our years of research using BBN, we have only observed metastasis in one inguinal lymph node (Figure 5), in a female Wistar rat after 20 weeks' exposure to BBN and 8 weeks' of water, and in the uterine wall of another animal submitted to the same protocol.

In mice, we observed lung metastasis in one ICR male mouse submitted to 12 weeks of BBN followed by 8 weeks of water (Figure 6), and alterations in the urethra of seven mice subjected to the same protocol, consistent with the pre-neoplastic and neoplastic lesions induced by BBN (Figure 7). As yet, we have not been able to find reports demonstrating alterations of normal urethral cells as a direct effect of BBN. All metastases found were associated with invasive urothelial carcinomas.

During our experimental studies using BBN, the mortality rate in mice and rats has been very low. When death happens, it is generally associated with hydronephrosis, which is diagnosed at necropsy.

BBN-induced Urothelial Tumours: Applications

The similarity between bladder cancer in humans and rodents enables the investigation of several areas that cannot be studied under clinical conditions, such as pharmacokinetics and

toxicity. Rodent models of bladder cancer chemically-induced by BBN are useful in the evaluation of new bladder cancer therapies. Such models can be used to determine the efficacy of therapeutic drugs that are intravesically instilled (6) or administered by other routes, such *per os* (103) or intraperitoneally (86, 87). However, before beginning treatment, it is absolutely necessary to confirm tumour uptake. Physical examination by means of bladder palpation and urine inspection are classically reported methods that can be used in mice and rats. However, haematuria and palpable mass might represent the latter stages of cancer. *In vivo* imaging systems that can monitor tumour development are useful. Non-invasive methods such as ultrasonography (104), endoscopy (105), cystoscopy (106) and magnetic resonance imaging (MRI) (107, 108), computerized axial tomography (CAT) (109) and bioluminescence (110, 111), can be applied with these aims in mind. The possibility of applying all these detection and measurement methods are very useful in animal models of human cancer, since they allow the reduction of the number of animals needed for such studies. If we are able to determine the presence of pre-neoplastic and neoplastic lesions without the need to sacrifice animals, we can progress to the treatment phase, and each animal will function as its own control (109).

Genetic Alterations in BBN-induced Urothelial Tumours

Bladder cancer in humans is associated with several genetic and chromosomal changes (112). In rats, chromosomal alterations associated with oral BBN exposure have been previously reported by our team (113) and others (114-117). In mice, there are some studies that describe several genetic and chromosomal changes (118-120). These genetic alterations are similar to those found in humans. BBN-induced rodent tumours, particularly murine tumours, have *p53* mutations, or mutations in genes related to the *p53* pathway, especially in high-grade tumours (3, 115). Numerous chromosomal abnormalities and mutations in other key growth control genes are frequently detected in these tumours, even at their early non-invasive stages (3). *H-ras* mutations are observed infrequently in mouse and rat models, although BBN-induced carcinogenesis occurs more efficiently in *H-ras* transgenic mice. BBN-induced tumours display elevated levels of Epidermal growth factor receptor (EGFR) (115, 120, 121). In rats, an up-regulation of *H19*, an imprinted maternally expressed oncofetal gene which is used as a tumour marker in human bladder cancer, has also been shown (116). Allelic losses within mouse chromosome 4 (syntenic to human 9p21-p22) are also common, mirroring the loss of 9p21-p22 that occurs in human bladder cancer (121).

These findings confirm the similarity of rodent models to human bladder cancer, and emphasize the applicability of such models for future therapeutic studies.

Other Chemicals

In the past, *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT) and *N*-methyl-*N*-nitrosourea (MNU), both genotoxic carcinogenic compounds, were widely used to induce bladder cancer in laboratory animals. However, nowadays, their use is very limited, taking into consideration the fact that both compounds have very particular handling requirements.

FANFT is a nitrofurane derivative, administered in the animals' diet, with specific bladder carcinogenicity for mice, rats, dogs (122-124) and hamsters (124, 125). FANFT is a dangerous compound that must be carefully manipulated, otherwise carcinogenic and teratogenic lesions may arise in the experimenters (6).

MNU is a direct- and complete-acting carcinogen causing tumour initiation and/or promotion in several organs, including the urinary bladder, lung, liver, thyroid, pancreas, prostate, intestine, forestomach, glandular stomach, alimentary tract, kidney, nervous system and hematopoietic system (126-128). In order for it to induce bladder tumours, it must be administered by the intravesical route. It decomposes spontaneously in aqueous solution at a rate proportional to the pH (129, 130).

Conclusion

The BBN-induced rodent model is an orthotopic model widely used in urinary bladder cancer research, especially for the evaluation of the preventive and therapeutic effect of drugs for human urinary bladder cancer. Mouse and rat models simulate the natural environment of bladder cancer, with intact pathological and immunological responses.

