CÁRMEN LÚCIA DE VASCONCELOS NÓBREGA

Mice urinary bladder chemical carcinogenesis: Evaluation of the therapeutic effect of gemcitabine, sirolimus and everolimus

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To my parents

To Prof. Dra. Paula Alexandra Martins de Oliveira

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RESUMO

O cancro é uma doença que afeta milhões de pessoas em todo mundo. De acordo com a Organização Mundial de Saúde uma em cada oito mortes é provocada por cancro. Numa escala global, esta doença provoca mais mortes do que a combinação de doenças como a síndrome de imunodeficiência adquirida, a tuberculose e a malária.

O cancro da bexiga é o sétimo tumor mais comum no homem e o segundo quando nos concentramos no trato urogenital, sendo precedido apenas pelo cancro da próstata. Exibe predisposição sexual, sendo o homem cerca de quatro vezes mais afetado do que a mulher. Em termos histológicos, a maioria dos tumores da bexiga são superficiais, limitados à mucosa, submucosa ou lâmina própria. O carcinoma invasivo é menos frequente, no entanto assume uma grande importância clínica, dado o seu difícil tratamento, a elevada possibilidade de metastização e em consequência, ter um prognóstico reservado.

O modelo animal de carcinoma invasivo da bexiga induzido pela administração prolongada na água de bebida da *N*-butil-*N*-(4-hidroxibutil)nitrosamina (BBN) a murganhos é excelente para a investigação em oncologia urológica, porque permite avaliar a ação de novos agentes terapêuticos ou profiláticos e estudar os mecanismos biopatológicos associados ao crescimento, invasão e metastização dos tumores. Foram objetivos desta tese de doutoramento avaliar o efeito terapêutico de alguns fármacos, isolados ou combinados (gencitabina, sirolimus, everolimus), no modelo invasivo do cancro da bexiga em murganhos; avaliar a ação *in vitro* do everolimus em linhas celulares humanas de tumores de bexiga (T24; HT1376; 5637); avaliar o stresse oxidativo hepático e mitocondrial induzido pela BBN e ainda estudar a expressão da caderina E e da β catenina nas lesões uroteliais induzidas pela BBN.

Para a realização da componente prática foram utilizados 106 murganhos macho da estirpe ICR, 86 dos quais foram expostos à BBN durante 12 semanas. Destes, 60 foram divididos em 4 grupos de 15 animais, para avaliar o efeito terapêutico dos fármacos acima mencionados. Os restantes 26 animais fizeram parte do grupo controlo. A gencitabina e o sirolimus foram administrados por via intraperitoneal e o everolimus foi administrado por via oral. Todos os animais foram sacrificados no final do trabalho experimental e submetidos a necrópsia.

Os resultados obtidos permitem-nos afirmar que o uso isolado da gencitabina e do sirolimus diminui a incidência das lesões uroteliais quimicamente induzidas, no entanto, a sua associação, no mesmo modelo, não teve qualquer efeito. O everolimus

não teve efeito significativo na diminuição da incidência das lesões uroteliais quimicamente induzidas pela BBN. Os resultados do ensaio *in vitro* obtidos com o everolimus foram variáveis, havendo resultados promissores apenas na linha celular 5637. No que diz respeito às alterações hepáticas induzidas pela BBN podemos afirmar que histologicamente não foram observadas lesões, no entanto, foi detetado um aumento do stresse oxidativo mitocondrial que poderá favorecer a hepatotoxicidade quando a BBN é associada a fármacos sujeitos a biotransformação hepática. Neste modelo, as informações relativas à expressividade da caderina E e β catenina, moléculas de adesão celular, são escassas. Nesse sentido, ambas as moléculas foram avaliadas em cortes histológicos do urotélio normal, de lesões pré-neoplásicas e neoplásicas. Na hiperplasia simples e nodular, predominou o padrão identificado no urotélio normal (marcação membranar). Nas lesões displásicas predominou o padrão citoplasmático. Nos carcinomas invasivos observámos um padrão heterogéneo, com a coexistência de marcação citoplasmática e nuclear e por vezes também membranar. A análise da expressividade destas moléculas permitiu-nos concluir que, à semelhança do que está descrito no Homem, também em murganhos estas podem ser consideradas como indicadoras do grau de agressividade e da evolução do tumor.

Palavras-chave: carcinogénese química; *N*-butil-*N*-(4-hidroxibutil)nitrosamina; carcinoma invasivo; murganho; modelos animais; sirolimus; gencitabina; everolimus; caderina E; B-catenina; hepatotoxicidade.

ABSTRACT

Cancer is a devastating disease affecting millions of people all over the world. According with World Health Association, worldwide one in eight deaths is due to cancer. In a global scale, cancer causes more deaths than acquired immunodeficiency syndrome, tuberculosis, and malaria combined.

Bladder cancer is the seventh most common tumour in man, and the second most common tumour of the genitourinary system, being preceded only for prostate cancer. It has sexual predisposition, with males being up to four times more affected than females. Histologically, the majority of bladder tumours are superficial, confined to mucosa, submucosa or lamina propria. Invasive carcinoma is less frequent, however it assumes great clinical importance, since it is difficult to treat, metastasis is a strong possibility and the prognosis is less favourable.

Mice model of invasive bladder cancer, induced by oral administration of *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) in drinking water, is a good and widely used research model for urologic oncology. It allows the evaluation of new therapeutic or prophylactic agents and also to study the basic mechanisms of tumour growth, invasion and metastasis.

In this thesis we evaluate the therapeutic efficiency of several drugs (gemcitabine, sirolimus, everolimus) alone or in combination in chemically induced bladder cancer in male mice. Everolimus was also analysed *in vitro*, in three different bladder cancer cell lines, two invasive (T24; HT1376) and one superficial (5637). Hepatic and mitochondrial oxidative stress induced by BBN and the analysis of E-cadherin and β-catenin were also executed.

106 male ICR mice were used during experimental work. 86 of them were exposed to BBN for 12 weeks, and from these, 60 were divided into four groups of 15 animals each, to analyse the therapeutic effects of drugs. The remaining 26 mice were used as control animals. Gemcitabine and sirolimus were administered by intraperitoneal route. Everolimus was administered by gavage. All animals were euthanized at the end of the experimental work and submitted to a complete necropsy.

According to our results, gemcitabine and sirolimus used as single agents, reduce the incidence of chemically induced urothelial lesions in mice. However, their combination did not add any improvement regarding urothelial incidence. Everolimus showed no significant effect in decreasing urothelial lesions incidence. *In vitro*, its effect on proliferation and apoptosis across different bladder cancer cell lines was heterogeneous. Promissory results were seen only in cell-line 5637.

BBN is metabolized mainly in liver. Regarding hepatic changes induced by BBN, we can say that liver histological evaluation did not show any microscopic changes compatible with liver stress, however mitochondrial oxidative stress was detected. This can lead to hepatotoxicity, when BBN is combined with drugs with liver biotransformation.

E-cadherin and β–catenin are adhesion molecules that were evaluated in control mice and in mice exposed to BBN. In simple and nodular hyperplasia, membrane staining was dominant. In dysplasia, cytoplasmic pattern was prevalent and in invasive carcinomas a heterogeneous staining was observed (cytoplasmic, nuclear and membrane staining). Like in man, also in mice these adhesion molecules can be considered as indicators of tumour aggressiveness and evolution.

Key-words: chemical carcinogenesis; *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine; invasive carcinoma; mouse; animal models; sirolimus; gemcitabine; everolimus; E-cadherin; β–catenin; hepatotoxicity.

LIST OF PAPERS, POSTERS AND ORAL PRESENTATIONS RELATED WITH THIS EXPERIMENTAL WORK

1.1 – FULL PAPERS PUBLISHED IN SCI INDEXED JOURNALS

- Vasconcelos-Nóbrega C, Costa C, Arantes-Rodrigues R, Henriques A, Vala H, Colaço A, Santos L, Lopes C, Oliveira PA. E-cadherin and β-catenin expression during urothelial carcinogenesis induced by N-butyl-N-(4-hydroxybutyl)nitrosamine in mice (accepted for publication in *Urologia Internationalis*).

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1.4- POSTER PRESENTATIONS IN NATIONAL CONFERENCES

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ΔΨ	-	Electrical potential
Akt	-	serine/threonine-specific protein kinase
BBN	-	*N*-butyl-*N*-(4-hydroxybutyl) nitrosamine
BCG	-	Bacillus Calmette-Guérin
BCMN	-	*N*-butyl-*N*-(carboxymethyl)nitrosamine
BCPN	-	N-butyl-N-(3-carboxypropyl)nitrosamine
BHCPN	-	*N*-butyl-*N*-(2-hydroxy-3-carboxy-propyl) nitrosamine
BOPN	-	*N*-butyl-*N*-(2-oxopropyl)nitrosamine
BSA	-	Bovine serum albumin
CAT	-	Catalase
CIS	-	Carcinoma *in situ*
DMN	-	Dimethylnitrosamine
DMSO	-	Dimethylsulfoxide
EDTA	-	Ethylenediaminetetraacetic acid
EGFR	-	Endothelial growth factor receptor
EGTA	-	Ethyleneglycol-bis(-aminoethyl ether) N,N,N',N'-tetraacetic acid
ELISA	-	Enzyme-Linked Immunosorbent Assay
FANFT	-	[4-(5-nitro-2furyl)-2-thiazolyl]formamide
GC	-	Gencitabine and cisplatin
GR	-	Glutathione reductase
GSH	-	Reduced glutathione
GSSG	-	Oxidized glutathione
GST	-	Glutathione S-transferase
Gy	-	Gray
HER2	-	Human EGF (Epidermal Growth Factor) Receptor 2 (Proto-oncogene)
HRP	-	Horseradish peroxidase
HVA	-	Homovanilic acid
KCl	-	Potassium cloride
KCN	-	Potassium cyanide
KH_2PO_4	-	Potassium di-hydrogen phosphate
LPO	-	Lipid peroxidation

MDA	-	Malondialdehyde
MNU	-	*N*-methyl-*N*-nitrosourea
MPT	-	Mitochondrial permeability transition
MPTP	-	Mitochondrial permeability transition pore
mTOR	-	Mammalian target of rapamycin
MTT	-	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
MVAC	-	Methotrexate, vinblastine, adriamycin and cisplatin
$Na_2S_2O_4$	-	Sodium hydrosulfite
NBT	-	Nitrotetrazolium blue chloride
NDEA	-	*N*-nitrosodiethylamine
NNK	-	4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone
PI3K	-	Phosphatidylinositol 3 kinase
PI3K/Akt/mTOR	-	Intracellular signalling pathway
PVDF	-	Polyvinylidene difluoride
PMS	-	Post Mitochondrial Supernatant
RCR	-	Respiratory control ratio
SD	-	Standard deviation
SDS	-	Sodium Dodecyl Sulfate
SE	-	Standard error
SOD	-	Superoxide dismutase
TPP^+	-	Tetraphenyl phosphonium-selective
UCC	-	Urothelial cell carcinoma
VDAC	-	Voltage-dependent anion channel
VEGFR	-	Vascular endothelial growth factor receptors

CHAPTER 1:
GENERAL INTRODUCTION

1. General introduction

1.1- Bladder cancer

Bladder cancer is the second-most common tumour of the genitourinary tract, with prostate cancer the leader in terms of morbidity and mortality among urogenital tumours (1, 2). An estimated 386,300 new cases and 150,200 deaths from bladder cancer occurred worldwide in 2008, with males more frequently affected than females (1). The highest rates of bladder cancer are found in Europe, North America and Northern Africa, and the lowest in Melanesia and Middle Africa (1). In Europe, bladder urothelial cancer accounts for 7% of all cancers (3), but since de 1990s, a decline in the mortality rate (particularly in men) has been observed, perhaps due to the reduction in smoking prevalence in western countries along with reductions in occupational exposures known to cause bladder cancer (4). Tobacco smoke and other chemicals, like specific industrial chemicals, dietary nitrates and arsenic represent the most important exogenous risk factors associated with bladder cancer, particularly in developed countries (1, 5-7). It has been shown that smoking increases the risk of bladder cancer by two- to fivefold (5, 6). Occupational hazards related to the exposure to certain chemicals, include hairdressers and allied occupations and workers in the petroleum industry (8-10). Individuals living in areas with residential exposure to trihalomethanes in treated water have an increased risk of bladder cancer. Bladder cancer risk was also significantly elevated among those reporting longer duration showers or baths as well as among individuals who “ever” swam in swimming pools (9). *ortho*-Toluidine used as a biological stain and also in the production of dyes, pigments and rubber chemicals and benzidine-based dyes, used mainly for colouring paper, textiles, and leather, are some examples of carcinogenic chemicals associated with bladder cancer (8-11). In developing countries, in the Middle East and Africa, *Schistosoma haematobium* is the main etiological agent for bladder cancer (1, 6).

Urinary bladder tumours remain one of the deadliest and expensive human tumours, with regard to both cost per patient per year and lifetime cost per patient (12, 13). Early detection of bladder cancer, through the use of cystoscopy, radiographic imaging and urine cytology, improves patient prognosis since it enables the identification of lesions at an early stage (14). The staging, treatment and prognosis of bladder cancer all depend on how deeply it has invaded the bladder wall. Surgery is the standard treatment, both in the form of transurethral endoscopic resection - mainly for superficial incidence of the disease, and in the form of radical cystectomy with

urinary diversion when the disease is muscle-invasive (2, 14-17). Intravesical administration of Bacillus Calmette-Guerin (BCG) has been employed after endoscopic resection as the most effective agent for both the prophylaxis of disease recurrence and preventing its progression from superficial to invasive disease (5).

Systemic therapy for bladder cancer can be seen in four contexts: neoadjuvant, adjuvant, bladder sparing with radiation and chemotherapy, and metastatic disease:

- Neoadjuvant therapy is considered to be a standard of care in muscle-invasive bladder cancers planned for surgical intervention (18, 19). The integration of chemotherapy in the pre-surgical phase offers early treatment of systemic micrometastases, potential down staging of the primary and regional disease, and an *in vivo* assessment of chemosensitivity. This approach also avoids a potential delay in systemic treatment due to postoperative complications, which are frequent in bladder cancer (18, 20);

- Adjuvant chemotherapy is a systemic treatment option for patients still with muscle-invasive bladder cancer after being submitted to surgery. Surgical intervention allows the removal of as much of a tumour as possible and the relief of symptoms. Furthermore, it allows the pathologic staging that more accurately assesses risk, since clinical staging is often innacurate (18). There are several protocols being used in this modality, like cisplatin as a single agent or in association with methotrexate, or methotrexate, vinblastine, adriamycin and cisplatin in association (MVAC) or an association of methotrexate, vinblastine, epirubicin and cisplatin (MVEC). However, while available data suggest a beneficial effect from adjuvant therapy, further studies and trials are needed;

- Bladder preservation may be accomplished in appropriately selected patients with muscle-invasive urinary bladder carcinoma, without compromising outcomes, using a trimodality approach: surgery (transurethral resection), chemotherapy and radiation therapy. Chemotherapy has been used in two phases of treatment: as radiosensitizers, given concurrently with radiation treatment, and as adjuvant treatment, recognizing that survival will only be improved by the successful treatment of micrometastases (21). Continued surveillance with cystoscopy both during and after chemoradiotherapy is mandatory (18).

- When metastatic disease is established, conventional cytotoxic chemotherapy, in the form of MVAC or gemcitabine and cisplatin (GC), is commonly used. However, the frequent occurrence of drug resistance and serious side effects has made treatment outcomes unsatisfactory (22, 23). Along with these, the median overall survival rates at 5 years in patients with metastatic urinary bladder cancer treated with

chemotherapy (15.2 months for MVAC and 14 months for GC) and the 5-year overall survival rates (15.3% for MVAC and 13% for GC) are poor (23).

1.2. Research in bladder cancer

Intensive research is being carried out in bladder cancer, to elucidate the reasons for the development of tumours, and to find out which factors determine the tumour progression. Bladder cancer is a recurrent and very prevalent cancer whose treatment involves high economic costs. This situation forces more investigation in the urological field, with the objective to do early diagnostics, to obtain better treatments and fewer side effects. The combination of *in vitro* and *in vivo* studies will provide insights into the biology of the tumour and is essential for the implementation of new therapeutic and/or preventive modalities (24, 25).

1.2.1. Cell lines

Tissue culture was first introduced in 1907 by Harrison. Since then, cell lines have evolved and have come to be widely used for research in several areas like biochemistry, virology, immunology and oncology.

Regarding cancer, cell lines have been very important in order to build our understanding of the disease's molecular pathophysiology and its treatment. The first continuous cancer cell line was HeLa cells, isolated from the aggressive glandular cervical cancer of a young woman approximately 60 years ago (26, 27). Nowadays, cancer cell lines are routinely used for various kinds of biomedical research, from drug-sensitivity tests to identifying potential therapy targets and pharmacologically useful compounds (27-29).

For the study of bladder cancer, several cell lines have been established. T24, HT1376, 5637, UM-UC-3 are some examples of human bladder cancer cell lines (27). Some have origin in superficial tumours but the majority are from invasive and metastatic ones (27, 28). Cell lines from experimental bladder cancer are fewer than those of human origin. AY-27 and NBT-II are bladder cancer cell lines derived from rats (27, 30-32). BTT-T739 and MB49 are examples of bladder cancer cell lines with origin in mice (27, 33).

In vitro studies with cell lines demand a great amount of care concerning the origin of cell lines. It is crucial to ensure that they are reliable, because cell cross-

contamination in cell cultures is a common problem during cell culturing and use. Cross-contamination provides misleading research results leading to unusable therapeutic products. The unwitting use of misidentified cell lines may, ultimately, expose patients to inappropriate, or even harmful, treatments.

Masters and co-workers (2001) (35), found a total of nine cell lines that were cross-contaminated with T24, a line started from human urinary bladder carcinoma in 1970. Four of the tested cell lines were 100% identical to T24, but traded under a different name. Van Bokhoven *et al*. (2001) (36), also tested two cell lines frequently used as models in prostate cancer (TSU-Pr1 and JCA-1) and concluded that they appear to be derivatives of the bladder carcinoma cell line T24 and not of prostatic origin, whereby they could no longer be used as models for the study of prostate cancer.

In urological research, cell lines, particularly human urothelial cell lines are well-established tools for preclinical trials. For most cytotoxic agents, if it does not work *in vitro*, it will most certainly not work *in vivo*. If it works *in vitro*, then there is the possibility it may be effective *in vivo*. They are a cost-efficient method of searching for drug activity and can further our understanding of drugs' action on several tumours. Other advantages in using cell lines can also be highlighted: they are easy to handle and can be replicated almost infinitely. If anything goes wrong, such as contamination or death of the cell culture, it can be easily replaced from frozen stocks. Additionally, they exhibit a relatively high degree of homogeneity. Cell lines, however, have some disadvantages. They are prone to genotypic and phenotypic drift during their continual culture. Subpopulations may arise and cause phenotypic changes over time by the selection of specific, more rapidly growing clones within a population (37).

1.2.2. Animal models and novel drugs for bladder cancer treatment

Animal models have greatly contributed to the understanding of bladder carcinogenesis. They are at the centre of experimental research and, at the same time, are the connection between experimental and clinical research (38). Animal models allow the investigation of aspects that cannot be studied under clinical conditions, such as the evaluation of new chemotherapeutic, immunotherapeutic or prophylactic agents, drug regimens, or other treatment methods and can also provide further information on basic mechanisms of tumour growth and spread (39, 40).

Although there is the possibility to use several animal species, rodents (rats and mice) are those most often used in animal experimentation (41-55).

Bladder tumours in rodents can be established subcutaneously (heterotopically) by transplantation of tumour cells, or intravesically (orthotopically) either by transplantation of tumour cells or by chemical induction (Figure 1) (33, 39, 47).

In the heterotopic model, the tumour is usually located in the flank or hind leg of the animal. This model can be syngeneic, when rodent urothelial cell carcinomas (UCC) tumour fragments or cells are inoculated via a small incision into an immunocompetent host of the same strain from which the tumour was originally derived. Or it can be a xenograft model, when human UCC fragments or cells are used in immunodeficient hosts (33). Heterotopic tumour models have been widely used, since tumour evolution is easily assessed. These models allow urinary tumour development to be studied *in vivo*, since the tumour tissue is developing within a healthy environment. However, it is questionable if the tumour development at these sites parallels the development of the tumour in the organ of its origin. Technically, they are easier to establish than orthotopic ones (33, 39, 47).

The orthotopic model can be divided into three different types: chemically induced bladder cancer models, the xenograft model (transplantation of human UCC into immunodeficient hosts) and the syngeneic tumour model (transplantation of carcinogen-induced bladder cancer into syngeneic immunocompetent hosts) (33, 48). Orthotopic tumour models have the great advantage of simulating the local cancer environment and resembling the behaviour of the disease in humans (33, 39, 47). One disadvantage of the xenograft model is that the immune response of intravesical therapy cannot be evaluated in mice with immune deficiencies (39, 48). An orthotopic model in immunocompetent animals has advantages over models of bladder cancer that either use immunodeficient rodents and/or heterotopic models. This is because the influence of the immune system and the anatomical and physiological factors of the tissue of origin, which undoubtedly influence the metastatic process, are not affected (49).

