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Targeting mTOR and p53 signaling inhibits muscle invasive bladder cancer *in vivo*

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Abstract

Urothelial tumors, accompanied by mutations of the tumor suppressor protein TP53 and dysregulation of mTOR signaling, are frequently associated with aggressive growth and invasiveness. We investigated whether targeting these two pathways would inhibit urothelial tumor growth and progression. Six-week-old transgenic UPII-SV40T male mice ($n=15$ /group) were fed control diet (AIN-76A) or experimental diets containing mTOR inhibitor (rapamycin, 8 or 16 ppm), p53 stabilizing agent (CP31398 [CP], 150 ppm), or a combination. Mice were euthanized at 40 weeks of age. Urinary bladders were collected and evaluated to determine tumor weight and histopathology. Each agent alone, and in combination, significantly inhibited tumor growth. Treatment with rapamycin alone decreased tumor weight up to 67% ($p<0.0001$). Similarly, CP showed ~77% ($p<0.0001$) suppression of tumor weight. The combination of low-dose rapamycin and CP led to ~83% ($p<0.0001$) inhibition of tumor weight. There was no significant difference in tumor weights between rapamycin and CP treatments ($p>0.05$). However, there was a significant difference between 8 ppm rapamycin and the combination treatment. Tumor invasion was also significantly inhibited in 53% ($p<0.005$) and 66% ($p<0.0005$) mice after 8 ppm and 16 ppm rapamycin respectively. While tumor invasion was suppressed in 73% ($p<0.0001$) mice when CP was combined with 8 ppm rapamycin. These results suggest that targeting two or more pathways achieve better treatment efficacy than a single-agent high-dose strategy that could increase the risk of side effects. A combination of CP and rapamycin may be a promising method of inhibiting muscle-invasive urothelial TCC.

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Keywords

Transitional Cell Carcinoma; UPII-SV40T; Rapamycin; CP-31398

Introduction

Urinary bladder cancer is the second most frequently diagnosed genitourinary cancer worldwide. In the United States, 74,000 new cases and 16,000 deaths from urinary bladder cancer are expected in 2015 (1). A significant portion of patients diagnosed with bladder cancer have muscle-invasive transitional cell carcinoma (TCC) that is life-threatening due to their high risk for metastasis (2). Moreover, ~15% of non-muscle-invasive TCC also progresses to the muscle-invasive form, with recurrence after surgery and the acquisition of additional genetic alterations. Compared with non-muscle-invasive tumors, muscle-invasive TCC are difficult to treat and have a low 5-year survival rate of only 6% in cases with distant metastasis (1).

Research toward understanding this disease led to the identification of major risk factors and important molecular changes underlying urothelial tumor initiation and progression. p53 is known to be mutated in over 50% of all human cancers, including invasive TCC (3). Altered p53 expression levels are associated with tumor recurrence, lower survival rates (4), and poor prognosis in bladder cancer patients (5). Similarly, mammalian target of rapamycin (mTOR) is reported to be frequently hyperactivated in many cancers, and is a clinically validated target for drug development (6–7). Accumulating evidence also suggests that activation of the mTOR pathway plays a role in the oncogenesis of bladder cancer (8–10). Activation of the PI3K / Akt / mTOR pathway is correlated with tumor progression and reduced survival in patients with urothelial carcinoma of the urinary bladder (10–11). Dysregulation of the p53 and mTOR pathways generates a favorable oncogenic environment, such as an increase in cell-cycle progression (12) and activation of protein translation (13). In view of the key role played by these two molecular targets in urothelial tumorigenesis and invasion, it is crucial to investigate a combination of agents that can modulate these two pathways, to determine the effects on tumor growth and disease progression.

The mTOR inhibitor rapamycin is known to inhibit cell proliferation, migration, and invasion (14). Rapamycin has been reported to have anticancer effects in *in vitro* and *in vivo* models of urothelial carcinoma (15). CP-31398 (CP), a synthetic styrylquinazoline, is documented to restore the p53 pathway by stabilizing both mutant and wild-type p53 protein (16). Several *in vitro* and *in vivo* studies have successfully demonstrated the anti-tumor potential of this agent in the treatment of many cancers (17–19). In the present study, we determined the anti-tumor effects of CP and rapamycin, either individually or in combination, using a transgenic mouse model of urinary bladder cancer (UPII-SV40T); this model has histopathology and molecular features similar to a highly invasive variant of human urinary bladder cancers (20). The use of this mouse model has played a key role in the elucidation of the mechanisms underlying urothelial tumorigenesis (21–22) and in the identification of anti-tumor agents for the treatment of bladder cancer (23–25).