Stable, reliable and reproducible orthotopic animal models are critical, as they provide the opportunity to study pathogenesis mechanisms and enable the research and development of novel therapeutic agents. Developing therapeutic agents for bladder cancer requires adequate *in vivo* models. The BBN model of urothelial tumours includes the vascular and stromal environment that is crucial to determine the efficacy of therapeutic drugs. Moreover, rodents have a lower urinary tract which is comparable to humans, and neoplasms in the bladder are morphologically very similar, with a similar phenotype to human urinary carcinoma with respect to tumourigenesis and gene expression. This experimental model of urinary bladder tumour *in vivo* enables the study of the biology and evolution of tumours subjected to different agents, using imaging, genetic, molecular and histological techniques.

Acknowledgements

This study was supported by a grant from the Fundação para a Ciência e Tecnologia (FCT), Ministério da Ciência e Ensino Superior, Portugal (SFRH/BD/40896/2007) and by the strategic research Project Pest-OE/AGR/UI0772/2011 also financed by FCT.

References

- Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. *CA Cancer J Clin* 61: 69-90, 2011.
- Kompier LC, Lurkin I, van der Aa MNM, van Rhijn BWG, van der Kwast TH and Zwarthoff EC: *FGFR3*, *HRAS*, *KRAS*, *NRAS* and *PIK3CA* mutations in bladder cancer and their potential as biomarkers for surveillance and therapy. *Plos One* 5(11): 1-13, 2010.
- Cohen SM: Comparative pathology of proliferative lesions of the urinary bladder. *Toxicol Pathol* 30(6): 663-671, 2002.
- Grignon DJ: The current classification of urothelial neoplasms. *Mod Pathol* 22: S60-S69, 2009.
- Petraki CD and Spikas CP: Non-papillary urothelial lesions of the urinary bladder: morphological classification and immunohistochemical markers. *In Vivo* 22: 493-502, 2008.
- Oliveira PA, Colaço A, De la Cruz Palomino LF and Lopes C: Experimental bladder carcinogenesis – rodent models. *Exp Oncol* 28: 2-11, 2006.
- Arentsen HC, Hendricksen K, Oosterwijk E and Witjes JA: Experimental rat bladder urothelial cell carcinoma models. *World J Urol* 27: 313-317, 2009.
- Clayson DB, Fishbein L and Cohen SM: Effects of stones and other physical factors on the induction of rodent bladder cancer. *Food Chem Toxicol* 33(9): 771-784, 1995.
- van Moorselaar RJA, Ichikawa T, Schaafsma HE, Jap PHK, Isaacs JT, van Stratum P, Ramaekers FCS, Debruyne FMJ and Schalken JA: The rat bladder tumor model system RBT resembles phenotypically and cytogenetically human superficial transitional cell carcinoma. *Urol Res* 21: 413-421, 1993.
- Martin-Sanz P, Mayoral R, Casado M and Bosca L: COX-2 in liver, from regeneration to hepatocarcinogenesis: What we have learned from animal models? *World J Gastroenterol* 16(12): 1430-1435, 2010.
- Steele VE and Lubet RA: The use of animal models for cancer chemoprevention drug development. *Semin Oncol* 37(4): 327-338, 2010.
- Zhang ZT, Pak J, Shapiro E, Sun TT and Wu XR: Urothelium-specific expression of an oncogene in transgenic mice induced the formation of carcinoma *in situ* and invasive transitional cell carcinoma. *Cancer Res* 59: 3512-3517, 1999.
- Johnson AM, O'Connell MJ, Messing EM and Reeder JE: Decreased bladder cancer growth in parous mice. *Urology* 72(3): 470-473, 2008.
- He F, Mo L, Zheng XY, Hu C, Lepor H, Lee E Y-HP, Sun T-T and Wu XR: Deficiency of *pRb* family proteins and *p53* in invasive urothelial tumorigenesis. *Cancer Res* 69(24): 9413-9421, 2009.
- Stone R, Sabichi AL, Gill J, Lee I-I, Adegboyega P, Dai MS, Loganantharaj R, Trutschl M, Cvek U and Clifford JL: Identification of genes correlated with early stage bladder cancer progression. *Cancer Prev Res (Phila)* 3(6): 776-786, 2010.
- Grippio PJ and Sandgren EP: Highly invasive transitional cell carcinoma of the bladder in a Simian virus 40 T-antigen transgenic mouse model. *Am J Pathol* 157(3): 805-813, 2000.
- Iida K, Itoh K, Kumagai Y, Oyasu R, Hattori K, Kawai K, Shimazui T, Akaza H and Yamamoto M: Nrf2 is essential for the chemopreventive efficacy of oltipraz against urinary bladder carcinogenesis. *Cancer Res* 64: 6424-6431, 2004.