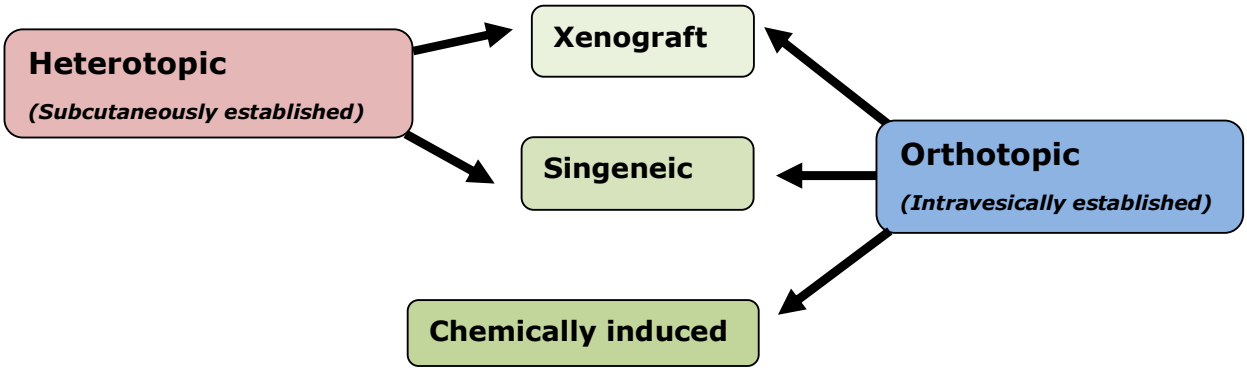

Figure 1: Animal models of bladder cancer (33, 39, 47).

The mouse model of chemically induced bladder cancer was the model used in this study, and it is widely utilized by investigators all over the world in bladder cancer research. It has enormous applications. It can be used to test prophylactic drugs, to test therapeutic drugs and also to define the impact of chemical carcinogens on other organs. There are several chemical carcinogens that can be used to induce bladder cancer in experimental animals. Nitrosamines, *N*-[4-(5-nitro-2furyl)-2-thiazolyl]formamide (FANFT) and *N*-methyl-*N*-nitrosourea (MNU), are some examples (50-54).

Apart from these models, there are also transgenic models for bladder cancer, that are genetically altered animals (lacking genes, expressing genes or having mutated genes) (54-58) that exhibit increased carcinogenicity (59).

The use of animal models, allowed a growing understanding of tumour molecular biology, and also enabled the identification of the signalling pathways involved in bladder tumorigenesis and progression. This important knowledge opened the door for the discovery of new therapeutic pathways. The PI3K/AKT/mTOR pathway and the *RAS-MAPK* pathway, involved in bladder cancer, are now identified as important targets for the development of new therapeutic drugs (60-62). Endothelial growth factors, that promote angiogenesis and tumorigenesis, are also possible targets for therapeutic antiangiogenic intervention in cancer (63, 64).

Among therapeutic agents targeting the PI3K/AKT/mTOR pathway, mTOR inhibitors are the most developed. Rapamycin, also known as sirolimus, and its derivatives such as everolimus, tensirolimus and deforolimus, have antineoplastic activity when it comes to a variety of solid tumours - including bladder cancer - and are being used for clinical trials. Other agents like endostatin, bevacizumab and cetuximab that can target vascular endothelial growth factor and, therefore, tumour angiogenesis are also being subjected to clinical trials to analyze the action they have against bladder cancer (65, 66). To target EGFR and HER2/*neu,* two of the generally overexpressed genes, there are monoclonal antibodies such as cetuximab and panitumumab (35, 65). Tipifarnib, a farnesyltransferase inhibitor, is able to inhibit farnesylation, an important step in activating *Ras* oncogene. Sorafenib (BAY43-9006) is an oral, dual inhibitor of *Raf* and vascular endothelial growth factor receptors (VEGFR). Other agents that have shown promising results regarding bladder cancer (vinflunine, celecoxib, TKI-258 and AEZS-108), are currently under clinical trials (15, 66, 67). This entire therapeutic advance would not be possible without animal models;

however, more data concerning drugs action within monotherapy or politherapy regimens and any eventual side effects are still needed.

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CHAPTER 2:

AIMS

2. Aims

Over the years, bladder cancer research has contributed to a better knowledge in bladder cancer biology, treatment and prevention. Although most superficial bladder cancers can be transurethrally removed with excellent clinical outcomes, muscle-invasive disease can represent a major clinical challenge since it is associated with postoperative recurrence, metastasis and a worse prognosis. Regardless of all the investigation, current treatments are not good enough. To improve survival, it is mandatory to search for new therapeutic options, using new drugs or old drugs in new regimens.

With this thesis we had the ultimate goal of bringing new contribution to bladder cancer basic research. This was supported in four lines of action:

1) The first one was to gather all the information concerning *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) as a urothelial carcinogen, sharing the knowledge gained over many years of research using this chemical in experimental urinary bladder cancer research with rodents.

2) The second one was to test the therapeutic effect of several drugs, *in vivo* and *in vitro*:

- *In vivo*: Using the mice model of bladder cancer chemically induced by BBN that mimics the human invasive bladder cancer in a normal microenvironment, we intended to test the efficacy of gemcitabine, sirolimus and their association, and also to test the efficacy of everolimus;

- *In vitro*: to evaluate everolimus, in three different cell lines (T24; HT1376; 5637).

3) The third one was to determine any eventual liver damage induced by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine;

4) The fourth objective was to study E cadherin and β catenin expression in normal mice urothelium and in preneoplastic and neoplastic lesions induced in ICR mice by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine.

CHAPTER 3:

BBN AS A UROTHELIAL CARCINOGEN

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3. BBN as a urothelial carcinogen

3.1. Introduction

Bladder cancer is among the most common malignancies worldwide, and it assumes greater prominence in developed countries. It 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 bladder-cancer patients, 75-85%, are diagnosed with superficial tumours while the remaining 15% to 25% are 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 ones originate as flat lesions like hyperplasia, dysplasia and carcinoma *in situ* (CIS) 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).

3.2. Experimental models of bladder cancer

The need for experimental models of tumours that are as similar as possible to human tumours has prompted researchers to attempt various approaches. Animals such as dogs, rabbits, guinea pigs and hamsters have all been used to induce and study urinary bladder carcinogenesis (6). However, for ethical questions and due to the conditions in animal facilities, dogs cannot be used in experimental carcinogenesis models. Animal facilities 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 humans, and neoplasms in their bladders are morphologically very similar (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 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, 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 develop 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 present in bladder carcinogenesis (11-14), allowing it to be determined if genetic changes, including oncogene over expression/mutation or tumour-suppressor gene loss, could increase the risk of neoplastic progression (13, 15).

Table I - Genetically altered animals used in carcinogenesis studies.

Transgenic animals	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 Nrf2 allele	mouse	Higher sensitivity to BBN than the wild type (20-22)
H-Ras	activated Ha-ras oncogene harboring a point mutation at codon 61 of the second exon converting CAG to CTG	mouse	Urothelial hyperplasia followed by superficial papillary tumours of the bladder (12, 23, 24)
H-Ras	$H\text{-}Ras^{Q61L}$ mutation	mouse	Develop early urothelial lesions (25)
$p53^{+/-}$	null mutation in one p53 allele	mouse	Higher sensitivity to BBN than the wild type (26,27)
$p53^{flox/flox}$; $Pten^{flox/flox}$	conditional alleles for both p53 and Pten	mouse	CIS; high-grade invasive carcinoma with areas of transitional cell; squamous; sarcomatoid carcinoma (28)
$p27^{kip1}$	null mutation in both p27 allele	mouse	Higher sensitivity 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)

These models are useful for extrapolating the results from animal carcinogenicity studies into human risk assessment (15) and also provide *in vivo*

platforms for testing preventive bladder-cancer strategies (14, 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 (15, 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 like 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 (1966), 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 increased 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 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 (20Gy) 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, period of exposure and time of follow-up (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 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 a chemical substance, 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, to avoid non-organ specificity of the carcinogenic/mutagenic effect. In recent decades, several chemical compounds have been proved to be particularly effective in bladder-cancer induction. When administered via the appropriate route, at the appropriate doses and in the appropriate strain of animal, all produce 100% incidence of bladder tumours.

3.3. 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, 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). Currently, there are approximately 50 human bladder cancer cell lines established (64). Mouse and rat cell lines availability for the study of bladder cancer is considerably lower (65, 66). However, some authors question the relevance of rodent cell lines to normal and malignant human urothelial cells (67).

3.4. 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. That 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).

3.4.1. *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (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 dibutylnitrosamine (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 successfully 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-glucoronide by uridine diphosphate-glucuronosyltransferase-catalyzed conjugation, but this metabolite does not possess carcinogenic properties. BCPN, unlike BBN-glucoronide, 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-carboxy-propyl)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 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).

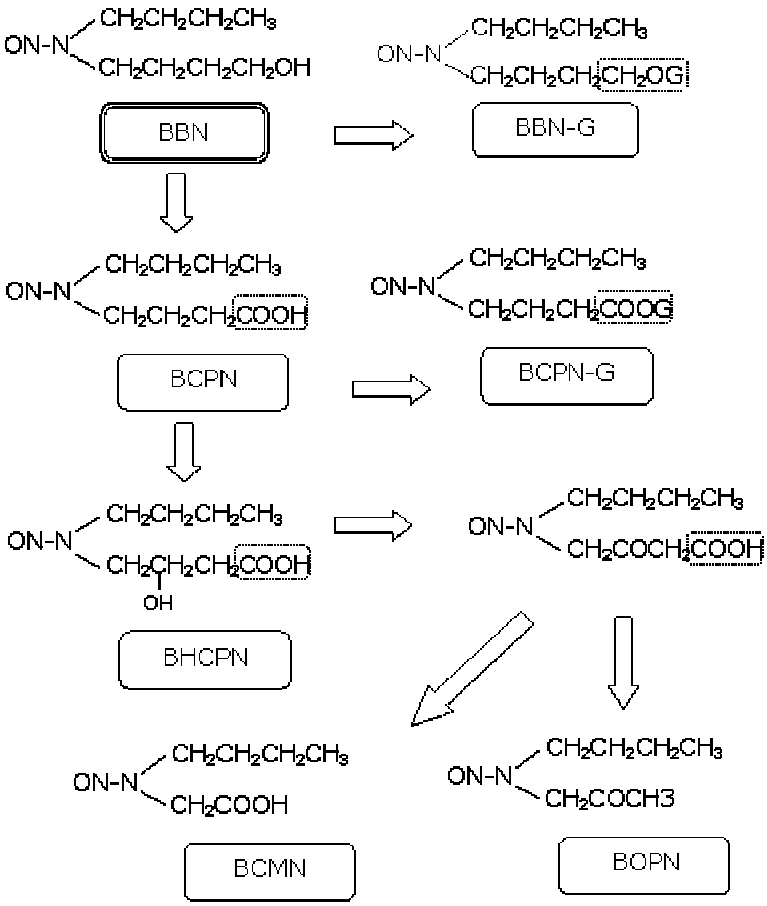

Figure 1: Metabolism of *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN). (BBN-G: BBN-glucoronide; BCPN: *N*-butyl-*N*-(3-carboxypropyl)nitrosamine; BCPNG: BCPN-glucoronide; BHCPN: *N*-butyl-*N*-(2-hydroxy-3-carboxy-propyl) nitrosamine; BCMN: *N*-butyl-*N*-(carboxymethyl)nitrosamine; BOPN: *N*-butyl-*N*-(2-oxopropyl)nitrosamine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

3.4.1.1. 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 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.

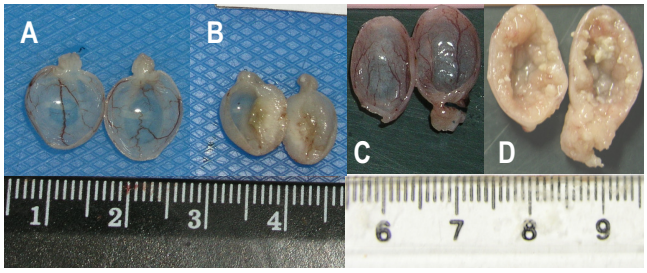

Figure 2: Macroscopic appearance of the mouse (A, B) and rat (C, D) bladder. B and D were exposed to *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine. A: Normal bladder; B: Invasive carcinoma; C: Normal bladder; D: Papillary neoplasm.

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.

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_2O (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 Wistar (♀)	5 weeks	0.05% (drinking water)	20 weeks	20 W BBN + 1W H_2O (n=10)	0 (0%)	8/10 (80%)	9/10 (90%)	9/10 (90%)	9/10 (90%)	0 (0%)	6/10 (60%)	5/10 (50%)	5/10 (50%)	1/10 (10%)	3/10 (30%)	1/10 (10%)	Data not published
		___	___	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 Wistar (♀)	5 weeks	0.05% (drinking water)	20 weeks	20 W BBN + 8 W H_2O (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
		___	___	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_2O (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_2O (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	7.25mg/mice (gavage)	10 weeks	10 W BBN + 10W H_2O (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	7.25mg/mice (gavage)	10 weeks	10 W BBN + 10W H_2O (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 – Papilar hyperplasia; CIS – Carcinoma *in situ;* PAP – Papilloma; PTLMP – Papillary tumour of low malignant potencial; LGPC – Low-grade papillary carcinoma; HGPC – High-grade papillary carcinoma; IC- Invasive carcinoma; SqM – Squamous carcinoma

Mice usually develop dysplasia, with or without hyperplasia, carcinoma *in situ* and invasive carcinoma (Figure 3).

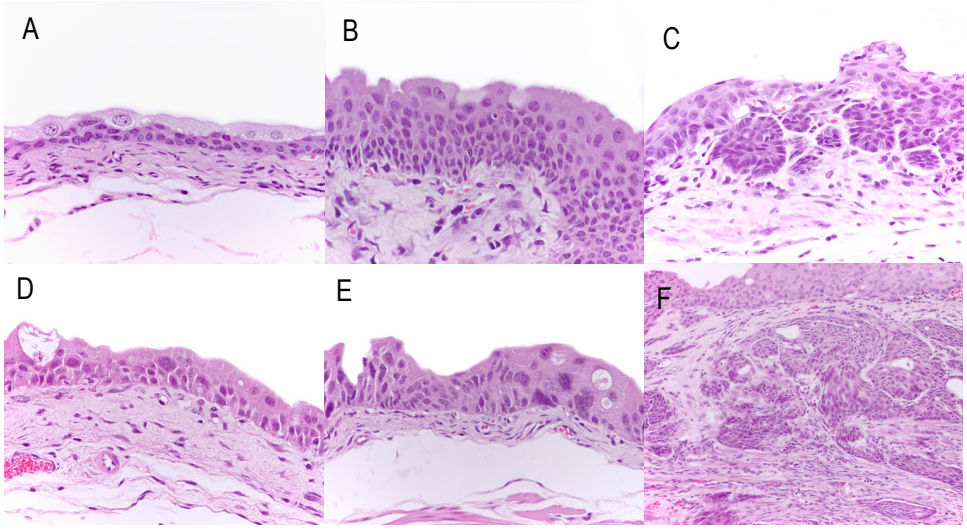

Figure 3: Histopathological evaluation of lesions identified in mouse urothelial carcinogenesis induced by BBN. A: Normal urothelium (control group, H&E, x400). B: Simple hyperplasia (H&E, x600). C: Nodular hyperplasia (H&E, x400). D: Dysplasia (H&E, x600). E: Carcinoma *in situ* (H&E, x400). F: Invasive carcinoma with squamous differentiation (H&E, x400).

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, 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 that observed in human cases (98, 99).

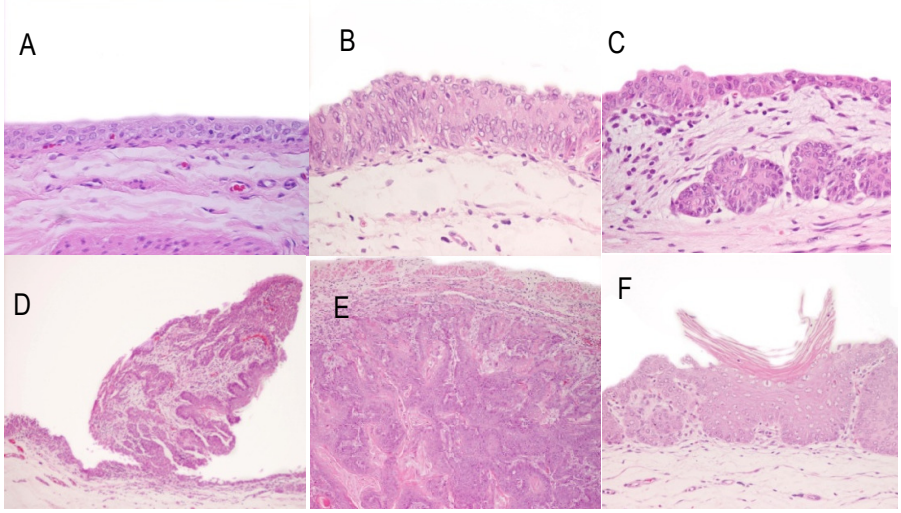

Figure 4: Histopathological evaluation of lesions identified in rat urothelial carcinogenesis induced by BBN. A: Normal urothelium (control group, H&E, x400). B: Simple hyperplasia (H&E, x200). C: Nodular hyperplasia (H&E, x200). D: Papilloma (H&E, x100). E: Invasive carcinoma (H&E, x200). F: Squamous metaplasia (H&E, x100).

3.4.1.2 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.

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

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-neoplasic and neoplasic 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.

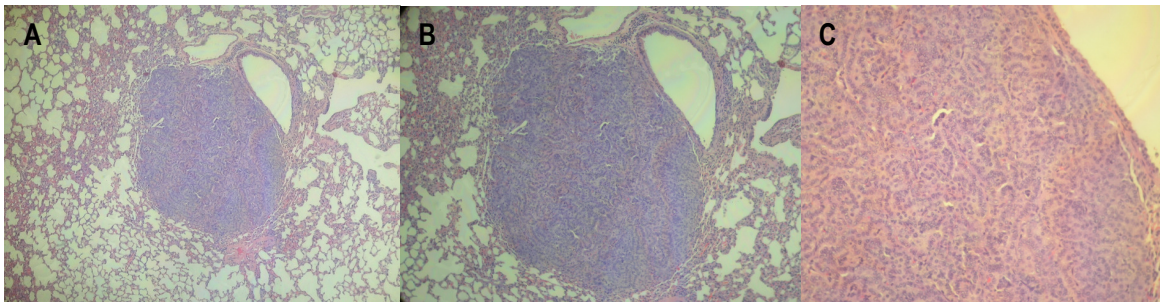

Figure 6: Lung metastasis in a male ICR mouse treated with *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine with invasive urothelial carcinoma (H&E) (A: x100; B: x200; C: x400).

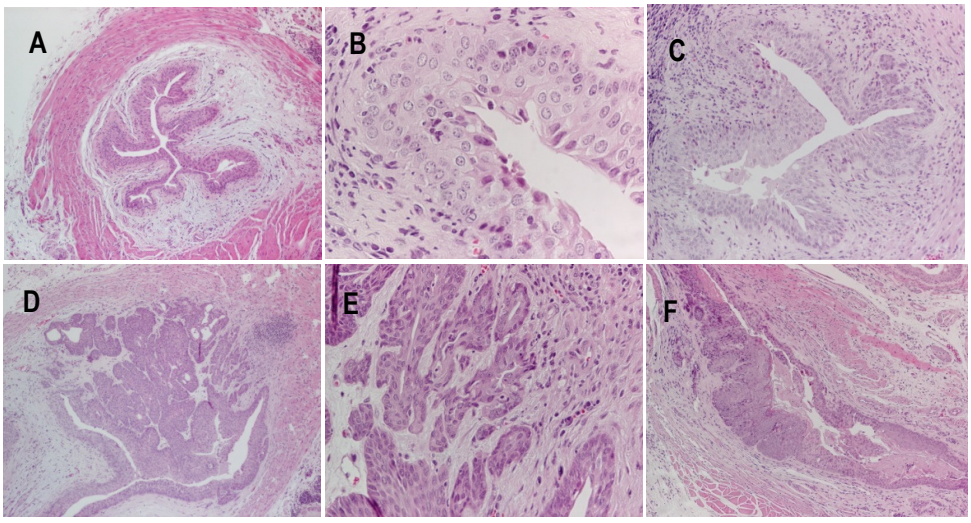

Figure 7: Histopathological evaluation of lesions identified in mouse urethra after BBN treatment. A: Normal urethra (transverse section) (H&E, x100). B: Dysplasia (H&E, x600). C: Nodular hyperplasia (H&E, x200). D: Invasive carcinoma (H&E, x100x). E: Invasive carcinoma (D) with higher magnification (H&E, x400). F: Squamous metaplasia (H&E, x100).

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.

3.5. 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 take. 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 of 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-neoplasic and neoplasic lesions without the need to sacrifice animals, we can progress to the treatment phase, and each animal will function as its own control (109).

3.6. 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.

3.7. Other chemicals

In the past, *N*-[4-(5-nitro-2furyl)-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 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 can arise in the experimenters (6).

MNU is a direct- and complete-acting carcinogen causing tumour initiation and 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). 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).

3.8. Conclusions

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 tumorigenesis 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.