Materials and methods

Animals, diet, and care

All animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC). Transgenic mice (UPII-SV40T) expressing a Simian Virus 40 large T antigen (SV40T) specifically in urothelial cells under the control of the Uroplakin II (UPII) promoter and reproducibly developing high-grade carcinoma in situ (CIS) and invasive tumors throughout the urothelium (20–21, 23–25) were used. The required number of UPII-SV40T transgenic mice were generated by breeding as described earlier (25). Animals were housed in ventilated cages under standardized conditions (21°C, 60% humidity, 12h-light/12h-dark cycle, 20 air changes per hour) in the University of Oklahoma Health Sciences Center rodent barrier facility. Semi-purified modified AIN-76A diet ingredients were purchased from Bioserv, Inc. Rapamycin and CP were procured from the National Cancer Institute chemoprevention drug repository. Appropriate amounts of these agents were premixed with small amounts of casein and were then blended into the diet using a Hobart mixer. Both control and experimental diets were prepared weekly and stored in a cold room. Mice were allowed ad libitum access to the respective diets and to automated tap water purified by reverse osmosis.

Breeding and Genotyping

All mice were bred and genotyped as described earlier (25). In brief, male UPII-SV40T mice were crossed with wild-type females to generate offspring. Transgenic pups were confirmed by tail DNA extraction using the mini-prep kit (Invitrogen) and polymerase chain reaction (PCR). PCR for the SV40T gene was done using the specific primers (Supplementary Table 1) and amplification was performed under the following PCR conditions: denaturation at 95°C for 5 minutes, followed by 35 cycles at 95°C for 1 minute, 58°C for 45 sec, and 72°C for 45 sec. The PCR products, when separated on a 2% agarose gel, showed a ~570 bp band.

Bioassay

Genotyped UPII-SV40T transgenic mice were used in the efficacy study. The experimental protocol is summarized in Fig. 1A. Five-week-old mice were selected and randomized so that the average body weights in each group were equal ($n=15$ UPII-SV40T mice per group and $n=6$ wild-type mice per group). Mice were fed a modified AIN-76A diet for 1 week. At 6 weeks of age, mice were fed control or experimental diets containing 0, 8 or 16 ppm rapamycin, 150 ppm CP, or a combination of 8 ppm rapamycin and 150 ppm CP (Fig 1A) until termination of the study. Mice were checked routinely for signs of weight loss, toxicity, or any abnormalities. The food intake and body weight of each animal were measured once weekly for the first six weeks, and then once a month until euthanasia. After 34 weeks on experimental diets (i.e., at 40 weeks of age), all mice were euthanized by CO₂ asphyxiation and necropsied. Urinary bladders were collected and weighed to determine the tumor weights. Portions of the urinary bladders were fixed in 10% neutral-buffered formalin for histopathological evaluation. The remaining bladder tissues were snap-frozen in liquid nitrogen for further analysis.

Tissue processing and histological analysis

Formalin-fixed, paraffin-embedded tissues were sectioned (4- μ m) and stained with hematoxylin and eosin (H&E). Multiple sections from various depths of each urothelial tumor were evaluated histologically by a pathologist blinded to the experimental groups. Tumors were classified into non-invasive TCC (CIS) or invasive carcinomas (lamina propria invasive and muscularis propria invasive) types according to histopathological criteria, as previously described (24–25).

Magnetic Resonance Imaging (MRI) of Urothelial Tumors

MRI was performed using a 30-cm, horizontal bore, 7-T magnet (Bruker BioSpin MRI GmbH, Ettlingen, Germany) on forty-week-old wild-type and transgenic mice that were fed the control diets. The mice were anesthetized with 2% isoflurane and restrained in a cradle for MRI scans. During imaging, the animals' respiratory rates were monitored (SA Instruments), and body temperatures were maintained at 37°C with a water heating system (Gaymar T/Pump). Morphological and contrast-enhanced imaging with gadolinium-diethylene triamine pentaacetic acid (Gd-DTPA; 0.5 mmol/kg body weight, 50 microliter volume in sterile saline) was performed on these mice. Gd-DTPA was administered via an intravenous tail vein catheter.

