Chemoprevention of BBN-Induced Bladder Carcinogenesis by the Selective Estrogen Receptor Modulator Tamoxifen¹

Suraj Konnath George*, Veronica Tovar-Sepulveda*, Steven S. Shen[†], Weiguo Jian[‡], Yiqun Zhang[§], Susan G. Hilsenbeck[§], Seth P. Lerner[‡] and Carolyn L. Smith^{*,‡}

*Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX; [†]Department of Pathology and Genomic Medicine, The Methodist Hospital, Houston, TX; [‡]Scott Department of Urology, Baylor College of Medicine, Houston, TX; [§]Dan L. Duncan Cancer Center, Baylor College of Medicine, Houston, TX

Abstract

Bladder cancer is the fifth most frequent tumor in men and ninth in women in the United States. Due to a high likelihood of recurrence, effective chemoprevention is a significant unmet need. Estrogen receptors (ERs), primarily ER β , are expressed in normal urothelium and urothelial carcinoma, and blocking ER function with selective ER modulators such as tamoxifen inhibits bladder cancer cell proliferation *in vitro*. Herein, the chemoprotective potential of tamoxifen was evaluated in female mice exposed to the bladder-specific carcinogen, *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN). Carcinogen treatment resulted in a 76% tumor incidence and increased mean bladder weights in comparison to controls. In contrast, mice receiving tamoxifen concurrent (8–20 weeks) or concurrent and subsequent (8–32 weeks) to BBN administration had no change in bladder weight and only 10% to 14% incidence of tumors. Non-muscle-invasive disease was present in animals treated with tamoxifen before (5–8 weeks) or after (20–32 weeks) BBN exposure, while incidence of muscle-invasive bladder carcinoma was reduced. ER β was present in all mice and thus is a potential mediator of the tamoxifen chemoprotective effect. Surprisingly, ER α expression, which was detected in 74% of the mice exposed to BBN alone but not in any control mice, was correlated with tumor incidence, indicating a possible role for this receptor in carcinogen-induced urothelial tumorigenesis. Thus, these data argue that both ER α and ER β play a role in modulating carcinogen-induced bladder tumorigenesis. Administration of tamoxifen should be tested as a chemopreventive strategy for patients at high risk for bladder cancer recurrence.

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Introduction

As the fifth most common cancer in men and ninth most common in women in the United States, urinary bladder cancer accounts for significant morbidity and mortality. There are estimated to be more than 73,000 new bladder cancer cases in 2012 [1] with urothelial carcinoma accounting for approximately 90% of these; the remainder is composed of squamous cell carcinomas, adenocarcinomas, and other rare histologies [2]. Approximately 70% of all new bladder cancer cases are classified as non-muscle-invasive (Ta, T1, Tis), while the remaining 30% are muscle-invasive (T2–T4) and generally lead to cystectomy. Bladder cancer recurs at a very high rate of 50% to 90% depending on the tumor stage, grade, and number of primary tumors, and this necessitates lifetime monitoring for tumor recur-

rence. Therefore, there is a significant unmet need for approaches to reduce the extent of urothelial cancer recurrence and, more importantly, to improve survival and maintain patient quality of life.

Address all correspondence to: Carolyn L. Smith, PhD, Molecular and Cellular Biology, BCM130, One Baylor Plaza, Houston, TX 77030. E-mail: carolyns@bcm.tmc.edu

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There is a significant gender disparity in bladder cancer incidence with three to four times more diagnoses in men than women [1]. Although environmental factors, such as cigarette smoking and industrial or occupational chemical exposure, which are greater in men, contribute to this gender discrepancy, they do not fully account for the excess risk in males [3]. This raises the possibility that intrinsic gender differences between males and females, such as the sex steroid milieu, are a determinant of the relative preponderance of bladder cancer in men. There is some rodent evidence suggesting that androgens may promote bladder carcinogenesis [4,5], and androgen receptors are present in human urothelial carcinomas [6–9]. Conversely, examining bladder cancer incidence relative to reproductive events that alter exposure to estrogens and other reproductive hormones suggests that estrogens may limit risk of urothelial carcinoma in women. For instance, women who experience early menopause are at higher risk [3], while parous women have a lower risk of bladder cancer than nulliparous women [10–12]. In addition, use of oral contraceptives and hormone replacement therapy also has been associated with reduced bladder cancer risk [13,14]. Further support for estrogens hindering urothelial carcinogenesis is found in adult rodent models of bladder cancer, for both males and females [15,16]. Moreover, in a UPII-SV40T transgenic mouse model of bladder cancer, tumors were significantly larger in nulliparous *versus* parous animals [17]. Taken together, the cumulative evidence suggests that female sex steroids may contribute to the lower risk of bladder cancer in females than in males.

Estrogens mediate their pleiotropic and tissue-specific effects through estrogen receptors (ERs), which bind this class of sex steroids with high affinity and specificity. ER α (NR3A1) and ER β (NR3A2) belong to the nuclear receptor superfamily of transcription factors that regulate various aspects of normal physiology including development and function of the reproductive and other systems (e.g., skeletal and cardiovascular). In humans, early studies indicated ER α expression in smooth muscle of the trigone and the posterior region of the bladder neck [18,19]. More recently, approximately 50% of the benign urothelium from analyses of 141 male and female patients were positive for ER α with most showing weak expression [20]. In contrast, a smaller study of 17 postmenopausal women indicated that the bladder transitional epithelium was negative for ER α , while transitional epithelium of the urethra was positive [21]. Expression of ER β in the normal urothelium has also been evaluated in several studies, and the preponderance of results indicates that this tissue is largely positive for ER β [20–23] with the exception of one report that indicated that approximately one-third of the female specimens was ER β -positive, while this receptor was undetected in 58 male samples [8]. In rodents, there appears to be negligible ER α nuclear staining in the bladder [24,25], but ER β is expressed in the urothelium and smooth muscle in both sexes [26–31]. Thus, for both humans and rodents, ER β appears to be the predominant ER expressed in normal urothelium.