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CHAPTER 4:

EXPERIMENTAL STUDY OF THE ANTI-CANCER EFFECT OF GEMCITABINE COMBINED WITH SIROLIMUS ON CHEMICALLY INDUCED UROTHELIAL LESIONS

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4. Experimental study of the anti-cancer effect of gemcitabine combined with sirolimus on chemically induced urothelial lesions

4.1. Introduction

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. 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 a 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 on these disappointing results, the use of perioperative chemotherapy has been studied since the 1980s. However, only a marginal level of response was 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 anti-viral agent and later developed as an anti-cancer drug, has been used to treat solid tumours (pancreatic cancer, non-small cell lung cancer, breast cancer and ovarian cancer) (3, 4, 5). Gemcitabine is a prodrug that requires cellular uptake and a number of steps in order to be activated (4, 6). It is phosphorilated 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, 8, 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 biological properties including immunosuppressive, anti-fungal and anti-proliferative 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 mice model of invasive bladder cancer were investigated by our

team (18) and others (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.* (2009) and Hansel *et al.* (2010). Equally, when we put the results of these studies together, it reveals that sirolimus inhibits cellular proliferation in several lines of human invasive bladder carcinoma.

Rational politherapy presupposes that two anti-cancer drugs with different mechanisms of action may have superior anti-neoplastic effect than these agents when 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 looking into the combined effect of these drugs in an orthotopic pancreatic cancer model has been carried out (24). Taking into consideration our previous results on the effect of sirolimus on mice invasive bladder cancer model and the *in vitro* effects of gemcitabine, this study was carried out in order to evaluate the association of gemcitabine and sirolimus in mice urothelial lesions induced by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN).

4.2. Material and Methods

4.2.1. Chemicals

N-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Gemcitabine (Gemzar®) and Sirolimus (Rapamune®) were purchased from Lilly and Wyeth, respectively.

4.2.2. Animals

Four-week-old male ICR mice were obtained from Harlan Interfauna Inc. and housed in plastic cages with wood chips for bedding. All mice were acclimatized for one week under routine laboratory conditions before starting the experiments. They were placed in ventilated chambers, in wich the temperature (23±2ºC) and humidity (50±10%) were controlled, and a 12:12 hour light/dark cycle was maintained. All animals had access to a standard lab diet and water *ad libitum*, and all procedures

were performed in accordance with the European Communities Council Directive 86/609/ECC.

4.2.3. 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).

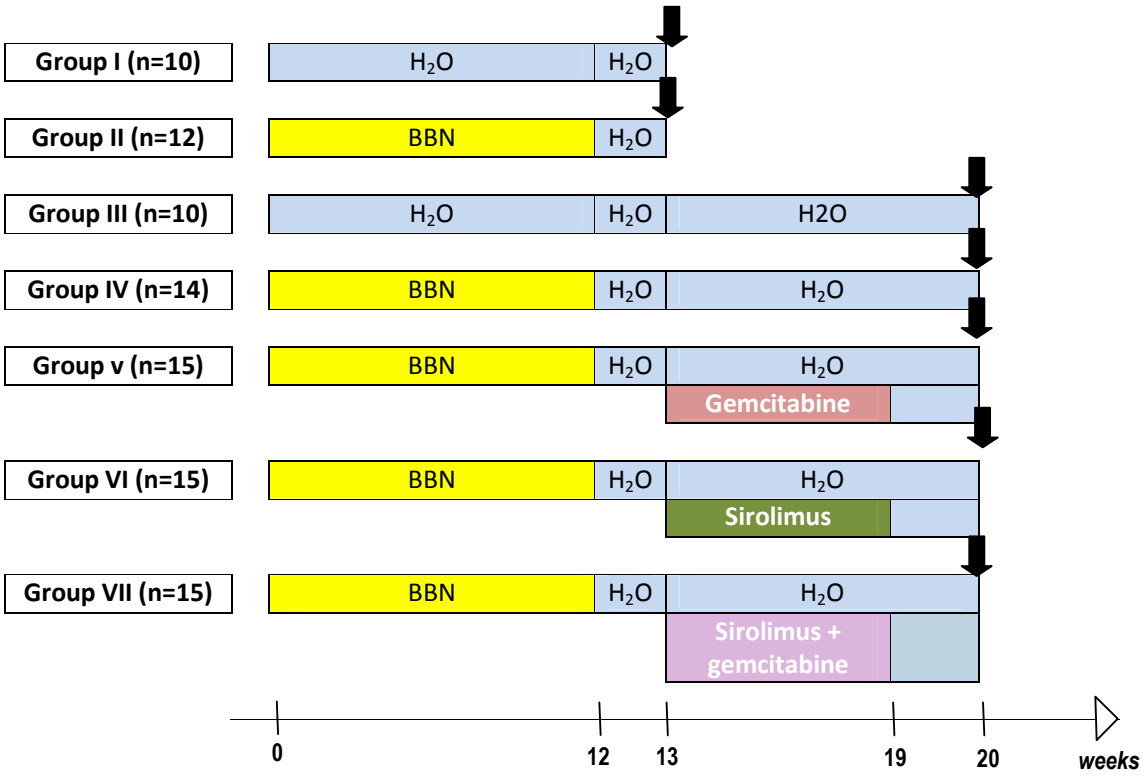

Figure 1: Experimental design (black arrows represent euthanasia).

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 euthanized, 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, the mice's treatment began. Animals in Group V were intraperitoneally administered with gemcitabine (50mg/kg) twice a week for six weeks. Animals in Group VI were intraperitoneally administered with sirolimus (1,5mg/kg), five days a week for six 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 treatment group. After ending their 12-week exposure to BBN, these animals were given normal tap water until the end of the experimental study.

The animal's drinking solution was 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, animals' body weights 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.

4.2.4. Evaluation of treatment

All the surviving animals were euthanized by means of pentobarbital overdose anaesthesia one week after the end of treatment. 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 mice's organs were then cut and, after this procedure, 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 understand the effects of treatments using gemcitabine and sirolimus.

Histology: Histological slides were observed under a light microscope by two different investigators, in a blind way. 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. 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, invasive urothelial carcinoma with squamous differentiation and epidermoid metaplasia.

4.2.5. Statistical analysis

A descriptive study was performed for all variables included in the study. Data was statistically analysed using SPSS 12. 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 + sirolimus and the controls. Parametric data was analysed via analysis of variance (ANOVA) with the Bonferroni correction multiple-comparison method; non-parametric data was computed using the $\chi 2$ test or Fisher's exact test with the Bonferroni correction. A *p* value of <0.05 was considered to be statistically significant.

4.3. Results

4.3.1. 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 subject to different treatments, we concluded that only Group VII (BBN+gemcitabine+sirolimus) is statistically different from 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.

Table I - Initial and final body weights and mean and relative weights of liver, kidney and bladder at the end of the study.

Group		H_2O	BBN	H_2O	BBN + H2O	BBN + Gemcitabine	BBN + Sirolimus	BBN+ Gemcitabine+ Sirolimus
		I	II	III	IV	V	VI	VII
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[1]	42.06±2.73[2]	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[b]	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[a]	1.95±0.28	2.3±0.25	2.08±0.25	2.29±0.35	2.27±0.23	2.08±0.24
Relative weight (g)	Bladder	0.063±0.0022	0.069±0.003	0.0038±0.0013	0.0095±0.007[c]	0.0045±0.0018	0.0049±0.002	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±0.004	0.0085±0.0008	0.0086±0.002	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

1) Mean final body weight is statistically different in Group III and VII (*p=0.002)*; 2) Mean final body weight is statistically different in Group IV and VII *(p=0.000);* a) Liver mean weight of Group I is different from Group II (*p <0.005)*; b) Bladder mean weight from Group IV is different from Groups III, V, VI, VII (*p <0.005)*; c) Bladder relative weight from Group IV is different from Groups III, V, VII (*p <0.005)*.

4.3.2. 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.

4.3.3. 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).

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/reduced by gemcitabine treatment. All other histological alterations, such as simple or nodular hyperplasia, CIS, low- and high-grade papillary tumours, also presented lower incidence levels in animals treated with gemcitabine than animals not treated (Group IV). However, these results were not statistically significant.

The incidence of urothelial lesions in animals treated with sirolimus (Group VI) was lower than in animals not treated. In this group, invasive urothelial carcinoma was reduced from 54% to 7.14% (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.

Table II- Incidence values of histological lesions.

Histological lesions	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII
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%)	2/13(15.38%)	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%)

a) Group IV is statistically different from group VI (p=0.035); b) Group IV is statistically different from group VI (p=0.008); c) Group VI is statistically different from group VII (p=0.037); d) Group IV is statistically different from group VI (p=0.031); e) Group IV is statistically different from group V (p=0.049).

Whatever the kind of pre-neoplastic and neoplastic lesions, we observed that the incidence of pre-neoplastic lesions was greater in animals treated, though the incidence of neoplastic lesions was greater in animals not treated (Table III).

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

	Group IV	Group V	Group VI	Group VII
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%)

4.3.4. Non-urothelial lesions

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

4.4. 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 (26, 27). To our knowledge, there have been no previous experimental studies designed to evaluate the efficacy of gemcitabine in mice invasive bladder cancer chemically induced.

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, decreases the incidence of chemically induced urothelial lesions in mice. The incidence of invasive bladder cancer in animals treated with sirolimus was statistically lower than 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 higher decrease in neoplastic

lesions than gemcitabine. The efficacy of 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 substances' different mechanisms of action, we were expecting additive or synergic results to come from this association. However, despite a reduction of neoplastic lesions, the results obtained were not as good as when each was used individually. This discrepancy between its susceptibility to 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 its companion drug (28) and by the expression of several enzymes involved in their mechanism of absorption, metabolization, distribution and elimination (4). Giovannetti *et al*. (2006) obtained similar results with the combination of gemcitabine and paclitaxel. They explain their results on the basis of drugs' cell-cycle effect, 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 for our results.

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

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CHAPTER 5:

IN VIVO AND *IN VITRO* EFFECTS OF EVEROLIMUS ON BLADDER CANCER

The content of this chapter was presented/ published in:

- 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, Oliveira PA (2011). *In vivo* and *in vitro* effects of RAD001 on bladder cancer. *Urol Oncol-Semin Ori*, Dec 9 (Full paper).

- Vasconcelos-Nóbrega C, Colaço AA, Santos LL, Vala H, Pinto-Leite R, Palomino LF, Lopes C, Oliveira PA (2010). mTor inhibitors Rapamycin and RAD001 in chemically induced bladder cancer. *International Journal of Molecular Medici*ne 26:S42-S42 (Abstract).

- Vasconcelos-Nóbrega C, Colaço AA, Santos LL, Vala H, Pinto-Leite R, Palomino LF, Lopes C, Oliveira PA (2010). mTor inhibitors Rapamycin and RAD001 in chemically induced bladder cancer. 15th WorldCongress on Advances in Oncology and 13th International Symposium on Molecular Medicine. October 7th - 9 th, Loutraki, Greece (Poster presentation).

5. *In vivo* and *in vitro* effects of everolimus on bladder cancer

5.1. Introduction

Cancer is a major public health problem worldwide. Bladder cancer is the second-most common tumour of the urinary tract (1, 2) and its incidence over the last half-century has increased. Urothelial tumours of the bladder are invasive in 20 to 30% of patients (3). The invasive and metastatic bladder cancers, represents the leading cause of morbidity and mortality among bladder-cancer patients, because the therapeutic protocols currently available fail to provide an effective response or to provide positive outcomes. New strategies and new therapeutic agents should therefore be tested.

Animal models of bladder cancer have been widely used in the pre-clinical development of new and more effective therapeutic agents, as well as to enable a deeper and more accurate understanding of the process of bladder carcinogenesis (4, 5). Oral administration over a period of 12 weeks (6) of BBN induces the development of invasive urothelial cancers that exhibit similar morphological characteristics and molecular events, such as their DNA content, proliferative index and *p53* overexpression, as human urothelial bladder cancer (7).

Activation of the PI3k/Akt/mTOR axis was associated with early events in carcinogenesis and the interruption of this pathway led to anti-proliferation, anti-survival, anti-angiogenic and pro-apoptotic effects (8).

Everolimus is an oral mTOR inhibitor with an anti-neoplastic activity similar to rapamycin (sirolimus), an anti-cancer agent used on solid tumours (9-13). Everolimus has a potent anti-proliferative effect when used against human breast cancer cell lines, lymphoma cells, prostate cancer cells and gastric cancer (14). Its therapeutic effects are under clinical evaluation for use on different types of solid cancer, including renal cell and endometrial cancer (15). It has a shorter half-life and greater bioavailability than rapamycin and after entering cells it binds with FKBP-12, an immunophilin. This forms a complex that binds to the mammalian target of rapamycin (TOR), thus inhibiting growth factor-induced transduction signals that mediate cellular division, retarding or arresting the cell cycle in G_1 phase or, in some cases, inducing apoptosis (12,14,16-19).

In this study, we evaluated the therapeutic effects of everolimus on mice with BBN-induced neoplastic urothelial lesions similar to those found in humans, as well as its effect *in vitro* on three human bladder-cancer cell lines (T24, HT1376 and 5637).

5.2. Material and Methods

5.2.1. *In vivo* studies

5.2.1.1 Chemicals

BBN reference is the same described in section 4.2.1.
Everolimus (Certican®) was purchased from Novartis.

5.2.1.2. Animals

Animals' procedures before the experiments were the same as described in section 4.2.2.

5.2.1.3. Animal experiments

After one week of quarantine, a total of 51 ICR mice were randomly divided into four groups (Figure 1) (group I: n=10; group II: n=12; group III: n=15; group IV: n=14), groups II, III and IV received BBN (0.05%) in drinking water for 12 weeks. Group I was used as a negative control group, drinking only tap water. One week after bringing BBN exposure to an end, groups I and II were euthanized by means of pentobarbital overdose anaesthesia to evaluate the presence of any preneoplastic and neoplasic lesions induced by BBN. After being treated with BBN solution for 12 weeks, groups III and IV were maintained with normal tap water during one additional week. Group III was treated with everolimus, two days a week (5mg/kg) (20) by oral gavage for six consecutive weeks, until the 19th week of the experiment, with group IV being used as the treatment control group.

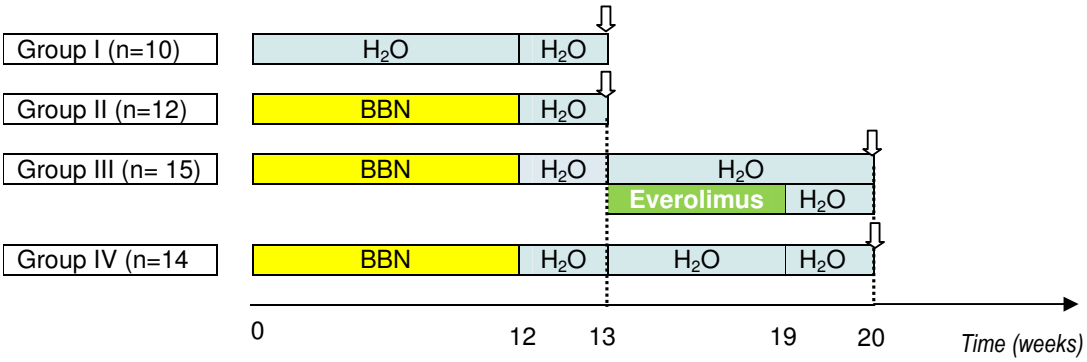

Figure 1: Experimental design (white arrows represent euthanasia).

For the duration of the study, the mice's state of health was monitored daily. The animals' drinking solution was changed once a week or earlier if necessary, and the volume drunk was recorded. Weekly food intake was also noted. Their body weights were initially measured once a week and then twice a week after treatment had begun, allowing us to adjust everolimus doses to individual weight variations.

5.2.1.4. Evaluation of treatment

All surviving animals from groups III and IV were euthanized, according to the technique described above, one week after ending everolimus treatment. Necropsies were carried out and the mice's urinary bladders were collected according to the technique described by Oliveira *et al.* (2009) (6). After overnight fixation, bladders were cut longitudinally and their mucosal surface was carefully examined for the existence of macroscopic lesions. After this procedure, they were embedded in paraffin and 2 µm sections were cut and stained with haematoxylin and eosin. All other organs were also examined macroscopically for any changes, and their weights were recorded.

5.2.1.4.1-Histology

Histological slides were observed under a light microscope by two investigators. All slides were examined without prior knowledge of the treatment given to the animals whose tissue samples were under investigation. Histological lesions found in different groups were classified and staged according to World Health Organization guidelines

(21). Urothelial lesions were categorized into either: simple hyperplasia, nodular hyperplasia, dysplasia, carcinoma *in situ*, papilloma, low-grade non-invasive papillary urothelial carcinoma, high-grade non-invasive papillary urothelial carcinoma, invasive urothelial carcinoma and squamous metaplasia.

5.2.1.4.2-Immunohistochemistry

The mTOR (7C10) rabbit mAb (#2983, Cell Signalling Technology) was used as the primary antibody. The immunohistochemistry assay was performed with an autostainer (LVI, Labvision Corporation) using the Ultra VisionHP-125-HL detection kit (Labvision Corporation). The staining procedure using this kit is based on the labelled streptavidin biotin method. Heat antigen retrieval was performed by slides pre-treatment in a jar with sodium citrate buffer pH6 and heated 3 times for 5 minutes in a microwave oven at 700 W. Slides were incubated 60 minutes at 37ºC with the primary antibody (1:50). Paraffin sections from human lung carcinoma were used as a positive control.

5.2.1.4.2-1: Immunohistochemical evaluation

Sections were examined under light microscopy. The immunohistochemical expression was classified as exhibiting positive staining if the pattern of immunoreactivity was identical to that of the cytoplasm staining of normal urothelial cells for mTOR activity. Negative staining was considered if lesions/tumours showed no immunoreactivity.

5.2.2. *In vitro* studies

5.2.2.1. Chemicals

Everolimus (Certican®) was purchased from Sigma.

5.2.2.2. Tumour cell lines and culture conditions

In vitro study was performed using two established human invasive bladder-cancer cell lines: T24 and HT1376 and one superficial urothelial bladder-cancer cell

line: 5637 (the first line was provided by DSMZ, Düsseldorf, Germany and the last two lines provided by Dr. Paula Videira of the Universidade Nova de Lisboa). All the cell lines were cultured as a monolayer in RPMI 1640 culture medium (PAA, Austria) supplemented with 10% heat-inactivated fetal calf serum (Biological Industries, Israel), 100U/mL penicillin (Biological Industries, Israel), 100µg/mL streptomycin (Biological Industries, Israel) and 2 mM L-Glutamine (Sigma Aldrich, EUA). Cells were maintained at 37ºC in a humidified atmosphere with 5% CO_2-95% O_2 and were routinely subcultured by trypsination. All experiments were performed during exponential cell growth. All bladder-cancer cell lines expressed higher amounts of VEGF-A, however the 5637 cell line had the highest VEGF-A expression of any cell line studied (22).

5.2.2.3. Drug exposure

Bladder-cancer cell lines were exposed to concentrations of everolimus (Rad001) ranging from 0 to 2 µM (0; 0.05; 0.1; 1; 2 µM) over the course of 72h to assess dose response-profiles. An untreated control group was processed in the same way as the treatment samples but in a drug free-medium, and the evaluation of the cytotoxicity effect was performed immediately after the end of drug exposure. The concentration of everolimus represented the average IC30 dose at 72h from each respective single concentration. IC30 is defined as the drug concentration that causes a 30% reduction in cell numbers, compared to that of the untreated control (IC30= 30% growth inhibition) (23).

5.2.2.4. Cell viability assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Sigma Aldrich, EUA) assay was used to assess the relative percentage of metabolically active cells relative to untreated controls (24). In brief, trypsinized tumour cells were re-suspended in a medium at 2-3x10^4 cells/mL, based on the growth characteristics of each cell line after verifying cell viability via trypan blue dye exclusion. One hundred µL of cell suspension were seeded into each well of a 96-well flat-bottom micro titer (Sarstedt, USA) and plates were incubated for 24h to allow adherent cell growth. After

overnight incubation, the medium was removed and 100 µL of the different reagent solutions in complete medium and medium control were distributed in each well and incubated for 72h. After incubation, 10 µL of MTT dye working solution (5 mg/mL) was added to each well. After four hours incubation, the supernatant in the wells was removed and replaced by 100 µL/well of dimethylsulfoxide (DMSO, Sigma Aldrich, USA). The absorbance (A) values of each well were recorded at 492 nm on an automatic ELISA plate reader (Multiskan EX, Labsystems). The medium containing tetrazolium dye but without cells (blank), was used as a negative control. After the blank correction, the relative viability was calculated as follows: Aexp group / Acontrol x 100 (20,25). In each experiment, triplicate wells were performed for each everolimus concentration and the assay was repeated in two independent experiment replications.

5.2.2.5. TUNEL assay

In order to detect and quantify DNA strand breaks in apoptotic bladder cells, TUNEL assay was performed. Cells ($2x10^4$) were then seeded and allowed to attach overnight. Subsequently, cells were treated with everolimus (2µM) and incubated for 72h. After treatment, cells were trypsinized, washed with PBS and fixed with 4% paraformaldehyde for 1h at 15 to 25°C. The *in situ* cell-death detection kit fluorescein (Roche, USA) was adapted from the manufacturer's instructions. Slides were observed using a fluorescence microscope (Nikon Eclipse E400). Cells were defined as apoptotic if the nuclear area of cells was labelled positively (green colour). The number of cells undergoing apoptosis was counted in random fields on each slide. At least 200 cells were counted (magnification 1000x) in random fields on each slide. The apoptotic index (AI) was determined as follows: AI (%)=(number of apoptotic cells/total number of cells)x100 (26).