- 18 Ding Y, Paonessay JD, Randall KL, Argoti D, Chen L, Vouros P and Zhang Y: Sulforaphane inhibits 4-aminobiphenyl-induced DNA damage in bladder cells and tissues. *Carcinogenesis* 31(11): 1999-2003, 2010.
- 19 Slocum SL and Kensler TW: Nrf2: control of sensitivity to carcinogens. *Arch Toxicol* 85(4): 273-284, 2011.
- 20 Zhang Z-T, Pak J, Huang H-Y, Shapiro E, Sun T-T, Pellicer A and Wu XR: Role of *Ha-ras* activation in superficial papillary pathway of urothelial tumor formation. *Oncogene* 20: 1973-1980, 2001.
- 21 Cheng J, Huang H, Pak J, Shapiro E, Sun T-T, Cordon-Cardo C, Waldman FM and Wu XR: Allelic loss of *p53* gene is associated with genesis and maintenance, but not invasion, of mouse carcinoma *in situ* of the bladder. *Cancer Res* 63: 179-185, 2003.
- 22 Ahmad, R Patel1, Y Liu, LB Singh, Taketo MM, Wu X-R, Leung HY and Sansom OJ: *Ras* mutation cooperates with *b-catenin* activation to drive bladder tumorigenesis. *Cell Death Dis* 2: e124, 2011.
- 23 Ozaki K, Sukata T, Yamamoto S, Uwagawa S, Seki T, Kawasaki H, Yoshitake A, Wanibuchi H, Koide A, Mori Y and Fukushima S: High susceptibility of *p53* (+/-) knockout mice in *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine urinary bladder carcinogenesis and lack of frequent mutation in residual allele. *Cancer Res* 58: 3806-3811, 1998.
- 24 Sills RC, French JE and Cunningham ML: New models for assessing carcinogenesis: An ongoing process. *Toxicol Lett* 120: 187-198, 2001.
- 25 Puzio-Kuter AM, Castillo-Martin M, Kinkade CW, Wang X, Shen TH, Matos T, Shen MM, Cordon-Cardo C and Abate-Shen C: Inactivation of *p53* and *Pten* promotes invasive bladder cancer. *Genes Dev* 23(6): 675-80, 2009.
- 26 Hikosaka A, Ogawa K, Sugiura S, Asamoto M, Takeshita F, Sato S Y, Nakanishi M, Kohri K and Shirai T: Susceptibility of *p27^{kpl}* knockout mice to urinary bladder carcinogenesis induced by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine may not simply be due to enhanced proliferation. *Int J Cancer* 122: 1222-1228, 2008.
- 27 Ota T, Asamoto M, Toriyama-Baba H, Yamamoto F, Matsuoka Y, Ochiya T, Sekiya T, Terada M, Akaza H and Tsuda H: Transgenic rats carrying copies of the human c-*Ha-ras* proto-oncogene exhibit enhanced susceptibility to *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine bladder carcinogenesis. *Carcinogenesis* 21(7): 1391-1396, 2000.
- 28 Tsuda H, Fukamachi K, Ohshima Y, Ueda S, Matsuoka Y, Hamaguchi T, Ohnishi T, Takasuka N and Naito A: High susceptibility of human c-*Ha-ras* proto-oncogene transgenic rats to carcinogenesis: a cancer-prone animal model. *Cancer Sci* 96(6): 309-316, 2005.
- 29 Reis LO, Pereira TC, Favaro WJ, Cagnon VHA, Lopes-Cendes I and Ferreira U: Experimental animal model and RNA interference: a promising association for bladder cancer research. *World J Urol* 27(3): 353-361, 2009.
- 30 Wu XR: Biology of urothelial tumorigenesis: insights from genetically engineered mice. *Cancer Metastasis Rev* 28: 281-290, 2009.
- 31 Gollapudi BB, Stott WT, Yano BL and Bus JS: Mode of action considerations in the use of transgenic animals for mutagenicity and carcinogenicity evaluations. *Toxicol Lett* 103: 479-484, 1998.
- 32 Garcia-Espana A, Salazar E, Sun TT, Wu XR and Pellicer A: Differential expression of cell cycle regulators in phenotypic variants of transgenically induced bladder tumors: implications for tumor behavior. *Cancer Res* 65(4): 1150-1157, 2005.
- 33 Hadaschik BA, Black PC, Sea JC, Metwalli AR, Fazli L, Dinney CP, Gleave ME and So AI: A validated mouse model for orthotopic bladder cancer using transurethral tumour inoculation and bioluminescence imaging. *BJU Int* 100: 1377-1348, 2007.
- 34 Chan E, Patel A, Heston W and Larchian W: Mouse orthotopic models for bladder cancer research. *BJU Int* 104(9): 1286-1291, 2009.
- 35 Jull W: The induction of tumours of the bladder epithelium in mice by the direct application of a carcinogen. *Br J Cancer* 5: 328-330, 1951.
- 36 Clayton DB and Cooper EH: Cancer of the urinary tract. *Adv Cancer Res* 13: 271-381, 1970.
- 37 Desesso JM: Confounding factors in direct bladder exposure studies. *Comm Toxicol* 3: 317-334, 1989.
- 38 Cohen SM and Lawson TA: Rodent bladder tumors do not always predict for humans. *Cancer Lett* 93(1): 9-16, 1995.
- 39 DeSesso JM: Proliferative lesions of the bladder: Their development and interrelationships. *Comm Toxicol* 3: 335-348, 1989.
- 40 Shirai T, Fukushima S, Tagawa Y, Okumura M and Ito N: Cell proliferation induced by uracil-calculi and subsequent development of reversible papillomatosis in the rat urinary bladder. *Cancer Res* 49: 378-383, 1989.
- 41 Fukushima S, Tanaka H, Asakawa E, Kagawa M, Yamamoto A and Shirai T: carcinogenicity of uracil, a nongenotoxic chemical, in rats and mice and its rationale. *Cancer Res* 52(7): 1675-1680, 1992.
- 42 Shirai T, Shibata M, Takahashi S, Tagawa Y, Imaida K and Hirose M: Differences in cell proliferation and apoptosis between reversible and irreversible mucosal lesions associated with uracil-induced urolithiasis in *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine-pretreated. *Carcinogenesis* 16(3): 501-505, 1995.
- 43 Fukushima S and Murrai T: Calculi, precipitates and microcrystalluria associated with irritation and cell proliferation as a mechanism of urinary bladder carcinogenesis in rats and mice. *IARC Sci Publ* 147: 159-174, 1999.
- 44 Jones RF, Debiec-Rychter M and Wang CY: Chemical carcinogenesis of the urinary bladder – a status report. *J Cancer Res Clin Oncol* 118: 411-419, 1992.
- 45 Cohen SM: Role of cell proliferation in regenerative and neoplastic disease. *Toxicol Lett* 82-83: 15-21, 1995.
- 46 Fukushima S, Shibata MA, Shirai T, Tamano S and Ito N: Roles of urinary sodium ion concentration and pH in promotion by ascorbic acid of urinary bladder carcinogenesis in rats. *Cancer Res* 46(4.1): 1623-1626, 1986.
- 47 Lina BAR and van Garderen-Hoetmer A: effect of urinary pH on the progression of urinary bladder tumours. *Food Chem Toxicol* 37: 1159-1166, 1999.
- 48 González AB and Darby S: Risk of cancer from diagnostic X-rays: estimates for the UK and 14 other countries. *Lancet* 363: 345-351, 2004.
- 49 Jung I, Messing E: molecular mechanisms and pathways in bladder cancer development and progression. *Cancer Control* 7(4): 325-334, 2000.
- 50 Jaal J and Dörr W: Radiation-induced damage to mouse urothelial barrier. *Radiother Oncol* 80: 250-256, 2006.