In human bladder cancer, most reports indicate a low percentage of tumors expressing ER α [32–36] with several studies failing to detect any ER α expression [37,38]. A recent report indicated weak expression of ER α in 27% of primary bladder cancer tissues, with no correlation between ER α and tumor recurrence, progression, or cancer-specific survival [20]. In contrast, strong ER β is detected in human bladder cancers in multiple studies, with up to 81% of tumors expressing this form of ER [8,9,20,22,35,39,40]. Expression of ER β appears to be greater in high-grade *versus* low-grade tumors [20,35] and is associated

with recurrence and progression of low-grade tumors, recurrence of muscle-invasive tumors, and reduced cancer-specific survival [20]. The greater expression of ER β in urothelial carcinoma and its association with poor outcome raises the possibility that blocking ER β function may be beneficial with respect to inhibiting bladder tumorigenesis and progression.

ERs can control proliferation, apoptosis, and migration/invasion with the net biologic outcome depending on the relative expression of ER α *versus* ER β , as well as whether the regulating ligand is an estrogen or antiestrogen [41]. In some bladder cancer cell lines, estradiol can induce DNA synthesis, suggestive of a growth stimulatory effect [42], while in others this estrogen was unable to induce cell proliferation [43]. In contrast, the antiestrogens raloxifene and tamoxifen inhibit *in vitro* growth of multiple bladder cancer cell lines representative of noninvasive and invasive tumors [35,43–45]. Moreover, tamoxifen and raloxifene inhibited the growth of 5637 transitional cell carcinoma xenografts in nude mice [44]. Collectively, this suggests that targeting ERs with antiestrogens limits the growth of existing urothelial carcinomas and raises the possibility that this growth-inhibitory potential could be employed in a chemoprotective manner relative to suppression of urothelial carcinogenesis in at risk populations and/or prevent bladder cancer recurrence.

In this report, the chemopreventive efficacy of the selective ER modulator (SERM) tamoxifen in bladder carcinogenesis was evaluated in a mouse model of bladder cancer induced with the organ-specific carcinogen, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN). This model shares morphologic [46], histologic [47,48], and genetic similarities with higher grades of human bladder cancer [49]. This experiment demonstrates that tamoxifen administered concurrently with carcinogen can significantly reduce the incidence of noninvasive and invasive bladder cancers in female mice, an effect associated with a reduction in urothelial proliferation and suppression of ER α expression induced by exposure to the BBN carcinogen, and suggests that SERMs such as tamoxifen should be evaluated in clinical trials testing a chemoprevention strategy to limit urothelial carcinogenesis in high-risk populations.

Materials and Methods

Experimental Design

Female C57BL/6J mice (Charles River Laboratories International, Inc, Wilmington, MA) were housed under specific pathogen-free conditions. Animals were divided into six groups ($n = 27$ –30/group; Figure 1). Group 1 served as the control, which received only tap water. Group 2 was pretreated with tamoxifen pellets for 3 weeks before BBN, group 3 had concurrent BBN and tamoxifen treatment for 12 weeks, group 4 had 12 weeks of BBN followed by tamoxifen for another 12 weeks, group 5 had concurrent BBN and tamoxifen followed by 12 weeks additional tamoxifen, while group 6 was treated with BBN alone. The BBN (TCI America, Portland, OR) carcinogen was supplied *ad libitum* at 0.05% in drinking water to mice from 8 to 20 weeks of age. Water consumption was recorded to determine BBN intake and compared between groups. Tamoxifen treatments (55 μ g/day) were administered by subcutaneous 90-day time-release pellets (Innovative Research of America, Sarasota, FL) that were implanted between the scapula. Body weights were measured at multiple time points between 8 and 32 weeks of age. Animals were monitored for tumor progression and survival and were killed after 32 weeks to obtain bladder and organ weights. Urinary bladders were processed for subsequent

Figure 1. Schematic for evaluation of tamoxifen in a mouse bladder carcinogenesis model. Female C57BL/6 mice were divided into six groups ($n = 27$ –30 mice/group), and the bladder-specific carcinogen, BBN, was administered to groups 2 to 6 from weeks 8 to 20 in drinking water, while control mice (group 1) received water alone. Tamoxifen (Tam; 55 μ g/day) was administered by slow-release pellets either before BBN (group 2), concurrent with BBN (group 3), after BBN (group 4), or during and after BBN (group 5). The experiment was concluded when mice reached 32 weeks of age.

histomorphologic and immunohistochemical analyses. To determine if BBN treatment increased ER α expression in male mice, 20 C57BL/6 male mice were treated with or without BBN (0.05%) in drinking water ($n = 10$ /group) from 8 to 20 weeks of age. Animals were killed at 27 weeks; bladders were isolated and split in half with one portion processed for histology and immunohistochemistry and the other flash frozen for RNA isolation. The earlier time point selected for terminating experiments with male mice is a reflection of the more rapid disease progression in male than female mice [50] and was selected to proceed the time point at which bladder obstructions may occur. All animal procedures were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine.