5.2.2.6. Cell-cycle analysis

Cells ($1x10^6$) were plated in 6-well plates and incubated for 24h. Then everolimus was added in serial dilutions. After 72h of incubation, cells were trypsinized, washed with PBS and fixed in ice-cold ethanol 70% for at least 1hour. Propidium iodide (PI, CycloscopeTM DNA Cytometry, Cytognos) was added in order to label total cellular DNA, and cell-cycle analysis was carried out using a Coulter® EPICSTM XL-MCLTM flow cytometer. DNA-content histograms were analysed with Modfit LT 3.0

software (VerityTM) to determine the percentage of cells in each cell cycle: G_0/G_1, S and G_2/M. Cells with DNA content less than that of G_0/G_1-phase cells were considered to be apoptotic (sub- G_0/G_1). Each independent experiment was performed in triplicate.

5.2.2.7. mTOR and Akt expression analysis

Whole cell extracts were obtained from mechanical homogenization of cell pellets in 1% SDS containing phosphatase inhibitors (P0044 and P5726, Sigma). Protein concentration was assayed by the colorimetric method "RC DC protein assay" (Bio-Rad) using bovine serum albumin (BSA) as standard. Samples from each cell line, with no treatment and with 2µM everolimus, were then diluted in Tris buffered saline (TBS; 100mM Tris, 1.5mM NaCl, pH 8.0) to obtain a final protein concentration of 0.4mg/mL and a volume of 100µL was slot-blotted into a nitrocellulose membrane (Whatman, Protan). Nonspecific binding was blocked with 5% (w/v) BSA in TBS-T (TBS with 0.5% Tween 20) for 1 hour and the membrane was then incubated with primary antibody (1:1000 dilution; rabbit anti-mTOR, #2983, rabbit anti-Phospho-mTOR, #2971, rabbit anti-Akt, #9272 or rabbit anti-Phospho-Akt, #4058 from Cell Signalling). After 1 hour incubation, the membrane was washed with TBS-T and incubated with anti-rabbit IgG peroxidase secondary antibody (1:1000; Amersham Pharmacia Biotech). Immunoreactivity was detected with enhanced chemiluminescence reagents (ECL, Amersham Pharmacia Biotech) according to the manufacturer's instructions and images were recorded using X-ray films (Kodak Biomax Light Film, Sigma). Films were scanned in Molecular Imager Gel Doc XR+ System (Bio-Rad) and analysed with QuantityOne software version 4.6.3 (Bio-Rad, Hercules, SA). Protein loading control of blotting membranes was performed by staining with Ponceau S.

5.2.3. Statistical analysis

Statistical analysis was carried out using the SPSS 17.0 statistical software (SPSS Inc. USA). Body weight differences and the mean and relative weights of the animals' livers, kidneys and bladders were assessed using ANOVA, with Bonferroni multiple comparison. Non-parametric data were computed using the $\chi 2$ test or Fisher's exact test with Bonferroni's correction. For *in vitro* studies ANOVA was performed, the equality of variances was tested by Levene F test and the statistical significance of

differences between the treatment and control groups were determined by Dunnett's Multiple Comparison post-hoc test for the MTT assay. The Pearson product-moment correlation coefficient was used to evaluate the correlation (linear dependence) of the cell cycle, apoptosis and everolimus concentration. *p* values of less than 0.05 were considered to be statistically significant.

5.3. Results

5.3.1. *In vivo* results

5.3.1.1. Animal growth and water and food consumption

The mean food and water intake was similar across the different groups throughout the whole study period (data not shown).

Two mice from groups III and IV died during the experiment and therefore only surviving animals were considered for histopathological examination. During the treatment period, animals exhibited normal cage activity. When comparing the body weight of mice treated and those not treated with everolimus, the differences were not statistically significant.

The mean weight of the animals' livers, kidneys and bladders, as well as the relative organ weights (calculated as the ratio of the mouse's organ weight by the mouse's weight) are shown in Table I.

Table I - Mean and relative weight of bladder, kidney and liver (value ±SD).

Group	Mean weight (g)				Relative weight (g)			
	Bladder	Right kidney	Left kidney	Liver	Bladder	Right kidney	Left kidney	Liver
I	0.280±0.113	0.327±0.018	0.341±0.042	2.32±0.41[c]	0.063±0.0022	0.0075±0.00054[f]	0.0078±0.001[g]	0.053±0.0091
II	0.269±0.115	0.347±0.041	0.343±0.051	1.95±0.28	0.069±0.003	0.009±0.001	0.0089±0.001	0.050±0.0051
III	0.167±0.05[a]	0.328±0.091	0.292±0.0107[b]	2.30±0.46[d]	0.004±0.001[e]	0.008±0.002	0.0072±0.0026	0.0566±0.0110[h]
IV	0.38±0.28	0.376±0.059	0.407±0.165	2.08±0.251	0.0094±0.0077	0.0089±0.001	0.0097±0.0042	0.0495±0.0047

a) Different from Group IV *(p*=0.01*)*; b) Different from Group IV *(p*=0.041*)*; c) Different from Group II *(p*=0.0024*)*; d) Different from Group IV *(p*=0.0139*)*; e) Different from Group IV *(p*=0.017*)*; f) Different from Group II *(p*=0.003*)*; g) Different from Group II *(p*=0.045*)*; h) Different from Group IV (*p*=0.033*)*. (SD- Standard deviation).

5.3.1.2. Macroscopic evaluation

Greyish-white urinary bladder masses which varied in size were observed in groups II, III and IV. The lesions were invasive or less frequently pedunculated. The lesions exhibited irregular surfaces and haemorrhage, while they were distributed randomly throughout the entire urinary bladder. No macroscopic urinary bladder changes were identified in the control group (I). Stone formation in the urinary bladder was not observed in any mice. No macroscopic changes were seen in the liver, lung, kidneys and gastrointestinal tract of the animals involved.

5.3.1.3. Microscopic evaluation

5.3.1.3-1: Histology

Effects of BBN on urothelial tumorigenesis: We conducted detailed histopathological examination to determine the degree of urothelial lesions induced by BBN. These occurred only in the groups drinking BBN solution. Animals from group I (control group) did not show any histological alterations i.e. 100% of them exhibited a normal urothelium. Table II summarizes the incidence of BBN-induced urothelial lesions in each group.

Effects of everolimus on urothelial tumorigenesis: The incidence of lesions between groups treated with everolimus and those not treated can be observed in Table II. Histological alterations, such as simple and nodular hyperplasia, dysplasia and carcinoma *in situ*, exhibited a lower incidence in animals treated with everolimus (group III) than animals not treated (group IV). There was a higher incidence of papillomas and low-grade non-invasive papillary urothelial carcinomas in animals treated with everolimus compared with animals not treated. The incidence of BBN-induced invasive urothelial carcinoma was slightly lower in mice treated with everolimus (group III) and these differences were not statistically significant.

Table II - Incidence of urothelial lesions in ICR mice exposed to *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine and treated with everolimus (Rad001)

Histological lesion	Group I H_2O (n=10)	Group II BBN (n=12)	Group III BBN+RAD001 (n=14)	Group IV BBN+H_2O (n=13)
- Normal urothelium	10/10 (100%)	0 (0%)	0 (0%)	0 (0%)
- Simple hyperplasia	0 (0%)	8/12 (67%)	9/14 (64.3%)	10/13 (76.9%)
- Nodular hyperplasia	0 (0%)	7/12 (58.3%)	8/14 (57.14%)	9/13 (69.23%)
- Dysplasia	0 (0%)	12/12 (100%)	12/14 (85.7%)	13/13 (100%)
- Carcinoma *in situ*	0 (0%)	4/12 (33%)	1/14 (7.1%)	2/13 (15.38%)
- Papilloma	0 (0%)	0 (0%)	1/14 (7.1%)	0 (0%)
- Low-grade non-invasive papillary urothelial carcinoma	0 (0%)	0 (0%)	3/14 (21.4%)	1/13 (7.69%)
- High-grade non-invasive papillary urothelial carcinoma	0 (0%)	0 (0%)	0 (0%)	1/13 (7.69%)
- Invasive urothelial carcinoma	0 (0%)	4/12 (33%)	6/14 (42.86%)	7/13 (53.84%)
- Squamous metaplasia	0 (0%)	8/12 (67%)	9/14 (64.3%)	10/13 (76.9%)

5.3.1.3-2: Immunohistochemistry

mTOR immunoreactivity was performed in urinary bladders samples from groups I, III and IV. The results of the mTOR immunoreactivity investigations are summarized in Table III and discussed below using the diagnostic categories reported on Table II. In normal urothelium (Figure 2-A) the cytoplasm was uniformly stained. Simple hyperplasia (Figure 2-B) and dysplasia (Figure 2-C) showed a similar mTOR pattern when compared with normal urothelium, with its expression confined to urothelium cytoplasm. On squamous metaplasia (Figure 2-D), we observed a homogeneous cytoplasm immunoreactivity pattern on basal layer, and loss of immunoreactivity in upper layers. mTOR immunohistochemical stain performed on invasive carcinoma from animals treated with everolimus (Figure 2-E) revealed no staining in 80% of the lesions observed. However, 75% of invasive carcinomas in animals not treated revealed positive cytoplasm immunoreactivity (Figure 2-F).

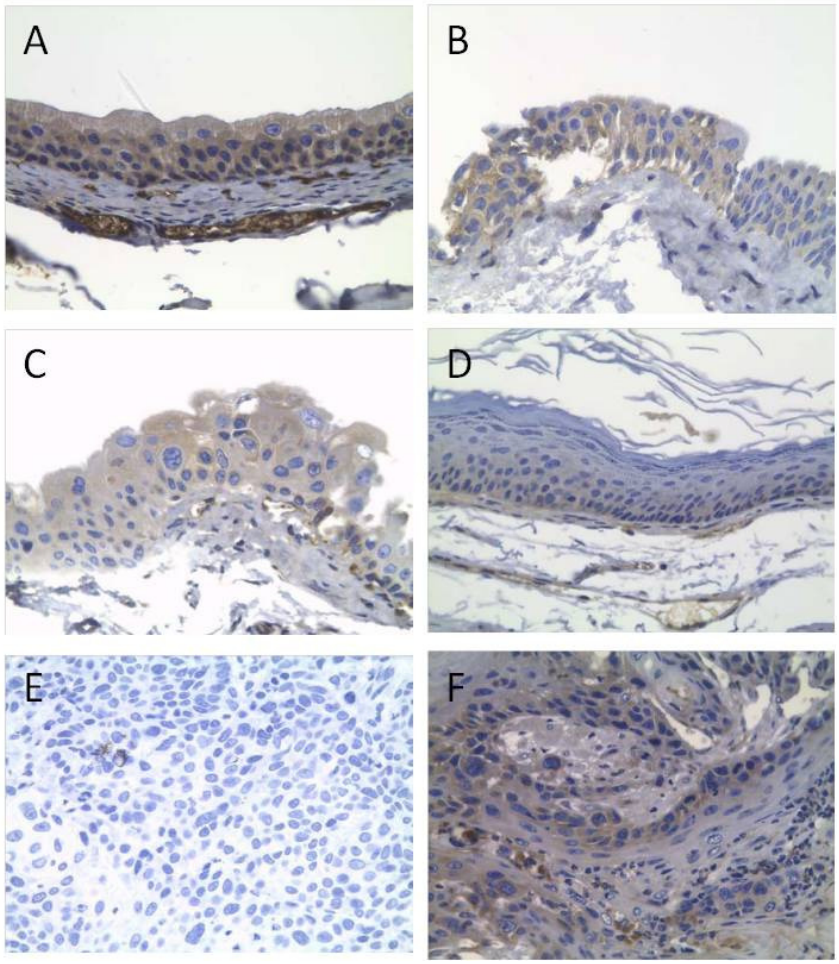

Figure 2: (A) mTOR expression in normal mouse urothelium (x400); (B) mTOR expression in simple hyperplasia (x400); (C) mTOR expression in dysplasia (x400); (D) mTOR expression in squamous metaplasia (x400); (E) absence of mTOR expression in invasive carcinoma (x400); (F) mTOR expression in invasive carcinoma (x400).

Table III - Cytoplasm immunoreactivity of urothelial lesions with mTOR antibody in ICR mice exposed to *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine and treated with everolimus.

Group	Histological lesion	Positive	Negative
Group I (H_2O)	Normal urothelium (n=10)	10 (100%)	-
Group III (BBN+ Everolimus)	Simple hyperplasia (n=9)	9 (100%)	-
	Nodular hyperplasia (n=5)	3 (60%)	2 (40%)
	Dysplasia (n=12)	12 (100%)	-
	Invasive urothelial carcinoma (n=5)	1 (20%)	4 (80%)
	Squamous metaplasia (n=6)	2 (33.3%)	4 (66.7%)
Group IV (BBN+H_2O)	Simple hyperplasia (n=9)	9 (100%)	-
	Nodular hyperplasia (n=3)	3 (100%)	-
	Dysplasia (n=8)	8 (100%)	-
	Invasive carcinoma (n=4)	3 (75%)	1 (25%)
	Squamous metaplasia (n=6)	5 (83.3%)	1 (16.7%)

5.3.2. *In vitro* results

5.3.2.1. Cytotoxic effect of everolimus against bladder-cancer cell lines

Everolimus was added to the three bladder-cancer cell lines and its proliferation was quantified 72h after plating. The cytotoxic activity of everolimus is shown in Figure 3. The IC values were determined by MTT assay, according to exposure time and dose of agent. Everolimus transiently inhibited bladder-cancer cell growth in a dose-dependent manner. Everolimus is more effective on 5637 cells and exhibits an IC30 at 1 μM. Among the three cell lines tested, T24 cells demonstrated significant resistance to everolimus exposition, as clearly documented by survival rates of 98.6, 98.5, 98.2 and 94.5%, and were the least sensitive to everolimus, when compared with HT1376 cell-line survival rates (99.2; 92.3; 92.4 and 87.5%). Statistically, the anti-proliferative activity of everolimus was only significant in the 5637 cell line, at all concentrations, when compared with the control group ($p<0.05$).

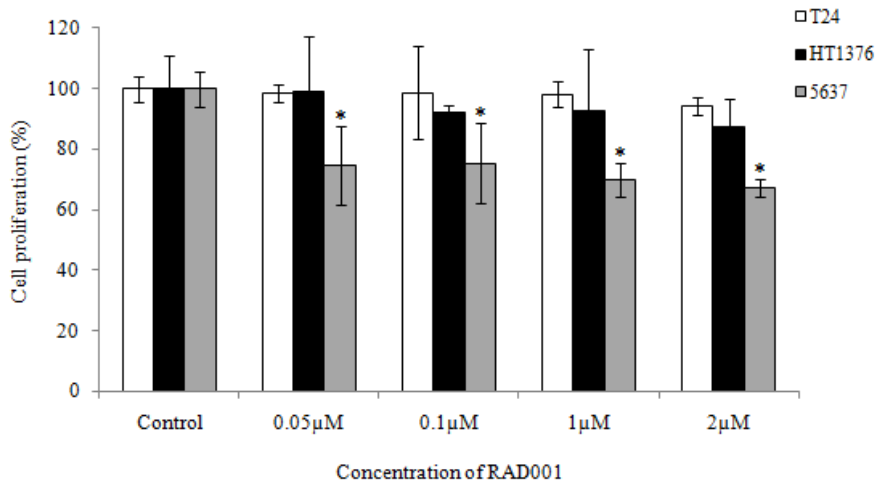

Figure 3: Cell proliferation of bladder cancer cell lines (T24; HT1376; 5637), treated with different doses of everolimus (RAD001) (cell proliferation was monitored using the MTT assay. The data shown and bars represent the mean values±SD. $p<0.05$ *versus* untreated controls).

5.3.2.2. TUNEL assay

To analyze everolimus induced apoptosis in bladder-cancer cells in vitro, TUNEL was carried out. Only an apoptotic index value of 6.5% in 5637 cell line was observed as a maximum apoptotic effect in this study. In T24 and HT1376 cell lines this effect was minimal (Table IV).

Table IV - Apoptotic index values (%) in the three bladder-cancer cell lines after exposure to everolimus.

Groups	**T24**	**5637**	**HT1376**
Control	1.5%	3%	1.5%
Everolimus (2µM)	2.5%	6.5%	1.5%

5.3.2.3. Cell cycle and apoptosis analysis

The comparative analysis of the percentage of cells in each cell-cycle phase, spanning the control experiment and the different drug concentrations tested, is summarized in Figure 4. Cell-cycle alterations were only observed in the 5637 bladder-cancer cell line, namely a decrease in the percentage of cells in S-phase fraction (p=0.029) between the control and treated cells. This effect on proliferative rate was inversely correlated to drug concentration (r=-0.978; *p*=0.004).

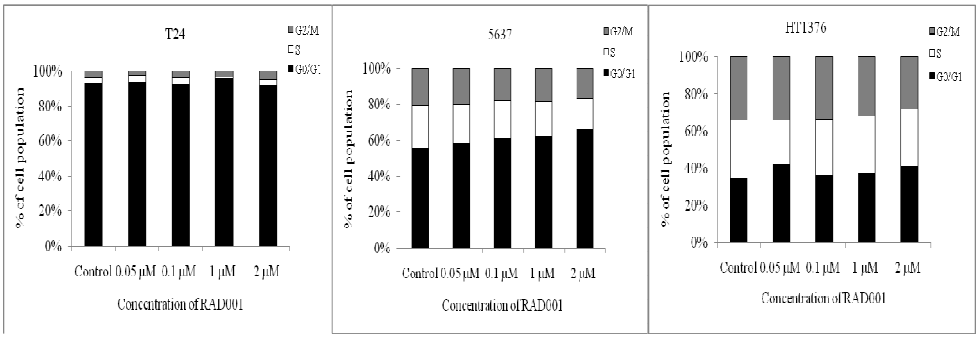

Figure 4: Cell-cycle analysis of the T24, 5637 and HT1376 bladder-cancer cell lines after exposure to different concentrations of everolimus (RAD001). The percentages of cells in each cell-cycle phases are mean±SD of three independent experiments. SD – Standard deviation.

Regarding the relationship between drug treatment and apoptosis, no effects were observed in invasive bladder-cancer cell lines T24 and HT1376 (Figure 5). Only in the 5637 superficial cell line was detected a slight, but statistically significant, increase in sub- G_0/G_1 fraction (*p*=0.001), an effect which was positively correlated with concentrations of everolimus (r=0.956; *p*=0.011).

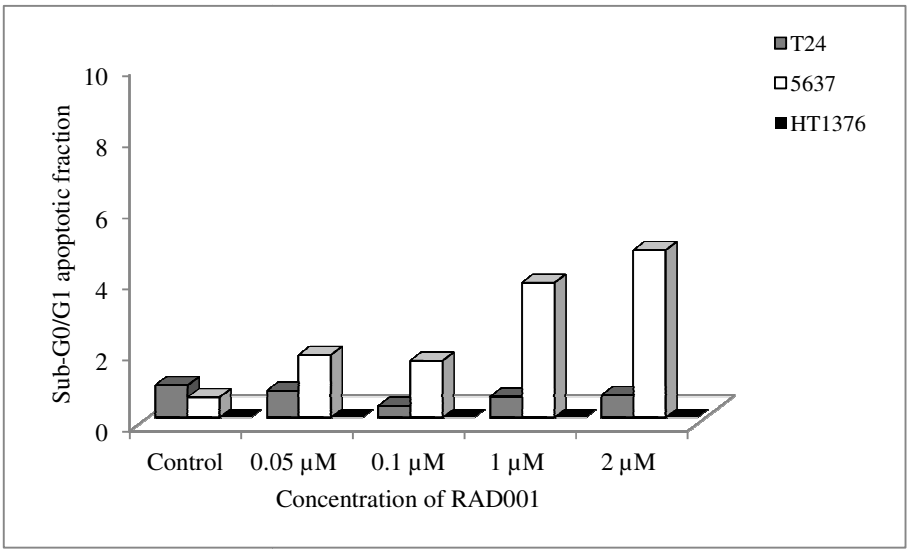

Figure 5: Sub-G_0/G_1 fraction on bladder-cancer cell lines T24, 5637 and HT1376, after exposure to different concentration of everolimus (RAD001). Sub-G_0/G_1 values are mean±SD of three independent experiments. For HT1376 the values were zero for all the experimental conditions. SD- Standard deviation

5.3.2.4. Effects of everolimus on mTOR and Akt activation

To analyse everolimus effect on mTOR activation, the expression of mTOR and its phosphorylated form was analysed in all the cell lines. As can be depicted in Figure 6, no significant expression differences of mTOR and on its phosphorylation at Ser2448 were found due to everolimus administration in cell lines T24 and HT1376. In cell line 5637 a significant decrease of mTOR expression was noticed, though not paralleled by a significant increase in its phosphorylation. Once the major PI3K pathway leads from Akt to mTOR, the expression of Akt was also evaluated by immunodetection of Akt and its phosphorylated form. Everolimus treatment resulted in a slight increase in the baseline expression of Akt in T24 and HT1376 cell lines and of its phosphorylation at Ser473 in HT1376 and 5637 cell lines.