- 51 Moon K, Stukenborg GJ, Keim J and Theodorescu D: Cancer incidence after localized therapy for prostate cancer. *Cancer* 107(5): 991-998, 2006.
- 52 Sountoulides P, Koletsas N, Kikidakis D, Paschalidis K and Sofikitis N: Secondary malignancies following radiotherapy for prostate cancer. *Ther Adv Urol* 2(3): 119-125, 2010.
- 53 Antonakopoulos GN, Hicks RM, Hamilton E and Berry RJ: Early and late morphological changes (including carcinoma of the urothelium) induced by irradiation of the rat urinary bladder. *Br J Cancer* 46: 403-416, 1982.
- 54 Stewart FA: Mechanism of bladder damage and repair after treatment with radiation and cytostatic drugs. *Br J Cancer* 53(Suppl. VII): 280-291, 1986.
- 55 Kunze E: Hyperplasia urinary bladder rat. In: *Monographs on Pathology of Laboratory Animals. Urinary System*. Second edition. Ed. Jones TC, Hard GC, Mohr U (eds.). Springer, New York, pp. 331-368, 1998.
- 56 Bulnes-Sesma S, Ullibarri-Ortiz de Zárate N and Lafuente-Sánchez JV: Tumour induction by ethylnitrosourea in the central nervous system. *Rev Neurol* 43(12): 733-738, 2006.
- 57 Pitot H and Dragan Y: Facts and theories concerning the mechanisms of carcinogenesis. *FASEB J* 5: 2280-2286, 1991.
- 58 Bryan GT: The pathogenesis of experimental bladder cancer. *Cancer Res* 37: 2813-2816, 1977.
- 59 Cohen SM: Promotion in urinary bladder carcinogenesis. *Environ Health Perspect* 50: 51-59, 1983.
- 60 Johansson SL and Cohen SM: Epidemiology and etiology of bladder cancer. *Semin Surg Oncol* 13: 291-298, 1997.
- 61 Cohen SM: Thresholds in genotoxicity and carcinogenicity: Urinary bladder carcinogenesis. *Genes Envir* 30(4): 132-138, 2008.
- 62 Burgués JP, Gómez L, Pontones JL, Vera CD, Jiménez-Cruz JF and Ozonias M: A chemosensitivity test for superficial bladder cancer based on three-dimensional culture of tumour spheroids. *Eur Urol* 51(4): 962-969, 2007.
- 63 Lin CW, Lin JC and Prout GR: Establishment and characterization of four human bladder tumor cell lines and sublines with different degrees of malignancy. *Cancer Res* 45: 5070-5079, 1985.
- 64 Hatina J, Huckenbeck W, Rieder H, Seifert HH and Schulz WA: Harnblasenkarzinomzelllinien Als modellsysteme zur pathobiologie des harnblasenkarzinoms. *Der Urologe* 6: 724-734, 2008.
- 65 Wang F, Wu J, Tian Y, Chen X, Hu H, Wu W, Li C and Huang Q: Role of VEGF in the growth and metastasis of a murine bladder carcinoma. *Chin Sci Bull* 48(22): 2404-2410, 2003.
- 66 Yang N, Li SH, Lü YZ, Chen LS and Ren DM: Attenuated *Salmonella typhimurium* carrying shRNA-expressing vectors elicit RNA interference in murine bladder tumors. *Acta Pharmacol Sin* 32(3): 368-374, 2011.
- 67 Crallan RA, Georgopoulos NT and Southgate J: Experimental models of human bladder carcinogenesis. *Carcinogenesis* 27(3): 374-381, 2006.
- 68 Lijinsky W: Chemistry and biology of *N*-nitroso compounds. In: *Cambridge Monographs on Cancer Research*. Cambridge University Press, Cambridge UK, 1992.
- 69 Druckrey H, Preussmann R, Ivankovic S, Schmidt CH, Mennel HD and Stahl KW: Selective induction of bladder cancer in rats by dibutyl- and *N*-butyl-*N*-butanol(4)-nitrosamine. *Z Krebsforsch* 2(66): 280-90, 1964.
- 70 Okada M and Ishidate M: Metabolic fate of *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine and its analogues: Selective induction of urinary bladder tumours in the rat. *Xenobiotica* 7(1): 11-24, 1977.