Histopathologic Evaluation and Tissue Microarray Preparation

To assess bladder histopathology, urinary bladders were first excised, cut in half longitudinally, and fixed in 10% buffered formalin. Formalin-fixed bladders were then paraffin embedded, sectioned, and stained with hematoxylin and eosin following standard protocols. Stained slides were histopathologically graded by an expert pathologist (S.S.S.), and bladders were categorized into normal or cancerous, invasive or muscle-invasive, bladders. These were then reviewed to mark the area for tumor for the construction of tissue microarrays. Tissue microarrays were made using 0.6-mm cylindrical cores punched out from the original paraffin blocks using a manual tissue arrayer (Beecher Instruments, Silver Spring, MD). Triplicate cores from individual blocks were made to enhance the representative reproducibility. Thus, a total of 540 cores representing 180 female mice were used to generate five master blocks. Five-micrometer sections were cut from these blocks and placed on charged slides (Fisher Scientific, Houston, TX) and stained appropriately. Briefly, these slides were deparaffinized, rehydrated, and pretreated by either microwave or proteinase K for antigen retrieval. Immunohistochemical staining was then performed using corresponding antibodies. The staining procedure was based on an indirect biotin-avidin system with a universal biotinylated Ig secondary antibody, DAB sub-

strate, and hematoxylin counterstain. A negative control slide was obtained after either omitting the primary antibody or incubating with an irrelevant antibody (mouse monoclonal Ig).

Tumor Cell Proliferation by Ki-67 Staining

Using the tissue microarrays generated above, sections were also stained for Ki-67 antigen assessed by immunohistochemistry using a monoclonal MIB-1 antibody (clone MIB-1, mouse IgG1, 1:100 from Dako North America Inc, Carpinteria, CA) that was incubated for 25 minutes in a TechMate 500 Plus (Dako North America Inc) and visualized with DAB. Images were captured using the Vectra scanner using the CRI multispectral camera with a $\times 20$ magnification objective (Caliper, Hopkinton, MA) for the entire tissue section. Image analysis was done using InForm 1.2 software. InForm was trained to count the Ki-67–positive cells in representative fields for each tissue section. From the images, areas of tissue other than urothelia were masked using Image-Pro Plus software (Media Cybernetics Inc, Bethesda, MD). The percentage of positively stained cells was calculated using images for the entire section of tissue.

Apoptosis Assays

Cell death was detected *in situ* by enzymatic labeling of DNA strand breaks using terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assays as described previously [51]. For negative controls, the terminal deoxynucleotidyl transferase was substituted by deionized water, while sections that were pretreated with 1.0 g/ml DNase I (DN 25; Sigma-Aldrich, St Louis, MO) were used for the positive controls. Images were captured using Vectra scanner as described above, and percentage of TUNEL-positive cells was determined using InForm 1.2 and Image-Pro Plus software.

ER Immunohistochemistry

For immunohistochemical staining of ER α , rabbit polyclonal antibodies against ER α (MC-20; Santa Cruz Biotechnology Inc, Santa

Cruz, CA) were used. For ER β , rabbit polyclonal antibody (PAI-310) was purchased from Affinity BioReagents, Inc (Golden, CO). Assessment of ER α and ER β expression was performed by a pathologist (S.S.S.) blinded to the tissue treatment using a modified version of Allred scoring [52]. The percentage of labeled cells was graded as follows: 0, no positive cells; 1, 1% to 25% labeled tumor cells; 2, 25% to 50% labeled tumor cells; 3, $\geq$ 50% positive tumor cells. The intensity of peroxidase deposits, ranging from light beige to dark brown, was scored as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). A composite score, ranging from 0 to 9, was obtained by multiplying the percentage grade by the intensity. ER expression scores were grouped as negative (0), low (<6), and high (≥ 6).

Analyses of ER mRNA Expression

Bladder specimens obtained from male mice were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA) and total RNA was extracted according to the manufacturer's recommendations. The RNA was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) and the resulting cDNAs were quantitated by quantitative real-time polymerase chain reaction (qPCR) analyses using TaqMan or Power SYBR Green Master Mix from Applied Biosystems (Foster City, CA). Primers and probe for measurement of mouse ER α (Mm00433149_m1) were purchased as a Taqman Gene Expression Assay (Applied Biosystems), while primers for mouse ER β (forward, 5'-GCAAGATCACTAGAACACACCTTGCC-3' and reverse, 5'-AGGACCAGACACCGTAATGATACCC-3') were purchased from Sigma (St Louis, MO). For normalization, levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Mm99999915_g1) were measured using primers and probe purchased from Applied Biosystems. Samples were run in a 7500 Fast Real-Time PCR System (Applied Biosystems) and the mRNA expression was analyzed by the $\Delta\Delta C_t$ method.

Statistical Analysis

Statistical analyses were performed using SAS 9.3 (SAS Institute Inc, Cary, NC). Wilcoxon Rank-Sum test was used to compare the difference between groups (pairwise) for bladder weight, Ki-67% positivity, and TUNEL% positivity since their distributions were highly skewed. We also performed analysis of variance with log transformation of the data. These two methods yielded essentially the same results. Fisher exact test was used to test for an association between treatment (tamoxifen or BBN treatment) and bladder histopathology, to compare ER α and ER β [negative, low (Allred score greater than 0 but <6) or high (Allred score ≥ 6)] between different treatment groups, and to test for an association between histologic classification (normal or tumor) and expression for ER α (ER α^+ and ER α^-). A two-sided 0.05 level was considered statistically significant.