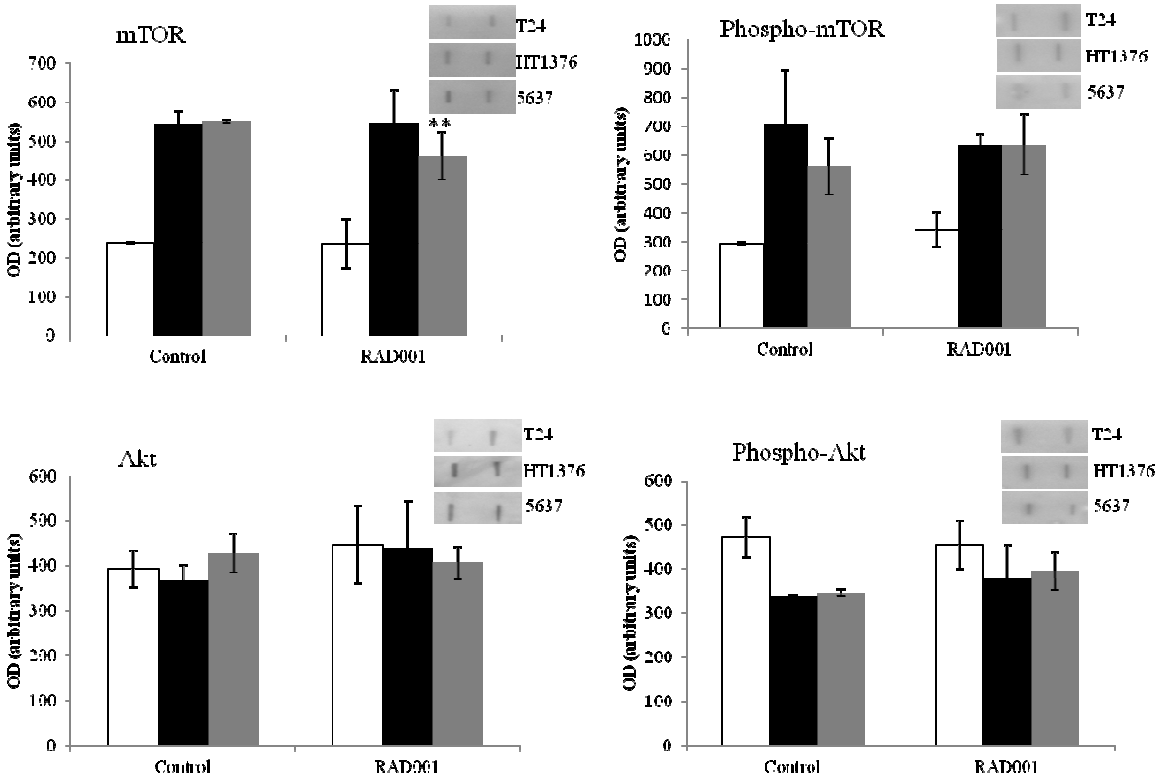

Figure 6: Effect of 2mM everolimus (RAD001) on the expression of mTOR, phospho-mTOR, Akt and phospho-Akt in bladder cell lines [T24 (white bars), HT1376 (black bars) and 5637 (grey bars)]. Representative immunoblots are presented above the corresponding graphs. Data shown and bars represent the mean values ± SD. ** $p<0.01$ *versus* untreated controls.

5.4. Discussion

The mouse model of invasive bladder cancer induced by BBN, as well as other animal models, plays an essential role in the evaluation of target cancer therapy. In this study, we investigated the effect of the mTOR inhibitor everolimus on BBN-induced bladder tumorigenesis in mice and human bladder-cancer cells. This investigation took into account our previous studies concerning the effects of sirolimus on the development of urothelial lesions induced by BBN in mice and the *in vitro* effects on the T24 cancer-cell line (24, 27). For this purpose, male ICR mice were exposed to BBN for 12 weeks and later treated with everolimus two times per week, for six consecutive weeks. Three human bladder-cancer cell lines were treated with various everolimus concentrations during a period of 72 hours.

According to our knowledge, this is the first report on the influence of everolimus on chemically-induced urothelial tumorigenesis in mice. The dosage used in our study seemed to be well tolerated and no macroscopic changes were seen in the liver, lungs, kidneys and gastrointestinal tract of the animals involved.

As determined by the observed results the development of BBN-induced urothelial lesions in mice was not significantly influenced by everolimus treatments. Although there were too few mice in each group, the incidence of urothelial preneoplastic lesions was slightly lower in those animals treated with everolimus compared to animals exposed to BBN but not treated. The incidence of invasive lesions was not affected by everolimus treatments. The rate of low-grade non-invasive papillary urothelial carcinoma was higher in the group treated with everolimus. In our previous study using the same murine model (animals and time of exposition to BBN) sirolimus was more effective, significantly reducing the development of invasive lesions in comparison to non-treated mice (6).

In the *in vitro* study, heterogeneous results were observed. Everolimus had no effect on mTOR and Akt expression, cellular proliferation and apoptosis of T24 and HT1376 cell lines. However, a significant S-phase decrease and a concomitant arrest of cells in G_0/G_1 cell-cycle phase were seen in the 5637 cancer cell line, the only cell line studied in which a significant decrease of mTOR expression was observed. These effects were correlated to drug concentration and consistent with those observed in the MTT assay. With regard to apoptosis, a slight but significant increase of the sub- G_0/G_1 fraction was only observed in the 5637 cancer cell line.

Mansure *et al*. (2009) (27) found that everolimus potently inhibits proliferation in some of several cell lines (UM-UC-3, UM-UC-5, UM-UC-6, UM-UC-9, and UM-UC-14) with different stages of bladder tumorigenesis *in vitro* and also significantly inhibits human bladder KU-7 tumour xenografts growing in nude mice, though no concomitant apoptotic tumour cells were observed. These results revealed a cytostatic effect with no significant change in the sub-G1 fraction and no cell loss in MTT assays.

Chiong *et al*. (2011) (28) also investigated the effect everolimus had on human bladder-cancer cells (UM-UC3, UM-UC13) *in vitro* and *in vivo*. They observed heterogeneity in terms of *in vitro* responses to everolimus. Regarding the expression of components of the PI3K/Akt/mTOR cascade, Mansure's study (2009) (27) and Gust and So (2009) (29) showed no association of expression with sensitivity to treatment. In the present study, the everolimus-related reduction of mTOR was only observed on 5637 cells and resulted in an increase of upstream phospho-Akt, in a similar way as previously reported for renal cell carcinoma. The effect of everolimus was augmented

by retreatment of the cells after three days. After retreatment, only sensitive cell lines showed G1 phase arrest, with no evidence of apoptosis. However, everolimus did significantly inhibit the growth of tumour cells that were subcutaneously implanted in mice. Both authors found that everolimus' anti-tumour activity is related to angiogenesis inhibition. This was evidenced by the decrease in VEGF production *in vitro*, and a significant reduction in microvessels.

Curiously, in a similar direction, we previously observed that 5637 cells, our most sensitive cell line, expressed nearly four times more VEGF-A than any other bladder-cell lines studied (22). Therefore, angiogenesis inhibition may be the predominant mechanism effect of sirolimus and its analogues (25, 30).

It is important to underline that changes in tumour volume and angiogenesis observed in xenografts (nude mice) induced by everolimus cannot be compared with the development of chemically induced bladder cancer in immune-competent mice. More studies including the evaluation of protein-synthesis inhibition through the S6K and 4E-BP1 pathways, in order to clarify everolimus' role in modulating the carcinogenesis process, are required using this murine model. In our opinion and according to our results it is important to evaluate the effect of everolimus prior to tumours being established, during the existence of pre-neoplastic lesions which means before 12 weeks of BBN exposition.

In summary, everolimus seems not to have a significant effect on chemically induced murine-bladder tumorigenesis. The treatment was well tolerated. The effect of everolimus on proliferation and apoptosis across different bladder-cancer cell lines is heterogeneous. Current knowledge suggests the possibility of everolimus integration in bladder-cancer treatment protocol. However, there needs to be further clarification in terms of the mechanism of mTOR inhibitors and their role in the treatment of bladder cancer.

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CHAPTER 6:

MITOCHONDRIAL AND LIVER OXIDATIVE STRESS ALTERATIONS INDUCED BY *N*-BUTYL-*N*-(4-HYDROXYBUTYL) NITROSAMINE – RELEVANCE FOR HEPATOTOXICITY

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6. Mitochondrial and liver oxidative stress alterations induced by *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine – relevance for hepatotoxicity

6.1. Introduction

Nitrosamines are widely present in the environment. Humans are exposed to these molecules from occupational and environmental sources, but also owing as due to *in vivo* formation of amines and nitrosating agents from ingested precursors (1). *N*-nitrosamines can be endogenously formed from the interaction of nitrate or nitrite with secondary amines, which are present in dietary components but can also be produced by intestinal microflora (2). The organic *N*-nitroso-compounds are characterized by the induction of marked acute liver toxicity, and chronic absorption of small amounts has been shown to result in cirrhosis in experimental animals (3).

N-nitroso compounds, or their metabolites, can react with cellular DNA or proteins to form adducts, which are a critical event in cancer induction (4, 5). The tumour induction by nitrosamines can result from exposure to a single large dose or from chronic exposure to relatively small doses (6).

Studies with *N*-nitrosofenfluramine have showed that the cytoxicity on isolated rat hepatocytes is characterized by the induction of the mitochondrial permeability transition (MPT), with loss of mitochondrial membrane potential ($\Delta\Psi$) (7). Failure in oxidative phosphorylation, with ATP depletion at an early stage, followed by increased lipid peroxidation at a later stage, was also observed (8). *N*-nitrosofenfluramine-induced toxicity depends on intracellular energy status, and the rapid depletion of intracellular ATP was associated with the induction of oxidative stress and morphological alterations (9). Studies performed with 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) have shown that mitochondria are an intracellular target (10). Janzowski and collaborators, (1994) (11) demonstrated that ω-carboxylated nitrosamines are substrates for mitochondrial enzymes of fatty-acid degradation, most probably following the degradation pathway of medium-chain fatty acids.

Liver is the principal organ responsible for detoxification processes, and mitochondria constitute the major energy-producing organelles of the hepatocyte. Any interference with mitochondrial bioenergetics is known to be a part of a cell-injury process caused by a multiplicity of mechanisms and assorted agents (12). In spite of this, mitochondria have proved to be an excellent model to evaluate the many xenobiotic effects on cell toxicity, and the obtained data are usually in good agreement with cytotoxicity parameters reported in cell cultures and whole organisms (13, 14).

The present study aims to describe the *in vivo* interaction of BBN with hepatic mitochondrial bioenergetics, since liver is the main organ responsible for BBN metabolism. Our results may help to better understand the relationship between nitrosamines' hepatotoxicity and the oxidative stress linked to mitochondrial dysfunction.

6.2. Materials and Methods

6.2.1. Chemicals

BBN reference is the same described in section 4.2.1.

Bovine serum albumin was purchased from Boeheringer Mannheim (Mannheim, Germany). Unless otherwise noted, chemicals used were of analytical grade and obtained from local commercial sources.

6.2.2. Animals

Animals' procedures before the experiments were the same as described in section 4.2.2.

6.2.3. Animal Experiments

Twenty-two four-week-old male ICR mice were randomly divided into two experimental groups: Group I (n=10) was the control group, drinking tap water; Group II (n=12) was exposed to BBN (0.05%) in their drinking water for a period of 12 weeks. The animals were observed daily by means of a health and body-weight check, while their food and water consumption was measured weekly. All the animals were sacrificed by means of pentobarbital overdose anaesthesia one week after BBN exposure ended. Necropsies were carried out and the mice's urinary bladders were collected in accordance with the technique described by Oliveira *et al*. (2009) (15). All other organs were also examined macroscopically for any changes, and their weights were recorded before immersion in 10%-buffered formalin. After overnight fixation, the bladders were cut longitudinally and their mucosal surfaces were carefully examined

for the existence of macroscopic lesions. Each mouse's liver, kidneys, lung and spleen were also dissected. After this procedure, all the organs were embedded in paraffin and 2 µm sections were cut and stained with haematoxylin and eosin. Histological slides were observed under a light microscope by two investigators and classified according to World Health Organization guidelines (16). All slides were examined without prior knowledge of the treatment given to the animals whose tissue samples were under observation. Urothelial lesions were classified into the following categories: simple hyperplasia, nodular hyperplasia, dysplasia, carcinoma *in situ*, invasive urothelial carcinoma and squamous metaplasia.

6.2.4. Isolation of mouse liver mitochondria and mitochondrial respiratory rates

Isolation was performed by conventional methods (17), with some minor modifications. The homogenization medium contained 0.25 M sucrose, 5 mM Hepes (pH 7.4), 0.2 mM EGTA [ethyleneglycol-bis(-aminoethyl ether) N,N,N',N'-tetraacetic acid] and 0.1% fatty-acid free bovine serum albumin (BSA). EDTA (ethylenediaminetetraacetic acid), EGTA, and BSA were omitted from the final washing medium, which was adjusted to pH 7.2. The final concentration of mitochondrial protein was determined by the biuret method (18), using BSA as standard. Mitochondrial respiration was followed using polarographic determination as described by Estabrook (1967) (19). The level of oxygen consumption by mitochondria was measured with a Clark-type oxygen electrode at 25 ºC, in a 1 ml water-thermostatic incubation-stirred chamber (CB1-D Hansatech). For this step, mitochondria were suspended in a buffer containing 100 mM KCl, 20 mM Tris, 3 mM KH_2PO_4, 3 mM $MgCl_2$, pH 7.2 on a basis of 0.8 mg mitochondrial protein per 1 ml buffer. After a two-minute equilibration period, the mitochondria were energized with pyruvate/malate (5 mM) or succinate (5 mM) as substrates. Pyruvate/malate indirectly supplies electrons to mitochondrial complex I and succinate directly reduces complex II. Rotenone (3 µM) was used to inhibit complex I and avoid the reverse flow of electrons from complex II to complex I. State 4 respiration corresponds to resting oxygen consumption after the phosphorylation of a small amount of ADP (25 µM). State 3 was achieved by the addition of 1.5 mM ADP. RCR, and ADP/O ratios were calculated, according to Chance and Williams (1956) (20), in mitochondria energized with pyruvate/malate and succinate.

6.2.5. Mitochondrial membrane potential measurements

The mitochondrial transmembrane potential (ΔΨ) was estimated with a tetraphenyl phosphonium-selective (TPP^+) electrode, according to the equation of Kamo *et al*. (1979) (21), without correcting for the "passive" binding contribution of TPP^+ to the mitochondrial membranes (the purpose of the experiment was to show relative changes in potential rather than absolute values). Reactions were carried out at 25ºC in 1 mL of the reaction medium (130 mM sucrose, 50 mM KCl, 2.5 mM KH_2PO_4, and 5 mM Hepes, pH 7.4) supplemented with 2 µM TPP^+, and 0.8 mg of mitochondrial protein. Calculations of the transmembrane potential were based on a mitochondrial matrix volume of 1.1 µL/mg protein.

6.2.6. Mitochondrial enzyme assays

Aliquots of mitochondrial suspensions were submitted to three cycles of freezing/thawing in order to disrupt intact mitochondria. The resulting mitochondrial membranes were kept at -80°C. NADH-dehydrogenase activity was measured spectrophotometrically at 25°C, by following the oxidation of NADH determined as a decrease in absorbance at 340 nm (22). The reaction was initiated by the addition of 50 µM coenzyme Q1 to 2 ml of the standard reaction medium, supplemented with 2 µg/ml antimycin A, 1 mM KCN, 150 µM NADH and 1 mg of mitochondrial protein.

Succinate-cytochrome c reductase activity was measured spectrophotometrically at 25°C by following the reduction of oxidized cytochrome c determined as an increase in absorbance at 550 nm (23). The reaction was initiated by the addition of 5 mM succinate to 2 ml of the standard reaction medium, supplemented with 0.3 mM EDTA, 1 mM KCN, 100 µM cytochrome c and 0.2 mg of mitochondrial protein.

Complex IV specific activity was determined as the rate of oxidation of reduced cytochrome c at 550 nm. Ferrocytochrome c was prepared by reducing ferricytochrome c with an excess of sodium dithionite ($Na_2S_2O_4$), followed by Sephadex G-25 chromatography to remove unreacted $Na_2S_2O_4$ (24). The reaction mixture, which consisted of 20 mM potassium phosphate buffer, pH 7.0, 0.45 mM n-dodecyl-β-D-maltoside and 15 µM ferrocytochrome c, was incubated at 25°C for one minute. Mitochondria (10 µg protein) were added to initiate the reaction. The reaction was of the first order with respect to cytochrome c.

6.2.7. Western Blot analysis

Alterations in protein expression were analyzed by Western Blot. After treatment with BBN, mitochondrial extracts of both experimental groups were collected. After denaturation in a Laemmli buffer at 95 ºC for five minutes (Bio-Rad, Hercules, CA), equivalent amount of proteins (50 mg) were separated by electrophoresis on 12% SDS-polyacrylamide gel (SDS-PAGE) (Bio-Rad, Hercules, CA) and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA). After blocking with 5% milk in TBST (50 mM Tris–HCl, pH 8; 154 mM NaCl and 0.1% Tween 20) for two hours at room temperature, membranes were incubated overnight at 4ºC with primary antibodies directed against two different subunits of mitochondrial complexes and against voltage-dependent anion channel (VDAC). NDUSF3 (0.75 µg/ml, mouse monoclonal from Abcam) is directed against the subunit of NADH dehydrogenase with the same name and MTCO1 (0.75 µg/ml, mouse monoclonal from Abcam) is directed against the subunit of cytochrome c oxidase bearing the same name. A VDAC antibody (1:100, rabbit polyclonal from Abcam) was used as a mitochondrial marker and for protein-loading control. The membranes were further incubated with alkaline phosphatase-conjugated secondary antibodies (1:2500, goat anti-mouse or goat anti-rabbit IgG from Jackson Immunoresearch Laboratories inc.) for one hour at room temperature. Membranes were reacted with the ECF detection system (Amersham, UK) and read with the Versa Doc imaging system (Bio-Rad, Hercules, CA). The densities of each band were calculated using Quantity One Software (Bio-Rad, Hercules, CA). Membranes were also stained with Ponceau reagent to confirm equal protein loading in each lane.

6.2.8. Calcium-induced mitochondrial permeability transition pore

Calcium-induced mitochondrial permeability transition pore (MPTP) was indirectly followed by the use of a tetraphenyl phosphonium-selective (TPP^+) electrode, as previously described. Mitochondria (1 mg protein) were incubated in 1 mL of the standard respiration medium supplemented with 3 µM TPP+ and 2 µM rotenone for two minutes at 25℃, prior to energization with 5 mM succinate. Calcium (100 nmol/mg protein) was added as indicated. Cyclosporin A (1 µM) was added to confirm the MPTP nature of calcium-induced membrane depolarization.

6.2.9. Hydrogen peroxide generation

Hydrogen peroxide release from mitochondria into the overlying medium was assayed using a modification of the method described by Valletta and Berton (1987) (25). This is based on the conversion of homovanilic acid (HVA) to its fluorescent dimer in the presence of H_2O_2 and horseradish peroxidase (HRP). Mitochondria were incubated in a buffer (130 mM sucrose, 50 mM KCl, 2.5 mM KH_2PO_4, and 5 mM Hepes, pH 7.4) supplemented with 5 mM succinate, 0.1 mM HVA and 3 U/mL HRP (25ºC). The assay was also performed in the presence of an inhibitor of complex I and III (2 µM rotenone, 20 µg/ml antimycin A). After 15 minutes the reaction was stopped by means of a 0.5 mL of cold glycine buffer (pH 12.0). The mitochondrial suspensions were centrifuged at 850g for ten minutes. The fluorescence of supernatants was measured at 312 nm as excitation and 420 nm as emission wavelengths. In each experiment, incubation was also performed with a control sample containing the reaction mixture alone (i.e., without mitochondria) to correct for any spontaneous dimerization of HVA. The peroxide generation was calculated using a standard curve of H_2O_2, and H_2O_2 levels were expressed as nmol H_2O_2 produced per miligram mitochondrial protein per 15 minutes.

6.2.10. Lipid peroxidation

Lipid peroxidation (LPO) was evaluated using thiobarbituric acid reactive products (MDA), as previously described (26). The amount of MDA formed was calculated using a molar extinction coefficient of $1.56 \times 10\ M^{-1}cm^{-1}$ and expressed as nmol MDA/mg protein.

6.2.11. Reduced glutathione quantification

Reduced glutathione (GSH) was determined by using the method of Jollow *et al.* (1974) (27). Post Mitochondrial Supernatant (PMS) was precipitated with 4% sulfosalicylic acid in a 1:1 ratio. The samples were kept at 4°C for one hour and centrifuged at 1500 rpm for 15 minutes at 4°C. The supernatant was used for GSH estimation. The assay mixture contained supernatant, 0.1 M phosphate buffer (pH 7.4) and 5-5'-dithiobis-2-nitrobenzoic acid, DTNB (stocks 100 mM in 0.1 M sodium phosphate buffer, pH 7.4; Sigma) in a total volume of 3 mL. GSH activity was

determined spectrophotometrically by measuring the reaction product at 412 nm and expressed as nmol of GSH consumed per miligram protein.