- 71 Suzuki E, Anjo T, Aoki J and Okada M: Species variations in the metabolism of *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine and related compounds in relation to urinary bladder carcinogenesis. *Gann* 74: 60-68, 1983.
- 72 Ito N, Shirai T, Fukushima S and Hirose M: Dose-response study of urinary bladder carcinogenesis in rats by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine. *J Cancer Res Clin Oncol* 108: 169-173, 1984.
- 73 Ohtani M, Kakizoe T, Nishio Y, Sato S, Sugimura T, Fukushima S and Nijima T: Sequential changes of mouse bladder epithelium during induction of invasive carcinomas by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine. *Cancer Res* 46: 2011-2004, 1986.
- 74 Wanibuchi H, Wei M, Salim EI, Kinoshita A, Morimura K, Sudo K and Fukushima S: Inhibition of rat urinary bladder carcinogenesis by the antiangiogenic drug TNP-470. *Asian Pac J Cancer Prev* 7: 101-107, 2006.
- 75 Hashimoto Y, Suzuki K and Okada M: Induction of urinary bladder tumors by intravesicular instillation of butyl(4-hydroxybutyl) nitrosoamine and its principal urinary metabolite, butyl(3-carboxypropyl)nitrosoamine in rats. *Gann* 65: 69-73, 1974.
- 76 Fuji K, Odashima S and Okada M: Induction of tumours by administration of *N*-dibutylnitrosamine and derivatives to infant mice. *Br J Cancer* 35: 610-614, 1977.
- 77 Tsuda H, Miyata Y, Hagiwara A, Hasegawa R, Shirai T and Ito N: Damage and repair of DNA in urinary bladder epithelium of rats treated with *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine. *Gann* 68: 781-783, 1977.
- 78 Cohen SM: Urinary bladder carcinogenesis. *Toxicol Pathol* 26: 121-127, 1998.
- 79 Kunze E, Schauer A and Schatt S: Stages of transformation in the development of *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine-induced transitional cell carcinomas in the urinary bladder of rats. *Z Krebsforsch Klin Onkol* 87(2): 139-160, 1976.
- 80 Atcha Z, Rourke C, Neo AHP, Goh CWH, Lim JSK, Aw CC, Browne ER and Pemberton DJ: Alternative method of oral dosing for rats. *J Am Assoc Lab Anim Sci* 49(3): 335-343, 2010.
- 81 Hoggatt A, Hoggatt J, Honerlaw M and Pelus LM: A spoonful of sugar helps the medicine go down: a novel technique to improve oral gavage in mice. *J Am Assoc Lab Anim Sci* 4(3): 329-334, 2010.
- 82 Oliveira PA, Pires MJ, Nóbrega C, Arantes-Rodrigues R, Calado AM, Carrola J, Ginja M and Colaço A: Technical report: Technique of bladder catheterization in female mice and rats for intravesical instillation in models of bladder cancer. *Scand J Lab Anim Sci* 36(1): 5-9, 2009.
- 83 Bonfanti M, Magagnotti C, Bonati M, Fanelli R and Airolidi L: Pharmacokinetic profile and metabolism of *N*-nitrosobutyl-(4-hydroxybutyl)amine in rats. *Cancer Res* 48: 3666-3669, 1988.
- 84 Mirvish SS: Role of *N*-nitroso compounds (NOC) and *N*-nitrosation in etiology of gastric, esophageal, nasopharyngeal and bladder cancer and contribution to cancer of known exposures to NOC. *Cancer Lett* 93: 17-48, 1995.
- 85 Iida K, Itoh K, Maher JM, Kumagai Y, Oyasu R, Mori Y, Shimazui T, Akaza H and Yamamoto M: *Nrf2* and *p53* cooperatively protect against BBN-induced urinary bladder carcinogenesis. *Carcinogenesis* 28(11): 2398-2403, 2007.