Results

Tamoxifen Conferred Protection against BBN-Induced Mouse Bladder Carcinogenesis

On the basis of prior work demonstrating the effectiveness of SERMs in inhibiting the growth of existing urothelial carcinomas [35,44,45], this study sought to evaluate whether tamoxifen administration either before, concurrent with, after, or during and after carcinogen exposure would impact BBN-induced carcinogenesis in

female mice (Figure 1). On the basis of prior experiments in female C57BL/6 mice [50,53], carcinogen was administered at 0.05% (vol/vol) in drinking water to animals aged 8 to 20 weeks, and the study end point of 32 weeks of age was selected to avoid bladder tumors progressing to an extent that would cause death. At euthanasia, bladders were dissected out and drained of urine, and wet weights were obtained; because of the variation in bladder weights obtained for BBN-treated mice, data are presented as box-and-whisker plots (Figure 2). The average wet weights of bladders obtained from animals treated only with BBN (group 6) were five-fold greater than for the control group (Wilcoxon Rank-Sum test, $P < .0005$). Two mice in this group were killed before the planned study end point of 32 weeks (one at week 31 and a second several days before week 32) due to large bladder tumors; these animals were included in all subsequent analyses. Mice treated with tamoxifen either before (weeks 5–8) or after (weeks 20–32) BBN exposure were also significantly larger than control bladders by 2.5-fold and 1.5-fold, respectively (Wilcoxon Rank-Sum test, $P < .0005$). In contrast, the average bladder weights of groups 3 and 5 that received tamoxifen concurrent to (weeks 8–20) or during and after (weeks 8–32) BBN were not different from control animals; rather, they were significantly smaller than the bladder weights obtained for mice exposed to BBN alone (Wilcoxon Rank-Sum test, $P < .0005$). Body weights were recorded at the end of 32 weeks, but there were no statistically significant differences between the groups (data not shown).

Histopathologic evaluation of all the bladders was employed to determine the effect of tamoxifen on BBN-induced carcinogenesis and revealed urothelial carcinoma in 76% of the animals treated with BBN alone; of these 21% had muscle-invasive and 55% had non-invasive carcinoma (Figure 3). This contrasted significantly from the control group in which no evidence of bladder cancer, as expected, was detected. There were fewer ($\leq 7\%$) cases of muscle-invasive urothelial

Figure 2. Effect of tamoxifen and BBN exposure on bladder weight. Mice in groups 2 to 5 were given BBN from weeks 8 to 20 and treated with tamoxifen for the indicated time periods. Bladders were obtained at 32 weeks and wet weights were determined. Data are expressed as log transformation of bladder weights (g) using box-and-whiskers plot. Statistical significances were determined by Wilcoxon Rank-Sum test.

Figure 3. Effect of tamoxifen and BBN treatment on bladder histopathology. Mice were given BBN with or without the indicated duration of tamoxifen, and bladder tissues were collected at 32 weeks and processed for histopathologic analyses. Data are expressed as percentage of animals in each group with urinary bladders graded into normal, non-muscle-invasive, and muscle-invasive tumors. For statistical significances conducted by Fisher exact test, non-muscle-invasive and muscle-invasive tumors were considered together in comparison to normal specimens.

carcinoma in mice treated with tamoxifen, regardless of when the SERM was administered, than in the BBN-only group (21%). Indeed, muscle-invasive carcinoma was not detected in mice treated with tamoxifen + BBN concurrently (Fisher exact test, $P = .024$) and was found at only low levels in groups treated with tamoxifen after (4%; Fisher exact test, $P = .10$) or during and after (3%; Fisher exact test, $P = .05$) exposure to BBN.

With respect to non-muscle-invasive carcinoma, 55 μ g/day tamoxifen administered concurrently with BBN (weeks 8–20) or during and after BBN exposure (weeks 8–32) yielded a very low level of disease, 14% and 7%, respectively, resulting in 86% and 90%, respectively, of the animals having bladders with normal histology (Figure 3). Thus, these treatment schedules were largely protective against BBN-induced bladder carcinogenesis. However, a 3-week pretreatment with tamoxifen before BBN exposure or tamoxifen treatment for 12 weeks after cessation of BBN exposure did not reduce the percentage of animals with non-muscle-invasive disease in comparison to the BBN-only group (57% and 63% versus 55% for the BBN-only animals). Overall, the histopathologic results were consistent with the bladder weight data (Figure 2) in which the control group was significantly different from the groups tamoxifen treated pre-BBN (weeks 5–8) or post-BBN (weeks 20–32) as well as the BBN-alone group (Fisher exact test, $P < .0001$). Likewise, groups treated with tamoxifen concurrently with BBN (weeks 8–20) or during and after BBN (weeks 8–32) were significantly different from the BBN-only group (Fisher exact test, $P < .0001$) but not from the control group. Taken together, these results indicate that tamoxifen inhibits progression of tumorigenesis from non-muscle-invasive to muscle-invasive carcinoma regardless of when tamoxifen is administered. Moreover, tamoxifen treatment concurrently with BBN exposure inhibits early stages of carcinogenesis induced by the nitrosamine compound.