6.2.12. Antioxidant enzymatic activities

Superoxide dismutase (SOD) activity was assayed according to Paya *et al.*, (1992) (28) with minor modifications (29). Nitrotetrazolium blue chloride (NBT) was used as the detection molecule instead of cytochrome c. Assays were conducted in the presence of a potassium phosphate buffer (100 mM, pH 7.0), hypoxanthine (10 mM), and NBT (10 mM). The reaction was initiated by the addition of xanthine oxidase (0.023 $Umol^{-1}$) to enzymatic extract at 25°C. Activity was reported by its ability to inhibit 50% reduction of NBT and the result is expressed as units per minute per milligram protein.

Catalase (CAT) activity was assayed polarographically. Assays were conducted in 0.05 M phosphate buffer (pH 7.4) with 0.03% of triton X-100. The reaction was carried out at 25°C and was initiated by the addition of 12 mM hydrogen peroxide. CAT activity was expressed in terms of µmol H_2O_2 consumed/min/mg protein.

Glutathione reductase (GR) activity was assayed using the method outlined by Carlberg and Mannerviek (1975) (30), as modified by Mohandas *et al*. (1984) (31). The reaction system consisted of 0.1 M phosphate buffer (pH 7.4), 0.5 mM EDTA, 1 mM oxidized glutathione (GSSG), 0.1 mM NADPH and 10% PMS. Enzyme activity was quantified at 25°C by measuring the disappearance of NADPH at 340 nm and expressed as nmol NADPH oxidized per minute per milligram protein.

Glutathione S-transferase (GST) activity was measured according to Habig *et al*. (1974) (32) with some modifications. The reaction mixture contained 2 ml of potassium phosphate buffer 100 mM, triton X-100 10%, CDNB 100 mM, and GSH 100 mM. The reaction was started at 25°C by adding the sample and the absorbance was monitored at 340 nm. The GST activity was expressed according to Uguz *et al*. (2003) (33).

6.2.13. Statistical analysis

Data are represented as means ± SE and were compared using the Mann-Whitney test. A value of $p<0.05$ was considered to be significant. Analyses were conducted using GraphPad Prism 4.0 (GraphPad Software).

6.3. Results

6.3.1. Animal growth, water and food consumption

ICR male mice were exposed to BBN (0.05%) for 12 weeks, and were sacrificed one week later. All the animals survived the study period. Water and food consumption decreased during the experimental study for those animals exposed to BBN, with these parameters lower in comparison to the control group by the end of the experimental period (data not shown). During the treatment period, the animals exhibited normal cage activity. When comparing the body weight of those mice exposed to BBN to the control group, the differences were statistically significant. However, no statistically significant difference was found in the relative liver weights (calculated as the ratio of the mouse's liver weight by the mouse's weight) (Table I).

Table I - Body weight, mean and relative weight of bladder, kidney and liver (value ±SE).

Group	Mean weight (g)				
	Body weight	Bladder	Right kidney	Left kidney	Liver
I (H_2O) (n=10)	42.29±1.28**	0.270±0.031	0.327±0.009	0.341±0.013	2.32±0.11*
II (BBN) (n=12)	38.5±0.85	0.263±0.028	0.347±0.011	0.343±0.014	1.96±0.06
Group	**Relative weight (g)**				
	Bladder	Right kidney	Left kidney	Liver	Bladder
I (H_2O) (n=10)	0.063±0.0007	0.0075±0.00034*	0.0078±0.0003*	0.053±0.0026	0.063±0.0007
II (BBN) (n=12)	0.069±0.0008	0.009±0.0003	0.0089±0.0003	0.050±0.0014	0.069±0.0008

Values are means±SE for animals treated with BBN (0.05%) for 12 weeks and control animals. Statistically significant compared with control group (* $p<0.005$, ** $p<0.05$).

6.3.2. Macroscopic and microscopic evaluation

No macroscopic changes were seen in the liver, lung, kidneys and gastrointestinal tract of the animals involved. We conducted detailed histopathological examinations to determine the degree of liver lesions induced by BBN. No microscopic

hepatic lesions were observed in control (Figure 1A) or BBN-exposed animals (Figure 1B).

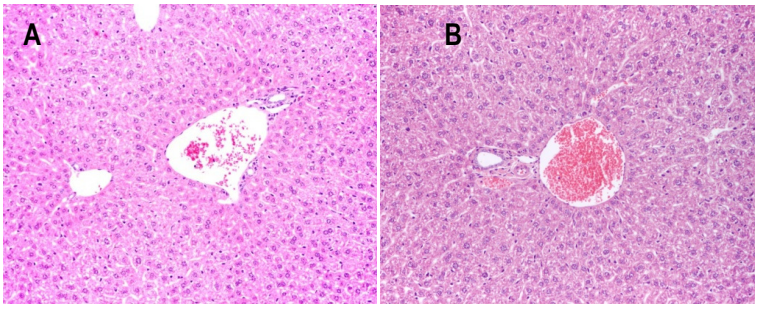

Figure 1: A: Normal mouse liver from the control group (H&E, x200); B: mouse liver from the *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) group, without any histological change (H&E, x200).

Table II summarizes the incidence of BBN-induced urothelial lesions in groups I and II. Animals from group I (control group) did not show any urothelial alterations, i.e. 100% of them exhibited a normal urothelium.

Table II - Incidence of urothelial lesions in ICR mice exposed to *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine and control animals.

Histological lesion	**Group I (H_2O)** (n=10)	**Group II (BBN)** (n=12)
Normal urothelium	10/10 (100%)	0 (0%)
Simple hyperplasia	0 (0%)	8/12 (67%)
Nodular hyperplasia	0 (0%)	7/12 (58.3%)
Dysplasia	0 (0%)	12/12 (100%)
Carcinoma *in situ*	0 (0%)	4/12 (33%)
Invasive urothelial carcinoma	0 (0%)	4/12 (33%)
Squamous metaplasia	0 (0%)	8/12 (67%)

6.3.3. Mitochondrial Bioenergetics

The transmembrane electrical potential (ΔΨ) is the main component of the electrochemical gradient, accounting for more than 90% of the total mitochondrial proton motive force. Alterations in the ΔΨ induced by xenobiotics contribute to alterations of mitochondrial and cellular bioenergetics.

The maximum ΔΨ generated after the addition of pyruvate/malate was higher in the control group when compared with the BBN-treated group (p= 0.0051) (Table III), although the maximum ΔΨ developed, with succinate, was not significantly different (p= 0.6943) between the BBN-treated group and the control group (Table III). When measuring ADP-induced depolarization and the phosphorylative lag phase, no differences were observed between the two groups regardless of the substrate used (Table III), suggesting that phosphorylation efficiency is not affected by BBN treatment.

Respiratory control ratio (RCR) is the ratio between mitochondrial respiration during state 3 (active ATP production) and state 4 (consumption of oxygen after ADP phosphorylation), and measures the coupling of mitochondrial substrate oxidation and ADP phosphorylation. Liver mitochondria from BBN-treated mice presented a lower RCR value only when pyruvate/malate was used as a substrate (p=0.0114) (Table III).

When measuring the ADP/O, no statistical differences were found between the control and BBN-treated group (Table III). The result confirms that BBN treatment does not significantly interfere with the efficiency of the phosphorylation system.

Respiration in state 4 was significantly increased in mitochondria isolated from mice treated with BBN, both for complex I (p= 0.0046) and complex II (p= 0.0007) substrates (Figure 2A). Nevertheless, no alterations were observed for state 3 respiration for both respiratory substrates (Figure 2B).

Table III - Effects of BBN treatment on mitochondrial membrane potential membrane and respiratory indexes.

		Group I (Control)	Group II (BBN)
Mitochondria energized with Pyruvate/malate	Maximum Potential (ΔΨ in mV)	-187.17±0.33	-177.47±0,97**
	ADP-induced depolarization (ΔΨ in mV)	-23.9±4.75	-22.4±2.85
	Repolarization potential (ΔΨ in mV)	-186.65±0.12	-173.00±1.68**
	Lag phase (min)	2.71±0.27	2.99±0.13
	RCR	1.93±0.19	1.27±0.07*
	ADP/O	1.43±0.07	1.21±0.11
Mitochondria energized with Succinate	Maximum Potential (ΔΨ in mV)	-193.63±2.02	-191.76±4.66
	ADP-induced depolarization (ΔΨ in mV)	-26.8±2.19	-24.0±2.21
	Repolarization potential (ΔΨ in mV)	-192.92±4.11	-190.83±3.02
	Lag phase (min)	1.36±0.08	1.23±0.11
	RCR	5.44±0.42	4.72±0.21
	ADP/O	3.12±0.10	2.96±0.22

Values are means ±SE (n=6). Statistically significant compared to control group (* $p < 0.05$; ** $p < 0.005$).

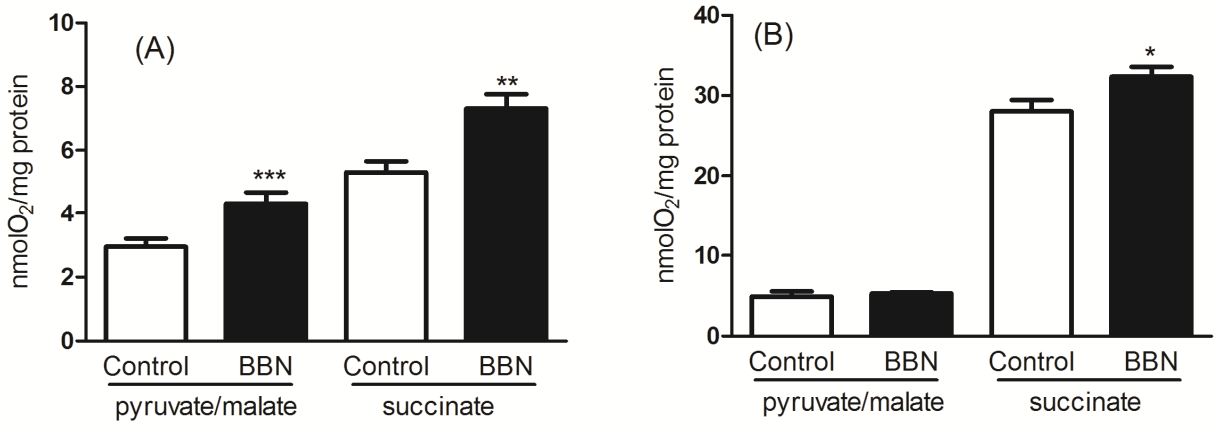

Figure 2: Effect of *in vivo* treatment with *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) (0.05%) on respiratory rates of liver mitochondria. Values statistically different from control group (**$p<0.01$; ***$p<0.001$).

The action of BBN treatment on the enzymatic activities of mitochondrial redox components was also studied in mitochondrial membrane preparations, as documented in table IV. BBN treatment caused a significant inhibition of NADH-ubiquinone oxirreductase (complex I) (36.5%; p= 0.0411), as well as in cytochrome c oxidase (complex IV) (23%; p= 0.0086). However, no significant effect was observed for succinate-cytochrome c reductase (complex II and III).

Table IV - Effect of BBN-treatment on the activities of liver mitochondrial complex I, II-III and IV, expressed as % of control values.

	Complex I	**Complex II-III**	**Complex IV**
Group I (Control)	100.0	100.0	100.0
Group II (BBN)	63.5±17.5*	92.0±7.7	76.7±4.3*

Values are means ±SE (n=4-6). Statistically significant compared to control group (* $p < 0.05$).

6.3.4. Mitochondrial complex protein levels

Since a decrease in complex I and complex IV activities was observed, we measured the amount of selected protein components from complex I and IV, in order to compare protein levels with the activity observed. Loading controls were performed by staining membranes with Ponceau reagent. As a negative control, alterations in the content of the voltage-dependent anion channel (VDAC) were measured. NDUSF3 antibody was used against one subunit of NADH dehydrogenase, which is codified by nuclear DNA. Figure 3 shows a decrease in the content (22.1%) of this subunit of mitochondrial complex I when mice were treated with BBN. MTCO1 antibody is specific to a subunit of cytochrome c oxidase codified by mitochondrial DNA. In this case, BBN treatment resulted in a 30.5% decrease in this subunit, which was significantly different from control (p = 0.0082, Figure 3). No differences were observed in the mitochondrial content in VDAC.

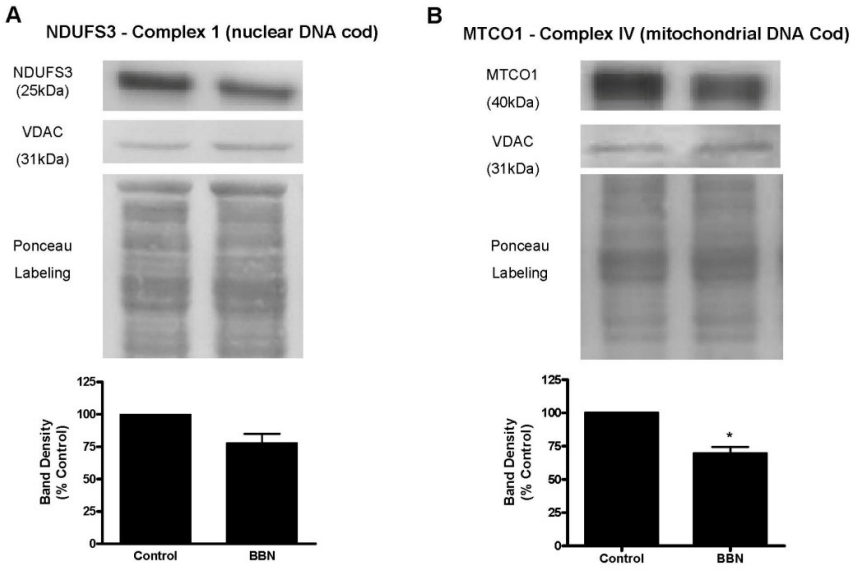

Figure 3: Effect of *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) treatment on the protein levels of mitochondrial subunits from respiratory complexes, assessed by western blotting. (A) Complex I subunit (NDUSF3 nuclear); (B) complex IV subunit (MTCO 1 mitochondrial). The voltage-dependent anion channel (VDAC) was also investigated. By staining membranes with Ponceau reagent, equal protein loading was confirmed. The results correspond to the means SE of four experiments obtained from different mitochondrial preparations. Values statistically different from control (*p<0.05).

6.3.5. Measurement of the mitochondrial permeability transition (MPT)

In order to follow MPT induction, we followed one end-point that is characteristic of that phenomenon, i.e. calcium-induced cyclosporin-A sensitive mitochondrial depolarization. Alterations of mitochondrial ΔΨ were followed by using a TPP^+-selective electrode. The results obtained indicate that BBN treatment increased the susceptibility of the pore to calcium, when compared to the control group (Figure 4). Mitochondrial incubation with Cyclosporin A, a specific MPT inhibitor (34), allows us to conclude that the ΔΨ collapse observed by calcium addition is specific to the opening of the MPT pore. The trace obtained when mitochondria were previously incubated with Cyclosporin A was similar for both control and BBN (Cyclosporin A+BBN).

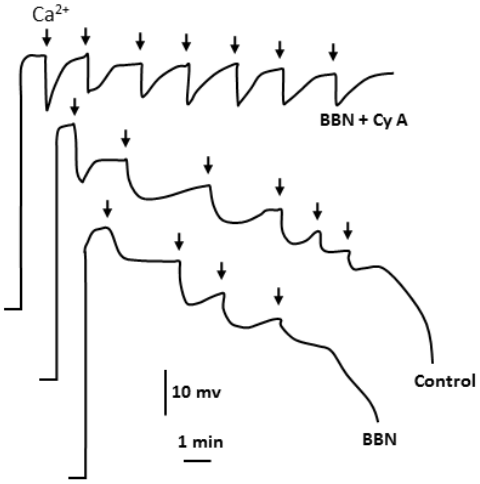

Figure 4: Effect of treatment with *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN; 0.05%) on the mitochondrial permeability transition pore induced by calcium. (The traces represent typical recordings from several experiments with four different mitochondrial preparations).

6.3.6. Effect of BBN treatment on mitochondrial and liver oxidative stress

The effect of BBN on liver mitochondrial oxidative stress was evaluated by measuring H_2O_2 generated by mitochondria, GSH content, lipid peroxidation (LPO) and by the activity of different antioxidant enzymes (SOD, CAT, GST and GR).

The production of H_2O_2 by the mitochondrial respiratory chain gives an indication of the propensity of mitochondria to contribute to cell oxidative stress. As we can see from table V, mitochondrial H_2O_2 production was significantly stimulated (46.6%) in hepatic mitochondria from mice treated with BBN (p= 0.008), although no significant alterations in GSH levels were measured (Table V).

LPO measured as malondialdehyde production showed a significant increase after BBN treatment (p=0.007), when compared to the control group (Table V). In a second method used to follow LPO, i.e. by monitoring oxygen consumption after ADP/Fe^{2+} addition (Figure 5), it can be observed that treatment with BBN promotes increased susceptibility to LPO. When mitochondria were obtained from mice treated with BBN, the extent and rate of oxidation, as measured by increased oxygen consumption, was higher.

Table V- Effect of BBN treatment on mitochondrial H_2O_2 production, GSH and MDA contents.

	H_2O_2	GSH	MDA
Group I (Control)	14.0±1.1	60.0±4.8	2.96±1.15
Group II (BBN)	20.5±1.7**	55.0±8.1	6.07±2.1**

H_2O_2: the results are expressed as the nmol H_2O_2/mg protein/15 min. GSH: the results are expressed as GSH µM/mg protein. MDA: the results are expressed as nmol MDA/mg protein. Values are means ±SE (n=4-6). Statistically significant compared to control group (** $p < 0.005$).

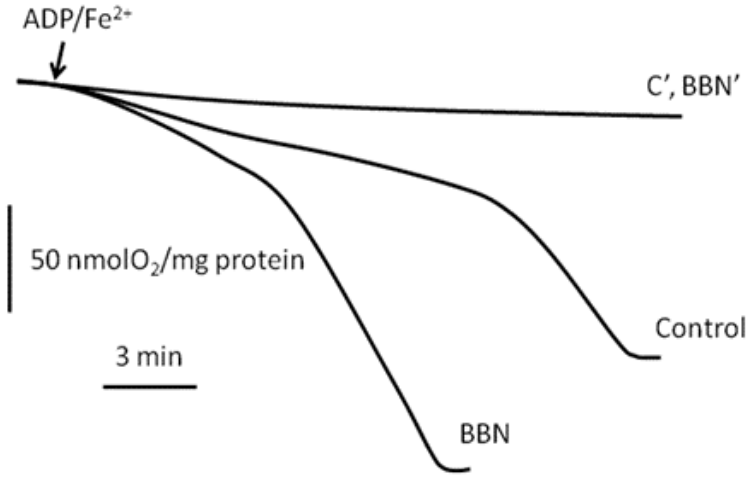

Figure 5: Rate and extent of mitochondrial oxidation induced by ADP/Fe^{2+} evaluated as oxygen consumption. Mitochondria were isolated from mice treated with *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN; 0.05%) and from control mice without BBN. (Traces represent typical direct recordings from four different preparations).

Table VI shows the results obtained for the activity of some antioxidant enzymes present in mouse liver homogenates. Liver homogenates obtained from mice

treated with BBN show a significant increase in the activities of SOD and CAT when compared to the control group (57% and 36.5% respectively), but not in the activities of GST and GR.

Table VI - Effect of BBN treatment on antioxidant enzymes in mice liver.

Antioxidant enzymes	**Group I** (Control)	**Group II** (BBN)
Superoxide dismutase (U/min/mg protein)	2.28±0.16	3.58±0.24*
Catalase (μmol H_2O_2/min/mg protein)	0.649±0.039	0.886±0.066**
Glutathione *S*-transferase (nmol CDNB /min/mg protein)	323.4±36.6	352.4±45.7
Glutathione reductase (nmol NADPH /min/mg protein)	32.4±1.4	32.6±1.5

Values are means ±SE (n=4-6). Statistically significant compared to control group (*p<0.05, **p<0.01).

6.4. Discussion

A number of studies indicate that mitochondria are the primary targets for chemical-induced cell injury, with mitochondrial dysfunction leading to cell death, involving both necrosis and apoptosis (35-37). Previous studies using nitrosamines showed that the exposure of hepatocytes to *N*-nitrosofenfluramine results in acute cell death, accompanied by an abrupt depletion of ATP through impairment of mitochondrial function, related to alterations in the mitochondrial $\Delta\Psi$ and/or oxidative phosphorylation at an early stage, with widespread LPO occurring at a later stage (8). In another study, *N*-nitrosofenfluramine elicited a concentration-dependent induction of mitochondrial swelling, which was dependent on MTP induction and prevented by pre-treatment with Cyclosporin A (7).

N-nitrosodiethylamine (NDEA) and *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) are two nitrosamine compounds that have already been demonstrated to present hepatotoxicity in rat (38, 39). Clearly, nitrosamines and nitrosamine precursors (36) are toxic for liver cells, which may result in abrupt changes in mitochondrial bioenergetics, increased oxidative stress, and therefore compromised cell biology.

Since the liver plays a pivotal role in the cellular mechanisms of biotransformation, representing a preferential target of toxicity, mitochondria isolated from this organ have been considered a good model for assessing the effects of xenobiotics on mitochondrial bioenergetics. Previous studies have demonstrated that

liver mitochondria preparations are a suitable method for studying the bioenergetic toxicity of xenobiotics (41, 42).