- 86 Suzuki E and Okada M: Metabolic fate of *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine in the rat. *Gann* 71(6): 856-862, 1980.
- 87 Mochizuki M, Suzuki E and Okada M: Structure and metabolic fate of *N*-nitrosodialkylamines in relation to their organotropic carcinogenicity with special reference to induction of urinary bladder tumors. *Yakugaku Zasshi* 117(10-11): 884-894, 1997.
- 88 Cohen SM, Ohnishi T, Clark NM, He J and Arnold LL: Investigations of rodent urinary bladder carcinogens: collection, processing, and evaluation of urine and bladders. *Toxicol Pathol* 35(3): 337-347, 2007.
- 89 Airoidi L, Magagnotti C, Bonfanti M and Fanelli R: Development of an experimental model for studying bladder carcinogen metabolism using the isolated rat urinary bladder. *Cancer Res* 47: 3697-3700, 1987.
- 90 Yamamoto K, Nakata D, Tada M, Tonoki H, Nishida T, Hirai A, Ba Y, Aoyama T, Hamada J, Furuuchi K, Harada H, Hirai K, Shibahara N, Katsuoka Y and Moriuchi T: A functional and quantitative mutational analysis of *p53* mutations in yeast indicates strand biases and different roles of mutations in DMBA- and BBN-induced tumors in rats. *Int J Cancer* 83: 700-705, 1999.
- 91 Oliveira PA, Palmeira C, Colaço A, de la Cruz LF and Lopes C: DNA content analysis, expression of *Ki-67* and *p53* in rat urothelial lesions induced by *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine and treated with mitomycin C and bacillus Calmette-Guérin. *Anticancer Res* 26(4B): 2995-3004, 2006.
- 92 Vasconcelos-Nóbrega C, Colaço A, Santos L, Vala H, Palomino LF, Lopes C and Oliveira PA: Experimental study of the anticancer effect of gemcitabine combined with sirolimus on chemically induced urothelial lesions. *Anticancer Res* 31(5): 1637-1642, 2011.
- 93 Oliveira PA, Arantes-Rodrigues R, Sousa-Diniz C, Colaço A, Lourenço L, De La Cruz LF, Da Silva VM, Afonso J, Lopes C and Santos L: The effects of sirolimus on urothelial lesions chemically induced in ICR mice by BBN. *Anticancer Res* 29(8): 3221-6, 2009.
- 94 Ito N, Fukushima S, Shirai T and Nakanishi K: Effects of promoters on *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine induced urinary bladder carcinogenesis in the rat. *Environ Health Perspect* 50: 61-69, 1983.
- 95 Ohtani M, Kakizoe T, Sato S, Sugimura T and Fukushima S: Strain differences in mice with invasive bladder carcinomas induced by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine. *J Cancer Res Clin Oncol* 112(2): 107-10, 1986.
- 96 Oyasu R: Epithelial tumours of the lower urinary tract in humans and rodents. *Food Chem Toxicol* 33(9): 747-755, 1995.
- 97 Oliveira PA, Colaço A, De La Cruz Palomino LF and Lopes C: Rat and mouse urothelial carcinogenesis. *Exp Pathol Health Sci* 2(1): 23-28, 2008.
- 98 Okajima E, Hiramatsu T, Hirao K, Ijuin M, Hirao Y, Babaya K, Ikuma S, Ohara S, Shiomi T, Hijioka T and Ohishi H: Urinary bladder tumors induced by *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine in dogs. *Cancer Res* 41: 1958-1966, 1981.
- 99 Hirao Y, Sasaki K, Sanma S, Ozono S, Babaya K and Okajima E: Scanning electron microscopy of changes in the urinary bladder in dogs treated with *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN). *Urol Res* 15(1): 25-9, 1987.
- 100 Higashi S, Murai T, Mori S, Kamoto T, Yoshitomi M, Arakawa Y, Makino S, Fukushima S, Yoshida O and Hiai H: Host genes affecting survival period of chemically induced bladder cancer in mice. *J Cancer Res Clin Oncol* 124(12): 670-676, 1998.