Tamoxifen Inhibition of Proliferation in BBN-Treated Bladder

The proliferative and apoptotic features of the bladder tumors were evaluated to determine if they were impacted by tamoxifen treatment. Apoptosis was assessed by TUNEL staining of tissue microarrays prepared from each of the bladders evaluated for the study. Quantitative analysis of three cores obtained from each bladder did not reveal any significant difference in the extent of apoptosis between the groups (Figure 4A).

Expression of the nuclear antigen Ki-67 was also evaluated by immunohistochemistry to assess the proliferative rates of the bladders in the different study groups. Initial assessments revealed significantly higher percentages of Ki-67-positive cells in each of the BBN-treated groups, ranging from 0.66% to 4.88% in comparison to the BBN-naïve group ($0.14 \pm 0.05\%$). As the distribution of tumor to normal histology differed widely between the BBN- and tamoxifen-treated groups, the average number of proliferating cells for bladders with normal urothelium was compared to the value obtained for bladder tumors within the same group. Thus, for mice exposed to BBN alone, the proliferation index in the tumors was 3.9 times greater than that determined for bladders with a normal histopathologic evaluation (Figure 4B). Indeed, comparing normal versus tumor (noninvasive and muscle invasive) in groups 2 to 6 by two-way analysis of variance reveals that proliferation of tumor tissues was greater than for the normal specimens ($P < .0001$).

Comparisons of proliferation determined for the tumors in each of the BBN-treated groups revealed high levels of Ki-67-positive cells in tumor tissues assessed for groups in which tamoxifen was administered before (weeks 5–8) or after (weeks 20–32) BBN exposure; this was consistent with the larger bladder weights and greater extent of tumorigenesis in these groups. In contrast, proliferation for tumors

in groups treated during and after (weeks 8–32; Wilcoxon Rank-Sum test, $P < .05$) BBN treatment were lower than for the BBN-only tumors, while the Ki-67 score for those exposed to tamoxifen concurrently with BBN (weeks 8–20) tended to be lower, but this did not reach statistical significance (Wilcoxon Rank-Sum test, $P = .082$). This was consistent with the protection afforded by these tamoxifen treatment schedules relative to bladder weight and links a reduced proliferation index to the reduction in the extent of BBN-induced tumorigenesis.

Expression of ERs in Bladder Tumors and Normal Urothelium

ERs are the primary mediators of tamoxifen action, and immunohistochemical detection of two ER subtypes, ER α and ER β , was therefore performed in mouse urinary bladders to examine their expression in control specimens and to assess the impact of BBN exposure with or without tamoxifen on their expression. Levels of

ER α and ER β were assessed by evaluating the percentage of positive cells and the extent (negative or weak→strong) of staining. A composite score, ranging from 0 to 9, was obtained by multiplying the percentage grade by the intensity; final ER expression scores were grouped as negative (0), low (<6), and high (≥ 6). Prior studies in rodents and humans indicated that ER β is the primary ER expressed in normal urothelium as well as urothelial carcinoma [27,30,35], and analyses therefore began with immunohistochemistry for ER β .

The ER β protein was detected in the superficial and basal layers of the normal urothelium and was primarily confined to the nucleus, although some minor staining was also detected in the cytoplasm (Figure 5A). Stromal, endothelial, and lymphoid cells were largely negative for ER β . While each bladder specimen examined, regardless of whether it was normal or carcinoma, was positive for ER β staining, there were modest changes in the distribution (high *versus* low) percentages of positive cells noted between the different treatment

Figure 4. Effect of BBN and tamoxifen treatments on apoptosis and proliferation in bladder specimens. Mice were given BBN with or without tamoxifen for the indicated duration, and bladder tissues were collected for the assessment of (A) apoptosis by TUNEL assay and (B) cell proliferation by Ki-67 staining. Three punches per animal were arrayed into tissue microarrays for the analysis. For TUNEL, data are expressed as percent TUNEL-positive cells determined using Image-Pro software. No significant differences were detected between the groups. For Ki-67 staining, data are expressed as the average $\pm$ SEM of percent Ki-67–positive cells for normal or tumor (noninvasive and muscle-invasive) histology. Statistical significances were determined by Wilcoxon Rank-Sum test.

Figure 5. Expression of ER β in bladder specimens obtained from female mice. Mice were given BBN with or without tamoxifen for the indicated duration, and bladder tissues were collected after 32 weeks for immunohistochemical staining for ER β . (A) Representative images of bladder sections obtained from control (group 1), BBN (group 6), and BBN + Tam (group 3) stained for ER β . (B) Data are expressed as percentages of animals with IHC scores for ER β grouped as low (Allred score greater than 0 but < 6) or high (Allred score ≥ 6). Statistical significance was determined by Fisher exact test.

groups. This was not related to a difference in staining between the invasive carcinoma and noninvasive disease. For the untreated control group, 79% (23/29) of the animals had high ER β expression with the remaining 21% having low ER β expression (Figure 5B). In the BBN-only group, ER β was expressed highly in 89% (24/27) of the specimens, with low expression levels in the remaining 11% of the animals; this was not significantly different from the control group. For groups treated with tamoxifen before (weeks 5–8), during (weeks 8–20) or during and after (weeks 8–32) BBN exposure, fewer animals expressed high levels of ER β in comparison to the BBN-only group. This was not, however, observed for the group treated with tamoxifen after BBN exposure (weeks 20–32), and thus, no correlation between ER β expression and tamoxifen-dependent protection from BBN-induced carcinogenesis could be detected.