Respiratory parameters were evaluated in the BBN-treated and control groups in order to identify some direct effects on the respiratory chain. A decrease in mitochondrial membrane potential, together with state 4 respiration stimulation observed in mice treated with BBN, indicates that BBN may contribute to increasing mitochondrial proton reflux through the mitochondrial inner membrane, either by acting as an uncoupler, owing to the existence of an acidic hydrogen from the hydroxyl group and oxygen from the nitro group that can be protonated, or simply by disrupting membrane integrity (Table III). The decrease in the RCR value, which is more noticeable with pyruvate/malate, is mostly caused by state 4 alterations and suggests some loss of coupling promoted by BBN. The data obtained in this study also indicate that the mitochondrial phosphorylative system is not affected, since the ADP/O value and other parameters related to ADP phosphorylation were not statistically altered (Table III). During the 12 weeks that BBN exposure took place, we identified by histological evaluation a large spectrum of urothelial changes in mice, however in these animals, no liver histological lesions were observed. In fact, BBN is known to be a potent carcinogen with selective tropism for the urinary bladder. The hydroxyl group of BBN is rapidly oxidized to a carboxyl group by the liver enzymatic system alcohol/aldehyde dehydrogenase. The metabolite formed, *N*-butyl-*N*-(3 carboxybutyl) nitrosamine (BCPN), is a bladder carcinogen and comes into contact with the urothelium via urine (43, 44). BCPN is a stable compound that binds covalently to cellular macromolecules and is ultimately responsible for the initiation of the carcinogenic process (44) and in some cases increased mitochondrial complexes activity (45). Huang *et al*. (1998) (45) observed a remarkable increase in the activity of NADH cytochrome c reductase, succinate cytochrome c reductase and cytochrome c oxidase during bladder tumorigenesis induced by BBN. However, we observed a decrease in NADH dehydrogenase and cytochrome c oxidase (Table IV) in the liver. The results obtained for the activity of NADH dehydrogenase and cytochrome c oxidase correspond to the results obtained by western blot analysis, where a decrease in the content of one subunit of complex I and one subunit of complex IV was observed (Figure 3). The results appear to indicate that a lower content in complex I and IV is observed, although apparently this did not translate into decreased mitochondrial respiration, at least as observed for state 3 respiration.

Calcium-accumulation capacity had decreased in mitochondria isolated from mice treated with BBN (Figure 4). The lower capacity for calcium accumulation could

result from altered calcium translocation or calcium release. Since the assay was performed with mitochondria energized with succinate, where no differences regarding ΔΨ existed, differences in ΔΨ magnitude was not a factor. Actually, the inhibitory effect of Cyclosporin A proved that the drop in the ΔΨ values caused by calcium was due to the stimulatory activation of MPTP by BBN treatment, as Cyclosporin A is considered to be a specific inhibitor of the pore (34).

BBN increases the susceptibility of MPT pore induction by calcium and this can be a consequence of increased reactive oxygen species (ROS) production, which was confirmed by measuring mitochondrial H_2O_2 production. Furthermore, MPT pore opening could induce cytochrome c release and consequently other factors that are apoptotic (42). When metabolized in a mouse's liver, *N*-nitroso compounds, such as dimethyl nitrosamine (DMN), stimulate Kupffer cells leading to the generation of ROS (48). ROS, such as hydrogen peroxide, superoxide anion and hydroxyl radicals, formed during the metabolism of nitrosamines, result in oxidative stress, which may be one of the key factors in promoting damage to liver cells and pathological conditions such as carcinogenicity, neoplasic changes and tumour formation (49). As seen from our results, BBN treatment stimulates mitochondrial H_2O_2 production and LPO. Consequently BBN can increase MPT induction by ROS-dependent mechanism, which can also trigger the release of pro-apoptotic proteins, leading to cell death (35).

Superoxide dismutase (SOD) and Catalase (CAT) are two major components in primary antioxidant enzyme system. Superoxide anions are dismutated by SOD to form H_2O_2, which is decomposed into oxygen and water by CAT. The liver SOD and CAT activities were significantly stimulated by BBN treatment, which can be seen as an attempt to counteract the oxidative stress induced by BBN metabolism (49). Nevertheless, this stimulation does not appear enough to avoid LPO. Furthermore, it is interesting to note that glutathione S-transferase (GST) and glutathione reductase (GR) activities were not affected, which is in agreement with unaltered GSH content. Despite the fact that some nitrosamines can cause a decrease in GSH content (38), our results are not surprising since it is known that the substitution of different groups on the nitroso group is capable of causing an alteration in such activities (2). Therefore, our data appear to indicate that the glutathione redox cycle is not involved in the metabolism of BBN, as neither it nor its metabolites are excreted in conjugation with GSH.

This study highlights that during BBN-induced urothelial carcinogenesis, the hepatic metabolism of BBN subjects the liver to intense stress through the involvement of mitochondria. However, liver histological evaluation did not show any microscopic

changes compatible with liver stress induced by BBN. Mitochondria functionality was altered by *in vivo* treatment with BBN, which translates into RCR decrease, especially when complex I substrates are used, and increased ROS production, LPO and MPT induction. These results lead to the conclusion that hepatic mitochondria can be one of the primary targets for BBN toxic action, wich would lead to cell death by apoptosis. These results can also help to better understand the mortality rate that occurs with several experimental protocols that associate the use of liver metabolized drugs with urothelial carcinogenesis induced by BBN (50).

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CHAPTER 7:

E-CADHERIN AND B-CATENIN EXPRESSION DURING UROTHELIAL CARCINOGENESIS INDUCED BY *N*-BUTYL-*N*-(4-HYDROXYBUTYL) NITROSAMINE IN MICE

The content of this chapter was submitted for publication in *Urologia Internationalis*:

- Vasconcelos-Nóbrega C, Costa C, Arantes-Rodrigues R, Henriques A, Vala H, Colaço A, Santos L, Lopes C, Oliveira PA. E-cadherin and β-catenin expression during urothelial carcinogenesis induced by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine in mice.

7. E-cadherin and β-catenin expression during urothelial carcinogenesis induced by *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine in mice.

7.1. Introduction

Cadherins and catenins are adhesion molecules.

The cadherin family is a group of glycoproteins, with a great deal of influence in the cell-cell adhesion process (1-5). E-cadherin is one of the members of this family, and is present in epithelial tissues. It is generally located on the lateral surfaces of epithelial cells in the *adherens* junction (1, 2, 6). It acts as intercellular glue, being responsible for homophilic, calcium-dependent cell-cell adhesion (7). E-cadherin has two portions: one extracellular and other intracellular (cytoplasmic). The extracellular one adheres to E-cadherin from neighbouring cells. The cytoplasmic portion connects to the actin cytoskeleton through catenin connection, thus contributing to cell and tissue integrity (1, 2, 4, 7-9). E-cadherin is regarded as a tumour-invasion suppressor (6, 10). Loss of cell adhesion may contribute to a loss of contact inhibition and thus play a role at an earlier stage of the neoplastic process (8). In addition, if this integrity is lost, cancer cells may cross normal tissue boundaries and metastasize (8, 11). E-cadherin is frequently altered in epithelial neoplasms. A loss or reduction in E-cadherin expression has been linked to the invasive phenotype of a wide variety of human neoplasms, including bladder tumours (5). Available data about the expression of this adhesion molecule in animal models of urinary bladder cancer is very scarce. To our knowledge, the only published study was made by our team (12) in rats with urinary bladder cancer that was chemically induced by use of *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN). In other animal models of bladder cancer, namely mice, where the administration of BBN induces invasive urothelial tumours that are similar to those in humans, this information does not exist.

Catenins, in addition to being a link between cadherin molecules and the cytoskeleton, also mediate the signal-transduction mechanisms that regulate cell adhesion, growth and differentiation (13). β-catenin belongs to the Wnt pathway and is involved in virtually every aspect of embryonic development, as well as controlling homeostatic self-renewal in a number of adult tissues (13, 14).

The association of catenins to cadherins is a key step in the function of intact adhesion complexes, and alterations in catenin molecules can lead to the disruption of cell-cell adhesion, resulting in tumour aggressiveness and invasiveness in neoplastic

disease (15, 16). The E-cadherin/catenin complex is important for cell polarity, maintenance of normal tissue morphology and cellular differentiation (17).

The objective of this study was to investigate E-cadherin and β-catenin expression in normal mice urothelium and during urothelial carcinogenesis induced by use of BBN in mice.

7.2. Material and methods

7.2.1. Chemicals

BBN reference is the same described in section 4.2.1.

7.2.2. Animals

Animals' procedures before the experiments were the same as described in section 4.2.2.

7.2.3. Animal experiments

Forty-five ICR mice were divided into four groups. Group I (n=12) and Group II (n=13), received *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) (0,05%) in drinking water during 12 weeks. Groups III (n=10) and IV (n=10) were control groups given only tap water to drink. One week after BBN exposure was brought to an end, Groups I and III were euthanized. Groups II and IV remained in the study until the 20th week, at which point they were also euthanized (Figure 1).

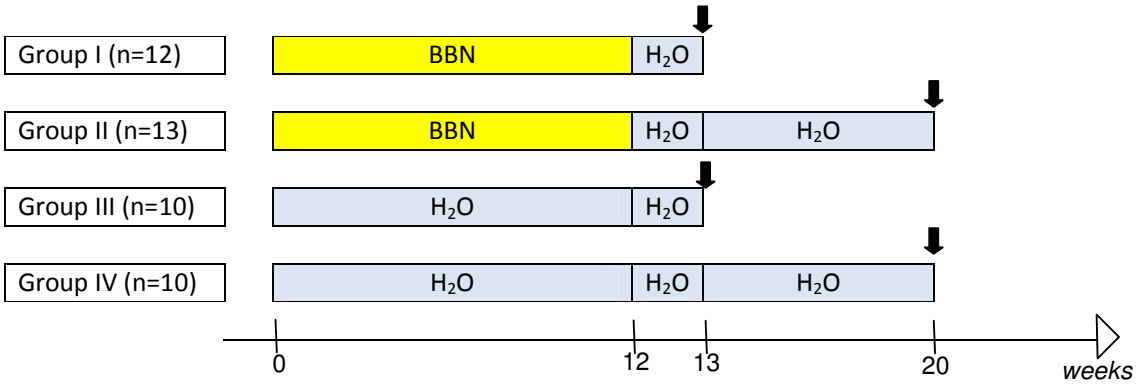

Figure 1: Experimental design (black arrows represent euthanasia points).

Euthanasia was performed by means of an overdose of pentobarbital anaesthesia. Necropsies were carried out and the mice's urinary bladders were collected. The urinary bladders were fixed *in situ* (formaline, 0.1 ml), and then collected and fixed in 10% buffered formalin, for 12 hours. After fixation, the bladders were cut longitudinally and their mucosal surface was carefully examined for the existence of macroscopic lesions. After this procedure, the urinary bladders were embedded in paraffin. All other organs were also examined macroscopically for any changes, and their weights recorded. The animals' lungs, heart, spleen, kidneys and liver were also submerged in formalin. Twelve hours later, all organs were once again carefully observed, cut and embedded in paraffin.

The animal's drinking solution was changed at least once a week (or earlier if necessary) and the volume drunk was recorded. Weekly food intake was also noted. The mice's state of health was monitored daily and their body weights were measured once a week.

7.2.4. Histological evaluation

Sections of 2 μm were cut and stained with haematoxylin and eosin. All slides were observed under a light microscope by two investigators. 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 (18). 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, invasive carcinoma with squamous differentiation and squamous metaplasia.

7.2.5. Immunohistochemistry evaluation

Formalin-fixed, paraffin-embedded tissue blocks were cut into 2-µm-thick sections for immunostaining and placed on adhesive-coated slides.

A standard Avidin-Biotin Complex (ABC) technique was used.

Sections were dewaxed, rehydrated and incubated for 20 minutes with 3% hydrogen peroxide in order to block endogenous peroxidase activity. Antigen retrieval was performed in a water bath at 98ºC following pre-treatment with a 0.01M pH6 citrate buffer. Tissue sections were washed in PBS/Tween and then covered with avidin-blocking solution for 15 minutes in a wet chamber. After two washings in PBS/Tween, biotin was added for 15 minutes. After these procedures, unspecific immunoreactivity was blocked by adding normal serum, according to the respective primary antibody, for a period of 20 minutes. The sections were incubated with the primary antibody, mouse monoclonal antibody against E-cadherin (DIL Zymed laboratories clone 4A2C7) or goat Anti-β-Catenin (DIL Zymed laboratories CAT-5H10) overnight at 4ºC, in a wet chamber. After washing in a PBS buffer, tissue sections were incubated in an appropriate biotinylated secondary antibody for 30 minutes at Room Temperature (RT). Following PBS washes, the section was reacted with 3.3'- diaminobenzidine tetrahydrochloride (DAB), followed by counterstaining with Harris haematoxylin, then it was dehydrated, coverslipped and reviewed under a light microscope.

Immunohistochemical expression was classified in all positive lesions. Staining patterns were classified according to Figure 2.

Membranar, cytoplasmic and nuclear patterns were attributed to lesions where only the membrane, cytoplasm or nucleus were stained. Membranar + cytoplasmic staining was the pattern given to lesions where more than 90% of cells had membranar staining and the rest had cytoplasmic staining. Cytoplasmic + membranar staining was attributed to lesions where more than 90% of cells had cytoplasmic staining and the rest had membrane staining. When lesions appeared with heterogeneous staining, where the membrane, cytoplasm and nucleus were simultaneously stained, the staining pattern was classified as membranar + cytoplasmic + nucleus.

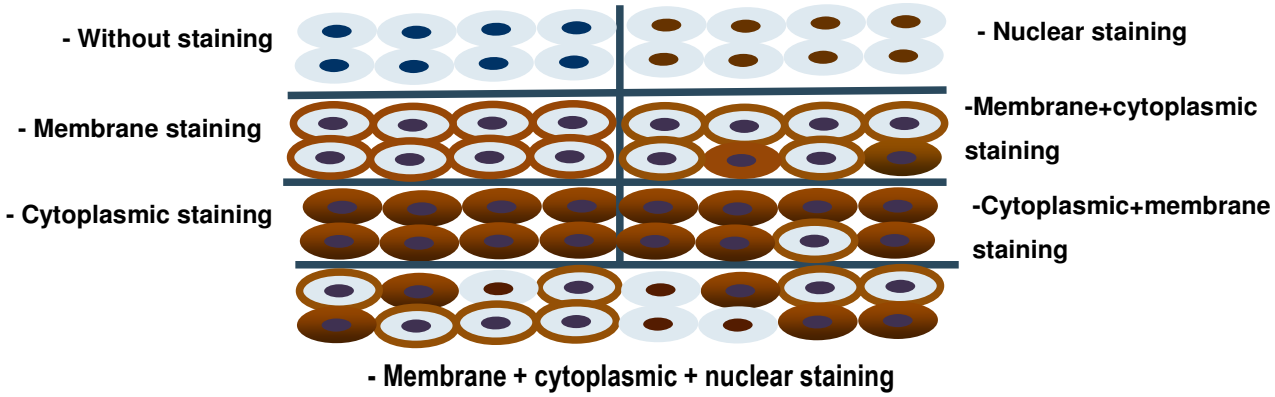

Figure 2: Schematic drawing representing staining patterns of E-cadherin and β-Catenin in the urothelia of mice submitted to BBN's action.

Following a qualitative classification proposed by Serdar *et al*. (2005) (19), the expression of E-cadherin and β-catenin in urothelial lesions were compared with that of normal epithelial cells. Cells that were stained with the same pattern as normal epithelial cells (membrane pattern) were defined as normal. All other patterns were classified as abnormal.

7.2.6. Statistical analysis

Statistical analysis was carried out using the SPSS 17.0 statistical software (SPSS Inc. USA). The Pearson product-moment correlation coefficient was used to evaluate the correlation (linear dependence) of E-cadherin and β-catenin expression. *p* values of less than 0.05 were considered to be statistically significant.

7.3. Results

7.3.1. E-cadherin

In normal urothelia (Figure 3A), the expression of E-cadherin was predominantly at the cellular membrane level (87.5%), reflecting the normal localization of the intercellular adhesion molecule; this served as an internal positive control.

A similar pattern was observed in simple hyperplasia (Figure 3B). Nodular hyperplasia (Figure 3C) showed normal membrane staining in 50% of lesions and abnormal staining in the other 50%, where a membrane and concomitant cytoplasmic pattern was observed. In 86.67% of all analysed dysplasia (Figure 3D), a cytoplasmic pattern was seen. On invasive carcinoma (Figure 3E), an abnormal staining pattern was present, with areas exhibiting weaker cell staining. However, the majority of invasive urothelial cells exhibited a pattern of membrane and cytoplasmic staining. On squamous metaplasia (Figure 3F), we also observed abnormal staining with heterogeneous immunoreactivity, with a membrane pattern on basal and intermediate layers and a loss of immunoreactivity in the most superficial ones.

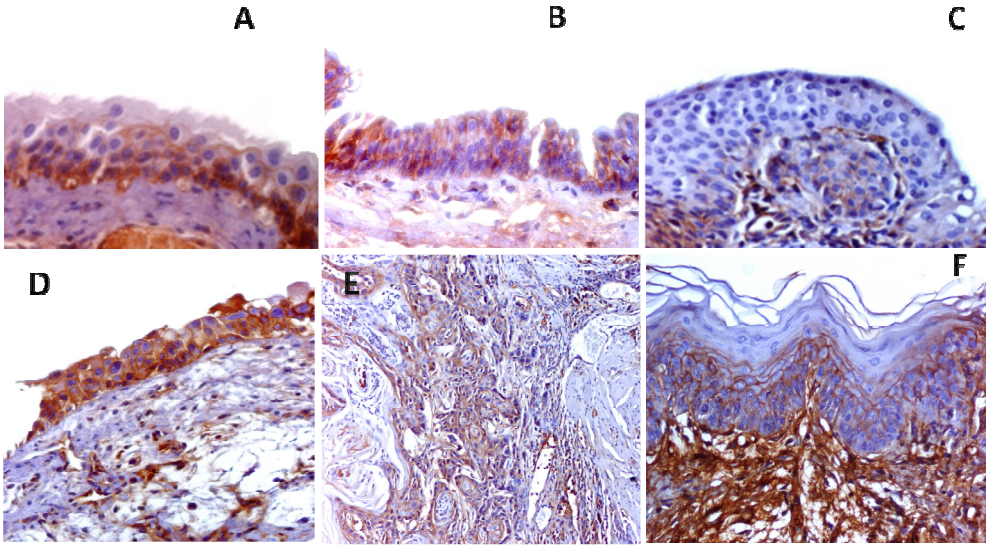

Figure 3: E-cadherin staining. A- Normal urothelium (x600); B- Simple hyperplasia (x600); C- Nodular hyperplasia (x600); D- Dysplasia (x400); E- Invasive carcinoma with squamous differentiation (x200); F- Squamous metaplasia (x400).

Table I summarizes results obtained wit E-cadherin that are also expressed in Figure 4.

Table I - E-cadherin expression pattern in normal urothelium and urothelial lesions (%).

E-cadherin expression pattern	Normal	Abnormal	Abnormal				
			Cytopl	Nuclear	Mb + Cytopl	Cytopl + Nuclear	Mb + Cytopl+Nuclear
Normal urothelium	87,5% (21/24)	12,5% (3/24)	4,17% (1/24)	0	8,33% (2/24)	0	0
Simple hyperplasia	78,57% (11/14)	21,43% (3/14)	7,14% (1/14)	0	14,29% (2/14)	0	0
Nodular hyperplasia	50% (1/2)	50% (1/2)	0	0	50% (1/2)	0	0
Dysplasia	13,33% (2/15)	86,67% (13/15)	86,67% (13/15)	0	0	0	0
Invasive carcinoma	0	100% (7/7)	0	0	57,14% (4/7)	14,29% (1/7)	28,57% (2/7)
Squamous metaplasia	90% (9/10)	10% (1/10)	0	0	10% (1/10)	0	0

Mb- membranar; Cytopl – cytoplasmic.

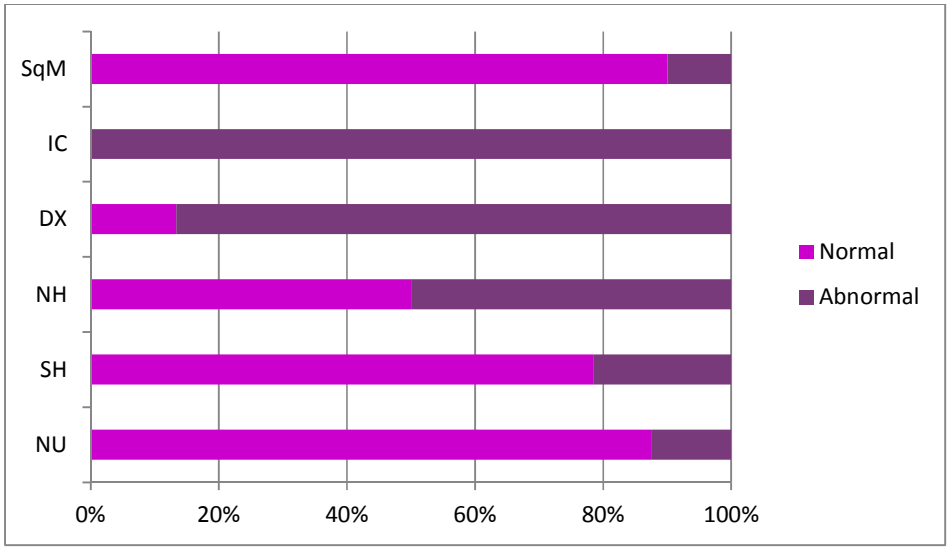

Figure 4: E-cadherin staining in different urothelial lesions (%). Nu: Normal urothelium; SH: Simple hyperplasia; HN: Nodular hyperplasia; Dx: Dysplasia; IC: Invasive carcinoma; SqM: Squamous metaplasia.