- 101 Boorman GA, Wood M and Fukushima S: Tumours of the urinary bladder. *IARC Sci Publ* 111: 383-406, 1994.
- 102 Murai T, Mori S, Hosono M, Takeuchi Y, Ohara T, Makino S, Takeda R, Hayashi Y and Fukushima S: Renal pelvic carcinoma which shows metastatic potential to distant organs, induced by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine in NON/Shi mice. *Jpn J Cancer Res* 82(12): 1371-1377, 1991.
- 103 Vasconcelos-Nóbrega C, Pinto-Leite R, Arantes-Rodrigues R, Ferreira R, Brochado P, Cardoso ML, Palmeira C, Salvador A, Guedes-Teixeira CI, Colaço A, Palomino LF, Lopes C, Santos L and Oliveira PA: *In vivo* and *in vitro* effects of RAD001 on bladder cancer. *Urol Oncol*, 2011 Dec 9. [Epub ahead of print].
- 104 Gofrit ON, Birman T, Dinaburg A, Ayesh S, Ohana P and Hochberg A: Chemically induced bladder cancer-a sonographic and morphologic description. *Urology* 68(1): 231-235, 2006.
- 105 Ohyama C, Kawamura S, Satoh M, Saito S, Yoshikawa K, Hoshi S and Orikasa S: Endoscopic observation for detection and monitoring of *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine induced bladder tumor in rats. *Urol Res* 25(3): 183-186, 1997.
- 106 Asanuma H, Arai T, Seguchi K, Kawauchi S, Satoh H, Kikuchi M and Murai M: Successful diagnosis of orthotopic rat superficial bladder tumor model by ultrathin cystoscopy. *J Urol* 169(2): 718-720, 2003.
- 107 Xiao Z, McCallum TJ, Brown KM, Miller GG, Halls SB, Parney I and Moore RB: Characterization of a novel transplantable orthotopic rat bladder transitional cell tumour model. *Br J Cancer* 81(4): 638-646, 1999.
- 108 Kikuchi E, Xu S, Ohori M, Matei C, Lupu M, Menendez S, Koutcher JA and Bochner BH: Detection and quantitative analysis of early stage orthotopic murine bladder tumor using *in vivo* magnetic resonance imaging. *J Urol* 170(4 Pt 1): 1375-1378, 2003.
- 109 Johnson AM, Conover DL, Huang J, Messing EM, Ning R, O'connell MJ, Rossi MA, Sun TT, Wood RW, Wu XR and Reeder JE: Early detection and measurement of urothelial tumors in mice. *Urology* 67(6): 1309-1314, 2006.
- 110 Chan E, Patel A, Heston W and Larchian W: Mouse orthotopic models for bladder cancer research. *BJU Int* 104: 1286-1291, 2009.
- 111 van der Horst G, van Asten JJ, Figdor A, van den Hoogen C, Cheung H, Bevers RF, Pelger RC and van der Pluijm G: Real-time cancer cell tracking by bioluminescence in a preclinical model of human bladder cancer growth and metastasis. *Eur Urol* 60(2): 337-343, 2011.
- 112 Fadl-Elmula I: Chromosomal changes in uroepithelial carcinomas. *Cell Chromosom* 4(1): 2005.
- 113 Oliveira PA, Adega F, Palmeira CA, Chaves RM, Colaço AA, Guedes-Pinto H, De la Cruz P LF and Lopes CA: DNA study of bladder papillary tumours chemically induced by *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine in Fisher rats. *Int J Exp Pathol* 88(1): 39-46, 2007.
- 114 Masui T, Mann AM, Macatee TL, Okamura T, Garland EM, Smith RA and Cohen SM: Absence of *Ras* oncogene activation in rat urinary bladder carcinomas induced by *N*-methyl-*N*-nitrosourea or *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine. *Carcinogenesis* 13(12): 2281-2285, 1992.
- 115 Masui T, Dong Y, Yamamoto S, Takada N, Nakanishi H, Inada KI, Fukushima S and Tatematsu M: *p53* mutations in transitional cell carcinomas of the urinary bladder in rats treated with *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine. *Cancer Lett* 105: 105-112, 1996.