Consistent with prior reports in rodents [24,25], ER α was not detected in the nucleus or cytoplasm of the urothelium or stroma of the control bladders (Figure 6A). Surprisingly, however, immunohistochemical analyses revealed nuclear ER α immunoreactivity in the basal urothelia of the urinary bladders of all groups treated with carcinogen with or without tamoxifen treatment (Fisher exact test, comparing to control, $P < .05$). In most positive cases, ER α expression was low (e.g., weak staining and/or a low percentage of positive cells). Positive ER α staining was also observed in noninvasive and muscle-invasive carcinoma. For animals treated with BBN alone, 74% (20/27) of the bladders revealed either high or low ER α expression (Figure 6B). A similar percentage of ER α -expressing bladders were also detected

for mice treated with tamoxifen before (weeks 5–8; 68%) and after (weeks 20–32; 65%) exposure to BBN. Interestingly, ER α expression was detected in only 39% (Fisher exact test, comparing to BBN-only group, $P < .05$) of the animals treated with tamoxifen concurrently with BBN, and for animals treated with the SERM during and after BBN exposure (weeks 8–32), the percentage of ER α -positive bladder specimens was reduced (40%; Fisher exact test, comparing to BBN-only group, $P < .05$).

Since reduced ER α expression was detected for the tamoxifen treatment groups with the lowest incidence of bladder carcinogenesis, the distribution of ER α positivity in normal *versus* tumor specimens was examined to determine if there was a correlation between ER α expression and BBN-induced bladder carcinogenesis. Overall, combining all the histologically normal bladders yields 106 specimens of which only 30% were ER α positive. In contrast, for the combined bladder tumor specimens from all groups ($n = 62$), a much higher proportion (76%) were found to express ER α (Figure 6C) and this was statistically correlated by Fisher exact test ($P < .001$). Taken together, these data suggest that induction of ER α expression as a consequence of BBN exposure in this model is associated with bladder carcinogenesis.

To determine if BBN induction of ER α expression was specific to female mice and thereby potentially contributing to gender differences in carcinogen-induced bladder cancer [16,50], levels of ER α were assessed in bladders obtained from male mice treated with BBN from age 8 to 20 weeks and killed at 27 weeks of age. Similar to our findings for females, immunohistochemical detection of ER α expression was

present only in animals exposed to BBN (Figure 7A). The numbers of ER α -positive specimens were lower in males (40%) than for the females (74%); it is unknown if this is a reflection of the relatively early assessment time point in males (27 weeks) *versus* females (32 weeks) or a gender-dependent difference (Figure 7B). The induction of ER α expression in BBN-treated animals suggested that the carcinogen treatment stimulated expression of the *ER α* gene. However, the similar levels of ER α mRNA detected for the control and BBN-treated groups (Figure 7C) suggested that the increase in ER α protein detected by immunohistochemistry was not due to activation of *ER α* gene expression but rather reflected an increase in the protein expression through a post-transcriptional mechanism(s). Not unexpectedly, levels of ER β mRNA were similar in control and BBN-treated groups.

Discussion

The ability of the SERMs, raloxifene and tamoxifen, to inhibit proliferation of human bladder cancer cell lines including 5637, RT4, and TSU-Pr1 *in vitro* as well as the growth of 5637 xenografts in nude mice demonstrated the potential of these agents to impede the progression of existing urothelial carcinoma [35,43–45]. Moreover, knock-down of ER expression blocks the ability of raloxifene to exert this inhibitory effect [43]. Recurrence of bladder cancer in patients following initial tumor resection is a significant medical problem and the goal of this study was to employ the BBN-induced urinary bladder tumor model to determine the ability of tamoxifen to prevent carcinogenesis in female mice. In our experiments, tamoxifen treatment regardless of when it was administered reduced the extent of invasive

urothelial carcinoma. In addition, tamoxifen when administered concurrently with carcinogen reduced both invasive and noninvasive diseases and largely protected female mice from urothelial carcinogenesis. This was reflected in reduced cell proliferation rather than an increase in apoptosis and is likely mediated by ER β that is present before and after BBN treatment in all animals, although the possibility of a contribution from ER α in some carcinogen-treated mice cannot be excluded. On the basis of the use of tamoxifen for other chemoprevention purposes (e.g., breast carcinogenesis), our data indicate that tamoxifen may be a practical approach to limit urothelial carcinogenesis in patient populations at high risk for bladder tumor recurrence.

BBN induces pathologic alterations in the bladder mucosa beginning from mucosal dysplasia, papillary or nodular dysplasia, and finally leading to invasive carcinoma in mice [50,54]. Initiation and promotion of bladder carcinogenesis by BBN is accompanied by increased cell proliferation in rodents and this is associated with changes in the expression of genes involved in cell cycle control [49,55–57]. Our data also indicate an increase in Ki-67–positive cells induced by BBN, and the reduced proliferation observed for animals treated with tamoxifen concurrent with BBN is consistent with this SERM’s protective effect. Although there is little information on tamoxifen effects in normal urothelium, estrogen treatment has been demonstrated to increase the proliferation of cultured urothelium cells *in vitro* [42,58] and over-expression of aromatase, the enzyme responsible for estradiol production, in transgenic mice also leads to increased proliferation in the urothelial layer [59]. Given the ability of estrogens to increase cell proliferation, it was not unexpected that tamoxifen, which was originally classified as an antiestrogen, was able to suppress cell proliferation in

Figure 6. Expression of ER α in bladder specimens obtained from female mice. Mice were given BBN with or without tamoxifen for the indicated duration, and bladder tissues were collected after 32 weeks for immunohistochemical staining for ER β . (A) Representative images of bladder sections obtained from control (group 1), BBN (group 6), and BBN + Tam (group 3) stained for ER α . (B) Data are expressed as percentages of animals with IHC scores for ER α grouped as negative, low (Allred score greater than 0 but <6), or high (Allred score $\geq$ 6). Statistical significance was determined by Fisher exact test; * $P < .05$ in comparison to all other groups. (C) Distribution of bladder specimens according to histologic classification (normal or tumor) and expression for ER α .