7.3.2. β-catenin

In normal urothelia (Figure 5A), the expression of β-catenin was predominantly confined to the cellular membrane (70%).

Simple hyperplasia (Figure 5B) showed cell-membrane staining in 66.67% of cases and cytoplasmic staining in the remaining ones. With regard to nodular hyperplasia (Figure 5C), only one case was observed, and this exhibited membrane staining. In 100% of dysplasia (Figure 5D), a cytoplasmic pattern was observed. On invasive carcinoma (Figure 5E), the staining pattern was generally heterogeneous with areas of weaker cell staining and others in which either the membrane, the cytoplasm or the nucleus was stained. Nucleus staining was always at a percentage that was lower than 10%. On squamous metaplasia (Figure 5F), the staining pattern was confined to the membrane, however, we observed a heterogeneous level of immunoreactivity. Basal and intermediate layers were uniformly stained, but most superficial cell layers lost their immunoreactivity. Following Serdar's *et al.* (2005) (18) qualitative classification, and in a similar way to what was done with E-cadherin, staining patterns were classified into two classes - normal or abnormal - for all urothelial lesions observed. A normal pattern was attributed when the stained area was exclusively in the cellular membrane. Results are summarized in Table II and expressed in Figure 6.

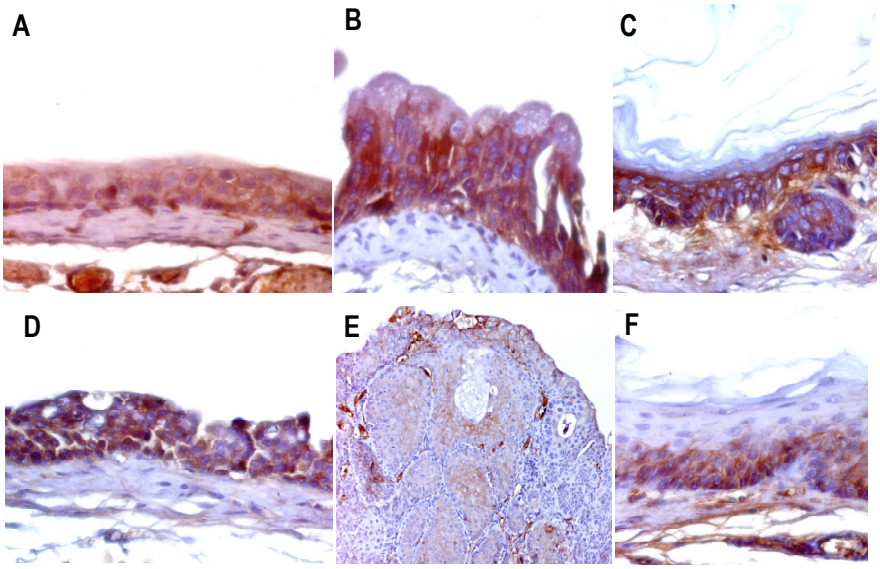

Figure 5: β-catenin staining. A- Normal urothelium (x600); B- Simple hyperplasia (x600); C- Nodular hyperplasia (x600); D- Dysplasia (x600); E- Invasive carcinoma (x200); F- Squamous metaplasia (x600).

Table II - β-catenin expression pattern in normal urothelium and urothelial lesions (%).

B-Catenin expression pattern	Normal	Abnormal	Abnormal				
			Cytopl	Nuclear	Mb + Cytopl	Cytopl + Nuclear	Mb + Cytopl+Nuclear
Normal urothelium	70% (7/10)	30% (3/10)	10% (1/10)	0	20% (2/10)	0	0
Simple hyperplasia	66,67% (4/6)	33,33% (2/6)	33,33% (2/6)	0	0	0	0
Nodular hyperplasia	100% (1/1)	0	0	0	0	0	0
Dysplasia	0	100 % (7/7)	100% (7/7)	0	0	0	0
Invasive carcinoma	0	100% (5/5)	0	0	40% (2/5)	20% (1/5)	40% (2/5)
Squamous metaplasia	100% (3/3)	0	0	0	0	0	0

Mb- membranar; Cytopl – cytoplasmic.

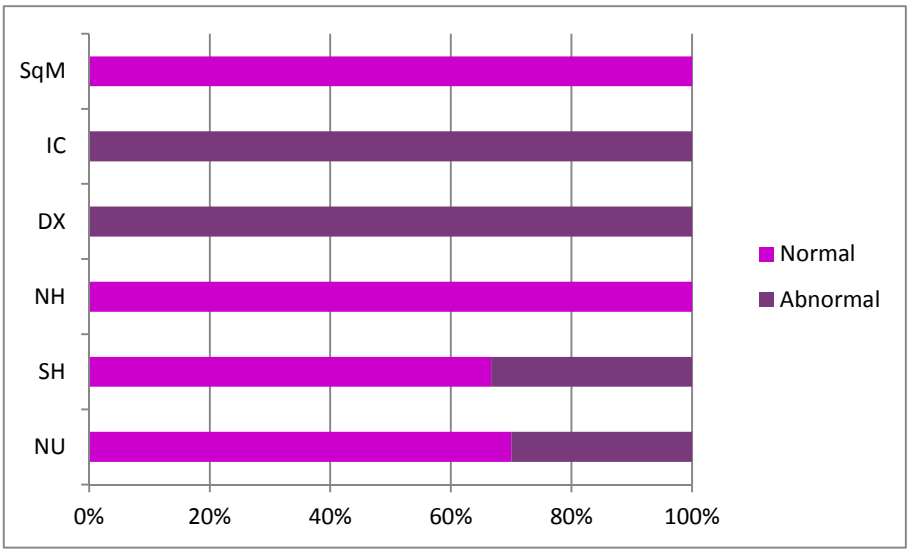

Figure 6: β-catenin staining in different urothelial lesions (%). Nu: Normal urothelium; SH: Simple hyperplasia; HN: Nodular hyperplasia; Dx: Dysplasia; IC: Invasive carcinoma; SqM: Squamous metaplasia

Statistic Analysis: The Pearson product-moment correlation coefficient of E-cadherin and β-catenin expression showed a strong positive correlation (r= 0.834572521; *p*=0.039).

7.4. Discussion

In this study, we have investigated E-cadherin and β-catenin expression in normal and transformed mice urothelia.

E-cadherin and β-catenin are both adhesion molecules and we were expecting that, in normal urothelia, they would only be found at membrane level. We observed that this is true in 87.5% of cases for E-cadherin and in 70% for β-catenin, but we also observed a mixed pattern featuring membrane and cytoplasmic staining (8.33% and 20%: E-cadherin and β-catenin respectively) and to a lesser degree, the exclusively cytoplasmic staining of normal urothelia in 4.17% and 10% (E-cadherin and β-catenin respectively) of cases.

When analyzing urothelial lesions we can divide them in preneoplastic and neoplastic lesions. Regarding preneoplastic ones, we can say that in simple hyperplasia and nodular hyperplasia, normal staining (membrane pattern) predominates, while dysplasia presents exclusively cytoplasmic pattern. Regarding neoplastic lesions, abnormal staining is clearly dominant. Invasive carcinomas exhibit heterogeneous staining where a cytoplasmic pattern is always present and associated with membrane and/or nuclear staining.

These results obtained for E-cadherin expression, are similar to those obtained by Oliveira *et al*. (2006) (12) where the expression of this adhesion molecule in rat urothelial preneoplastic lesions and tumours induced by oral administration of BBN, over the course of 10, 15 and 20 weeks, was determined. Simple hyperplasia and squamous metaplasia showed a similar E-cadherin pattern when compared with normal urothelia, with its expression confined to the cell membrane. Dysplasia and invasive carcinoma revealed an abnormal staining pattern with an increase in cytoplasm reactivity and a decrease in cell-membrane positivity. A strong correlation ($r=0.922$; $p=0.009$) was obtained between our results and those obtained by others (12).

E-cadherin and β-catenin expression in mice are positively correlated. This allows the conclusion that both adhesion molecules are valuable tools for investigating the cellular adhesion status of the urothelium. It is well recognized that a reduction or loss in E-cadherin expression is an important primary event in bladder tumorigenesis which is often linked to a poor prognosis (20-22). According to Garcia del Muro *et al*. (2000) (20), β-catenin, although to a lesser extent, can also be an important prognostic marker in patients with bladder carcinoma. Our results allow us to state that β-catenin,

similarly to E-cadherin, can be considered a good marker of the progression of bladder tumours that are chemically induced in mice by BBN.

These results also suggest that alterations in the expression of these adhesion molecules may be indicative of tumour progression in BBN-induced bladder cancer in mice.

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CHAPTER 8:

GENERAL DISCUSSION

8. General discussion

Oncologic research has contributed greatly to improve prevention, diagnose, treatment and survival of cancer patients. However, cancer is a health problem which is far from being solved. As the current population ages, and with the increase in life expectancy, the number of new cancer diagnoses in older people is expected to rise (1). Although malignant tumours occur at all ages, the highest incidence and mortality rates occur in those over 65 years old (2). With bladder cancer, the situation is similar. Below the age of 50, bladder cancer is uncommon (3), being 65 to 70 years old the median age at diagnosis (4). Apart from being one of the most common tumours of the genitourinary tract, bladder cancer is also one of the deadliest and expensive. By all these reasons, the research in this field is of extreme importance. In this thesis, we focus on invasive bladder tumour. Preclinical *in vitro* and *in vivo* experiments were performed, in order to determine the effect of different drugs. Additionally, toxicological and histological studies were done. Since it was used an animal model of bladder cancer chemically induced, we intended to investigate if the carcinogen used would damage the animal liver and by any mean, compromise hepatic function with consequences in the therapeutic action of the drugs. Finally, we also try to understand how urothelial adhesion molecules behave in the animal model used.

For ease of reference, the data will be discussed by category: Hystological; *in vitro*; toxicological and immunohistochemical results.

Hystological results

Hystological lesions were classified in accordance with the World Health Organization guidelines (5) and divided in two groups: preneoplastic and neoplastic lesions. The first group includes simple hyperplasia, nodular hyperplasia and dysplasia, and the second one includes all other lesions: carcinoma *in situ* (CIS), papillary neoplasms of low-malignant potential, low-grade papillary tumours, high-grade papillary tumours, invasive urothelial carcinoma, spinocellular carcinoma and epidermoid metaplasia. With the exception of everolimus, all other drugs or drug combination used (sirolimus, gemcitabine or their combination) reduced the incidence of neoplastic lesions, despite an increase in the incidence of preneoplastic ones. When comparing control group, where the percentage of neoplastic lesions was about 40.7% (22/54), with all the experimental groups, we can say that the greater decrease in neoplastic lesions was obtained with sirolimus that led to a reduction of 47.91%. Sirolimus treated group exhibited a total amount of 33 lesions, seven of which were neoplastic. In second, was the reduction obtained by gemcitabine, 37.1%, that showed 11 neoplastic

lesions in a total of 43, and finally the association of sirolimus and gemcitabine, that showed a decrease of 22.77% compared to control group, exhibiting 11 neoplastic lesions in a total of 35 urothelial lesions. Everolimus did not lead to any significant alteration, either in neoplastic or preneoplastic lesions, ie mice exposed to BBN and treated with everolimus and mice exposed to BBN and not treated, did not show significant differences. When analysing exclusively invasive carcinomas, results showed the same tendency. In control group, invasive carcinomas were observed in 7 out of 13 animals. The administration of sirolimus led to 86.7% reduction: only 1 out of 14 animals exhibited invasive carcinoma. A decrease of 63% was seen with the administration of gemcitabine, since that at the end of the experiment, 3 out of the 15 mice treated with gemcitabine showed invasive carcinomas. The association of both drugs (sirolimus and gemcitabine) result in a 22.77% reduction, with 5 animals out of 12 exhibiting invasive carcinomas. In this particular type of lesion, everolimus showed a reduction of 20.6% compared to the control group. In the 14 animals from the everolimus treated group, invasive carcinomas arose in 6 animals.

In previous studies with different mice models of bladder cancer, sirolimus showed promissory results, reducing the incidence of BBN-induced invasive urothelial carcinoma (6) and also preventing progression of non-muscle invasive CIS to invasive bladder cancer (7). Our results corroborate these findings. Sirolimus is an mTOR inhibitor. The mTOR pathway is an attractive target for clinical treatment of numerous cancers, namely bladder cancer, because it serves as a convergence point for many growth stimuli (8-10). When this pathway is blocked, cell growth and tumourigenesis are inhibited. Sirolimus derivatives, namely everolimus, which have greater bioavailability than sirolimus itself (11), emerged as a new drug that would have greater effects than sirolimus. However, our results contradict those expectations. Everolimus treated mice exhibited similar lesions to those not treated, wondering about the differences about sirolimus and its derivatives. Nevertheless, Chiong *et al*. (2011) (12) published a study, were everolimus effectiveness was assessed in an heterotopic model of bladder cancer. UM-UC-3, UM-UC-6, and UM-UC-9 cells were injected subcutaneously into the right flank of 4- to 6-week-old female nude mice, and obtained good results, confirming that mTOR inhibition by everolimus was effective in those conditions. Comparing our study and this one, where the difference was in the animal model used, we can question if the tumour development outside the bladder parallels the development of the tumour in the organ of its origin. Other important question regards the levels of mTOR, PI3K and Akt. Several studies have found that treatment with the mTOR inhibitor sirolimus actually results in increased PI3K activation by

negative feedback loops and phosphorilation of Akt (10, 13, 14). Our results do not lead us to suspect that this situation occurred with sirolimus, but eventually this could be an explanation for the bad results obtained with everolimus. On the other hand, mTOR inhibitors level must be constant for all the therapeutic period in order to continuously block mTOR. If mTOR is not permanently inhibited, therapeutic effect will decrease. Our schedule of administration of everolimus (twice a week) could be insufficient to maintain permanent mTOR inhibition. A new approach should consider to diminish the dosage, with a greater frequency of administrations. Gemcitabine is an antimetabolite drug that causes cell-growth inhibition by being incorporated into the DNA and RNA. It has been used to treat solid tumours of pancreas, non-small cell lung cancer, breast cancer and ovarian cancer. This, associated with the lack of cross-resistance observed with other anticancer drugs (15), made us think that it could represent an ideal candidate drug for combination chemotherapy. Given the different mechanism of action of gemcitabine and sirolimus we hypothesized that their association would bring additive or even synergic results to treat invasive bladder cancer, however, this was contradicted by our results.

In vitro results

In addition to *in vivo* experiment, everolimus was also tested *in vitro*, in three different cell lines of bladder cancer (T24; HT1376; 5637). Its effects were heterogeneous, with significant anti-proliferative activity of everolimus only observed in one of the cell lines tested (5637). Interestingly, this was the only superficial cell line. The two others were from invasive carcinomas. Additionally, mTOR and mTOR phosphorilation at Ser2448 activity was determined in these cell lines treated with everolimus. No significant expression differences were found in cell lines T24 and HT1376, but a significant decrease in mTOR expression was observed in 5637 cell line. Regarding Akt and Akt phosphorilation at Ser473 a slight increase was observed in the baseline expression of Akt in T24 and HT1376 cell lines and in the baseline expression of Akt phosphorilation at Ser473 for 5637 cell line. These results corroborate previous statements regarding Akt activation by feedback negative mechanisms (10, 13, 14). Akt activation (eventually by PI3K activation) leads to mTOR activation and all the downstream reactions, like the phosphorylation of p70 S6K and 4E-BP, increasing mRNA translation and also translation of proteins necessary for progression of the cell cycle contributing to tumourigenesis (9, 10, 14, 16). In 5637 cell line, mTOR reduction was very significant, leading to a better therapeutic response. Chiong *et al*. (2011) (12) presented a similar work, with different cell lines, but with the same heterogeneity of results. Earlier, Mansure and coworkers (2009) (17), had

already shown some heterogeneity in the everolimus action. They performed MTT assays on a panel of nine urothelial carcinoma cell lines to establish sensitivity by dose-response curves for everolimus, using five invasive carcinoma cell lines (UM-UC3; UM-UC5; UM-UC6; 253-JP; 253J-BV), two superficial bladder tumour cell lines (RT4; KU-7) and also two cell lines isolated from lymphatic metastases of bladder cancer (UM-UC1; UM-UC13). The most sensitive ones were UM-UC5, UM-UC6 and UM-UC1. 253-JP, 253J-BV and RT4 were moderately sensitive. The others (UM-UC3; UM-UC13; KU-7) were considered as relatively resistant to everolimus. These observations, in association with *in vivo* experiments and histological results, suggest that everolimus could have an eventual cytostatic potential and could be used prior to tumours establishment, over pre-neoplastic lesions, to prevent their progression to neoplastic ones, or even in superficial bladder tumours.

Toxicological results

Liver is an important organ responsible for several vital functions. Being the organ in charge for the biotransformation of all drugs and xenobiotics it is of extreme importance to evaluate any hepatotoxicity induced by BBN. Histological evaluation of the liver did not show any microscopic changes compatible with liver stress. Ito and colaborators (1983) (18) in a different study with rats, also reach to the same results. However, we also evaluated mitochondria functionality and concluded that it was altered by BBN. This is an important observation, especially in chemically induced disease models, when drugs under evaluation are biotransformed in liver. It is important to determine the normal hepatic function, otherwise unexpected results could be obtained, not associated with poor drug efficacy, but because of hepatic impairment, caused by the chemical used to induce the disease (19). BBN induced liver impairment is also referred by other authors, associated with higher levels of ALT and AST (20). To our knowledge, this was the first study to demonstrate liver stress and mitochondrial impairment associated with BBN oral administration to mice.

Immunohistochemical results

Mice and humans are very similar regarding bladder functions, urothelium characteristics and urothelial lesions. One of the biggest issues regarding bladder cancer is metastasis. Metastasis is a complex, multi-step process by which primary tumour cells invade adjacent tissue, enter the systemic circulation and finally proliferate from microscopic growths (micrometastases) into macroscopic secondary tumours (21). Metastasis and invasion of other organs are frequent when invasive bladder cancer is present, regulated by the expression of oncogenes and/or loss of expression of tumour suppressor genes. E-cadherin and β-catenin are proteins that regulate cell adhesion. Since in mice, available data about these adhesion molecules in the

urothelium were very scarce, we investigated how these molecules would behave. We concluded that a decrease in normal E-cadherin expression was significantly associated with high histological and invasion grades (87,5% for normal urothelium; 78,57% for simple hyperplasia; 50% for nodular hyperplasia; 13,33% for dysplasia; 0% for invasive carcinoma. Squamous metaplasia showed normal E-cadherin expression in 90% of the cases). β-catenin expression showed not the same tendency, but a similar one. In normal urothelium, β-catenin was confined to the cellular membrane level in 70% of the cases. In simple hyperplasia was observed a slight, non-significant reduction. The percentage of this lesion that exhibited normal pattern was of 66,67%. Surprisingly, nodular hyperplasia and squamous metaplasia showed 100% of normal pattern, with β-catenin expression confined to the cellular membrane. β-catenin expression was abnormal in all dysplasia and invasive carcinomas.

As in humans, loss of cell adhesion may play an important role in metastasis, allowing cancer cells to cross normal tissue boundaries and proliferate on other organs (22-24). Our results allow us to state that E-cadherin and β-catenin profile may be indicative of tumour progression in mice model of bladder cancer BBN induced

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CHAPTER 9:

FINAL CONSIDERATIONS

9. Final Considerations

Cancer is a devastating disease that can affect everyone, everywhere. About 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008 worldwide (1). Deaths related to cancer are projected to continue to rise to over 11 million in 2030 (2). Bladder cancer is one of the deadliest and most expensive human tumours (3, 4).

Most of our knowledge regarding carcinogenesis, profited greatly from the use of experimental models. Nowadays, more than 50 years after the publication in 1959, of "*The principles of human experimental technique",* animal experiments have evolved tremendous (5). The three Rs (Reduce; Refine; Replace) advocated by its authors, Russel and Burch, are a concern of every investigator, and in the XXI century rodent models of bladder cancer continue to be valid tools for urinary oncologic research.

Bladder cancer research has provided encouraging results in last decades. New drugs, new targets and new therapeutic regimens, as well as new applications for old drugs along to improvements in old therapeutic regimens, are trying to be achieved by investigators in urologic oncology research all over the world, with the objective to treat bladder cancer more effectively, to prolong overall survival and to reduce/eliminate drug resistence and the undesirable effects of drugs. To test the efficiency of drugs to prevent or treat cancer and to approve their use for human or animal use, is mandatory a long way process. Disparity between preclinical discovery and clinical findings is not uncommon during the development of chemotherapeutics. Therefore, after *in vitro* and *in vivo* evidence of drugs effectiveness, clinical trials are also necessary to identify susceptible tumour types (6).

Science is in constant evolution and there's been a revolution in understanding cancer at a basic molecular level. Future directions in the development of new therapeutic strategies are to identify genes expressed by tumours resistant to treatment, and then target their expression or activity. Other approach is to combine drugs with different mechanism of action, aiming to simultaneously block several key points in the complex tumourigenic process.

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