- 116 Ariel I, Ayesh S, Gofrit O, Ayesh B, Abdul-Ghani R, Pizov G, Smith Y, Sidi AA, Birman T, Schneider T, Groot N and Hochberg A: Gene expression in the bladder carcinoma rat model. *Mol Carcinog* 41: 69-76, 2004.
- 117 Yao R, Yi Y, Grubbsy CJ, Lubetz RA and You M: Gene expression profiling of chemically induced rat bladder tumors. *Neoplasia* 9(3): 207-221, 2007.
- 118 Yamamoto S, Chen T, Murai T, Mori S, Morimura K, Oohara T, Makino S, Tatematsu M, Wanibuchi H and Fukushima S: Genetic instability and p53 mutations in metastatic foci of mouse urinary bladder carcinomas induced by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine. *Carcinogenesis* 18(10): 1877-1882, 1997.
- 119 Ogawa K, Uzvolgyi E, St. John MK, Oliveira ML, Arnold L and Cohen SM: Frequent p53 mutations and occasional loss of chromosome 4 in invasive bladder carcinoma induced by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine in B6D2F1 mice. *Mol Carcinog* 21: 70-79, 1998.
- 120 Yao R, Lemon WJ, Wang Y, Grubbsy CJ, Lubetz RA and You M: Altered gene expression profile in mouse bladder cancers induced by hydroxybutyl(butyl)nitrosamine. *Neoplasia* 6(5): 569-577, 2004.
- 121 Wu XR, Sun TT, McConkey DJ, Shrader M and Papageorgiou A: Animal models of bladder cancer. *In*: Textbook of Bladder Cancer. Lerner SP and Schoenberg MP (eds.). Taylor & Francis, United Kingdom, pp. 157-169, 2006.
- 122 Niell HB, Mickey DD, Soloway LS and Wood CA: Growth characteristics of *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT)-induced mouse bladder tumor lines in a human tumor cell stem assay. *Cancer* 49: 323-330, 1982.
- 123 Drago JR: The Noble rat bladder cancer model – FANFT-induced tumours. *Cancer* 53: 1093-1099, 1984.
- 124 Swaminathan S and Bryan GT: Biotransformation of the bladder carcinogen *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide in mice. *Cancer Res* 44: 2331-2338, 1984.
- 125 Spry LA, Zenser TV, Cohen SM and Davis BB: Role of renal metabolism and excretion in 5-nitrofurantoin-induced uroepithelial cancer in the rat. *J Clin Invest* 76: 1025-1031, 1985.
- 126 Lijinsky W: Species differences in nitrosamine carcinogenesis. *J Cancer Res Clin Oncol* 108: 46-55, 1984.
- 127 Cui L, Shi Y, Daib G, Panb H, Chen J, Song L, Wang S, Chang HC, Sheng H and Wang X: Modification of *N*-methyl-*N*-nitrosourea initiated bladder carcinogenesis in Wistar rats by terephthalic acid. *Toxicol Appl Pharmacol* 210: 24-31, 2006.
- 128 Reis LO, Fávoro WJ, Ferreira U, Billis A, Fazuoli MG and Cagnon VHA: Evolution on experimental animal model for upper urothelium carcinogenesis. *World J Urol* 28: 499-505, 2010.
- 129 Swan PF: The rate of breakdown of methyl methanesulphonate, dimethyl sulphate and *N*-methyl-*N*-nitrosourea in the rat. *Biochem J* 110: 49-52, 1968.
- 130 Steinberg GD, Brendler CB, Ichikawa T, Squire RA and Isaacs JT: Characterization of an *N*-methyl-*N*-nitrosourea-induced autochthonous rat bladder cancer model. *Cancer Res* 50: 6668-6674, 1990.

Received January 24, 2012

Revised March 1, 2012

Accepted March 6, 2012