Figure 7. Expression of ERs in bladder obtained from male mice. Mice were given drinking water only (control) or water containing 0.05% BBN from weeks 8 to 20, and urinary bladders were collected at 27 weeks for analysis of ER expression. (A) Immunohistochemical staining and (B) quantitative IHC results for ERα expression determined for male mouse bladder specimens. (C) Levels of mRNA expression for ERα (left panel) and ERβ (right panel) were determined by reverse transcription-qPCR. Values represent $n = 10$ per group and are presented as the average $\pm$ SEM.

normal mouse bladders. Moreover, the ability of tamoxifen and raloxifene to reduce cell proliferation in bladder cancer cells suggests that SERMs may also limit proliferation after initial carcinogenesis events [35,43,44,60]. Estrogens may also increase urothelium proliferation in women as cellular proliferation in the lower urinary tract is also lower in postmenopausal *versus* premenopausal women or postmenopausal women taking hormone replacement [61], suggesting that tamoxifen may be similarly efficacious in high-risk patients.

Antiestrogens can interfere with growth of different cell systems through ERα and/or ERβ-dependent pathways and potentially via impacting other estrogen-regulated pathways that do not rely on nuclear receptors (e.g., GPR30-regulated proliferation; [62]). However, in bladder carcinoma cells like RT4 that express both ERs, either was competent to mediate growth inhibition by raloxifene [43]. In our studies, all the mouse bladder specimens expressed ERβ regardless of BBN or tamoxifen treatment, while ERα expression was present in 74% of the BBN-treated specimens but undetectable in BBN-naïve bladders. Although this raises the possibility that the protective effect of tamoxifen may be mediated by ERα and/or ERβ, there are multiple instances in which tamoxifen provided tumorigenesis protection in bladders that were ERα negative, suggesting that the protection is likely mediated by ERβ although the possibility that transient expression of ERα contributes to the chemoprotective effect of tamoxifen cannot be excluded.

While the available data do not indicate that ERα is obligatory for the protective effect of tamoxifen in this carcinogen-induced model, its expression in the majority of bladders obtained from BBN-treated animals and the absence of any detectable ERα staining in bladders from BBN-naïve animals raises the possibility that the induction of ERα expression following carcinogen exposure may contribute to tumorigenesis. Prior studies failed to detect ERα as a key player in BBN-induced bladder carcinogenesis in mice or rats [56,57,63]; instead, they identified pathways such as those involving epidermal growth factor receptor–Ras, cell cycle, transforming growth factor–β, c-Myc, apoptosis, and integrin-mediated cell adhesion in BBN-induced carcinogenesis. However, in a very different model of bladder cancer in which female mice are exposed to inorganic arsenic *in utero* followed by postnatal diethylstilbestrol, the resulting bladder transitional cell carcinomas are positive for ERα [64], raising the possibility that expression of this receptor may contribute to a cancer phenotype. The role of ERα as a positive regulator of proliferation and its association with inflammatory responses and the development of malignancy are well established [41]. Nonetheless, the importance of ERα to urothelial cancer in humans is unclear with most studies reporting little expression of the receptor, although a recent report linked a loss of ERα expression with higher grade tumors [20,32–36,38,65]. Future studies with BBN exposure of mice with a urothelial-specific knockout of ERα should be able to address potential roles of ERα in carcinogen-induced tumorigenesis.

The absence of ERα expression in BBN-naïve bladder urothelium of females is consistent with a prior report in which immunohistochemical staining for ERα in female mouse urinary bladder and urethra was negative [24]. Our finding of BBN induction of ERα protein expression in the urothelium of female and male mice indicates that this response is not gender-specific and raises the possibility of a contribution of ERα to BBN-induced carcinogenesis in male mice. The appearance of ERα protein detected by immunohistochemistry (IHC) was not reflected at the mRNA level, indicating that the induction of this receptor's expression is likely mediated through a post-transcriptional event rather than a significant increase in ERα gene expression. This would also explain why prior gene profiling studies did not detect an increase in ERα in this model [56,57,63]. There are multiple potential mechanisms by which ERα expression in the urothelium of BBN-treated mice may be increased following BBN exposure. Several microRNAs are known to target ERα in cell lines and tissues [66] and a reduction in their expression would be expected to increase the receptor's expression. Interestingly, expression of let-7c, a member of the let-7 microRNAs that target ERα [67], is decreased in bladder tumors arising in BBN-treated rats and postulated to play a significant role in oncogenesis [68]. Conversely, stabilization of ERα protein, potentially from a reduction in ubiquitin ligases (e.g., Mdm2) that target the receptor for degradation by the proteasome [69], could also contribute to increased protein expression in urothelium following carcinogen exposure.

The expression of ERβ in mice from all the treatment groups, even in the absence of ERα, raises the possibility that the former receptor mediates the chemoprotective effects of tamoxifen. ERβ is largely considered to be antiproliferative and proapoptotic, thereby exerting beneficial effects in multiple different cancers [41]. Although not fully elucidated, possible mechanisms for tumor suppression by ERβ include inducing G₁ arrest of the cell cycle by regulating cell cycle checkpoint genes [70], impeding G₂/M progression by inhibiting CDK1 activity [71] and suppressing inflammatory pathways [72]. In addition, where ERα and ERβ are co-expressed, the latter may block ERα-mediated

events such as estrogen-dependent cell proliferation [73]. Collectively, this may be the result of distinct profiles of genes regulated by ER α versus ER β [74], but it could also reflect a direct effect of ER β on ER α activity, potentially through formation of ER α /ER β heterodimers. Indeed, ER β can disrupt ER α recruitment to estrogen response elements and block ER α -dependent recruitment of other factors required for optimal gene expression [75]. There is also evidence that ER β can reduce ER α expression [75,76].

In general, tamoxifen appears to reduce the extent of invasive carcinoma in this carcinogen-induced mouse model and this bodes well for the use of tamoxifen in human chemoprevention trials where inhibition of the formation of invasive cancer is of primary concern. This would be of significant benefit to many patients who initially present with non-muscle-invasive disease that must undergo long-term monitoring for future development of invasive carcinoma. The reduction in invasive disease achieved by concurrent BBN and tamoxifen administration is not surprising, as the reduction in noninvasive cancer indicates that this tamoxifen treatment scheme inhibits early stages of carcinogenesis. However, administration either before or after BBN exposure reduced the extent of invasive carcinoma without influencing levels of noninvasive disease, suggesting that tamoxifen administered independently of carcinogen can inhibit later stages of carcinogenesis. In support of this, in other experiments in which we have evaluated BBN-treated mice by micro-ultrasound, dysplasia of the urothelium is evident during the latter third of the BBN exposure period (e.g., weeks 16–20; George & Smith, unpublished observations) consistent with prior findings [50]. Thus, SERM treatment begun after the cessation of BBN administration appears to be able to limit the progression from non-muscle-invasive to muscle-invasive carcinoma. While the receptor responsible for this apparent inhibition of invasion by tamoxifen is unknown in the BBN carcinogenesis model, it is interesting to note that ER β is associated with a block in epithelial-to-mesenchymal transition in mammary and prostate cancer [77,78] and stimulation of bladder cancer cell lines with the ER β -selective ligand diarylpropionitrile induces expression of E-cadherin while reducing N-cadherin expression [40].

In addition, administration of tamoxifen concurrently with BBN exposure reduced invasive as well as noninvasive carcinoma, suggesting an effect of concurrent tamoxifen treatment in preventing early stages of carcinogenesis. Whether this is due to a direct protective effect on the urothelium or an indirect effect, potentially through regulation of BBN metabolism, is unknown at the present time. The carcinogen BBN is subject to two possible metabolic fates. In the first one, BBN is metabolized to *N*-nitrosobutyl(3-carboxypropyl)amine and this product exerts carcinogenic effects on urothelial cells [79,80]. Alternatively, BBN is a substrate for several phase II drug metabolism enzymes, such as the UDP-glucuronosyltransferases (UGTs) that catalyze the glucuronidation of carcinogens making them water soluble for excretion [81]. A recent report indicates that estradiol can induce expression of the detoxifying enzyme UGT1a in immortalized SVHUC urothelial cells while reducing expression in 5637, UMUC3, and J82 bladder cancer cell lines [82], raising the possibility of ER-dependent chemoprotection through metabolism of BBN. Indeed, estrogen stimulation of UGT1a has been postulated to play a role in the lower incidence of bladder cancer in females versus males [82]. Tamoxifen alone was unable to alter UGT1a mRNA expression in these studies, and tamoxifen inhibition of endogenous estrogens would be anticipated to reduce UGT1a expression, thereby exacerbating BBN-induced carcinogenesis. The reduction in carcinogenesis achieved by tamoxifen in the present

study suggests that the protection conferred by this SERM is independent of UGT1a, although a potential contribution from alterations in other metabolic pathways cannot be ruled out at the present time.

Tamoxifen has a long history of use for treatment of breast cancer and, more recently, chemoprevention in women at high risk of developing this disease. Tamoxifen has also been employed in the treatment of other cancers including ovarian, hepatocellular carcinoma and malignant glioma [83]. Relative to bladder cancer, tamoxifen has been shown to enhance the sensitivity of human transitional carcinoma cells to standard chemotherapeutic drugs such as doxorubicin and cisplatin in *in vitro* studies [84,85]. Tamoxifen has also been evaluated for advanced stage bladder cancer patients in combination with cisplatin-based therapy. Although there was no chemotherapy only arm in this small study, the addition of tamoxifen was well tolerated and the combination therapy achieved responses comparable to historical controls [86]. A case report also describes the complete remission of a male patient with metastatic transitional cell carcinoma (TCC) given tamoxifen to treat gynecomastia [87]. These prior studies suggest a potential role for tamoxifen in treatment of existing bladder cancer, while our study addresses a chemoprevention application of this SERM in a well-established animal model with applicability to human disease. This may be useful in patients who are at high risk for recurrence following initial tumor resection with or without subsequent immunotherapy and is supported by the high incidence of ER β expression in normal urothelium as well as urothelial tumors of bladder cancer patients. Deciphering molecular targets through which the protective effects of tamoxifen are achieved may ultimately allow for selection of patients that would benefit from an SERM-based strategy for chemoprevention of bladder cancer.

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