Real-time PCR

Total RNA from urothelial tumor samples of male mice was extracted using the Totally RNA Kit per the manufacturer's instructions. Equal amounts of DNA-free RNA were used in reverse transcription reactions to make cDNA using SuperScript reverse transcriptase (Invitrogen). Real-time PCR reactions were done for rictor, raptor, sgk1, hif1 α , vegf, p53, pcna, cyclin D, AR, and actin using SYBR green and specific primers (Supplementary Table 1). Relative gene expression was calculated using the 2^{-CT} formula (26). All experiments were performed at least in triplicate using replicated tumor samples.

Immunohistochemistry (IHC)

Effect of treatments on the expression of mTOR, hif1 α , pcna, p16, cyclin A, and AR were evaluated by immunohistochemistry. Briefly, sections of paraffin-embedded tissues were deparaffinized in xylene, rehydrated using graded ethanol solutions, and washed in phosphate-buffered saline (PBS). Antigen retrieval was carried out by heating the sections in 0.01 mol/L citrate buffer (pH 6.0) for 30 minutes in a boiling water bath. Endogenous peroxidase activity was quenched by incubation in 3% H₂O₂ in PBS for 5 minutes. Nonspecific binding sites were blocked using Protein Block for 20 minutes. Then, sections were incubated overnight at 4°C with 1:300 dilutions of monoclonal antibodies against pcna (sc-56), p16, cyclin A, mTOR (bs-1992R), hif1 α (bs-0737R), and AR (bs-0118R). After several washes with PBS, sections were incubated with the appropriate secondary antibodies for 2 hours, and were then exposed to avidin-biotin complex reagent (Invitrogen). After rinsing with PBS, the slides were incubated with the chromogen 3,3'-diaminobenzidine for 3 minutes, and then counter stained with hematoxylin. Non-immune rabbit immunoglobulins were substituted for primary antibodies as negative controls. Specimens were observed using

an Olympus microscope IX71. Digital computer images were recorded with an Olympus DP70 camera.

Western blotting

Proteins (60 ug) in lysates from bladders of control and treated mice were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked with 5% nonfat milk (Biorad) in Tris-buffered saline (TBS) and were then incubated with antibodies for pcna (sc-56), Akt (sc-1619), p-Akt (sc-135650), p-mTOR (sc-101738), AR (bs-0118R), hif1 α (bs-0737R), and actin (sc-1616) overnight at 4°C. Subsequently, membranes were washed and incubated with HRP-secondary antibody (1:10,000 dilution) for 1 h. Protein was detected on BioMax MR film (Kodak) using chemiluminescence (Super Signal, Pierce Biotechnology). Equal protein loading was confirmed by detection of actin. ImageJ was used to perform image analysis.

Statistical Analysis

The data are presented as means $\pm$ standard errors (*SE*). Differences in body weights were analyzed by Analysis of Variance. Statistical differences between urothelial tumor weights in the control and treated groups were evaluated using unpaired t-test with Welch's correction. Tumor incidences (percentage of mice with urothelial tumors) were analyzed by Fisher's exact test. Differences between control and treatment groups were considered significant at $p < 0.05$. All statistical analysis was performed using Graphpad Prism 5.0 Software.

Results

General observations

All transgenic and wild-type mice were fed either modified AIN-76A diets containing rapamycin, CP, or a combination (Fig 1A). There were no significant differences in the bodyweights of control and drug-treated animals (Fig 1B). Examination of the gross anatomy of wild-type and transgenic mice revealed no visible evidence of any abnormality of the kidneys, liver, spleen, pancreas, intestines, heart, or lungs. Further, there were no significant differences in the weights of these organs in control and experimental-diet-fed mice (Fig 1B), indicating that the agents did not produce any overt toxicities. However, urinary bladders from control-diet-fed transgenic mice developed visible urothelial tumors and weighed significantly more than those from the control-diet-fed wild-type mice, which were completely free from tumor growth (Fig 1C). The urothelial tumors were visualized by MRI imaging. Histopathological analysis of formalin-fixed tumors confirmed the presence of muscle-invading TCC in the bladders of the transgenic mice (Fig 1C). Thus, we observed organ-specific tumor growth in the UPII-SV40T transgenic mice, which could be monitored for potential effects of the test agents administered in food.

Inhibition of urothelial tumor growth

Administration of rapamycin, CP, or a combination significantly inhibited urothelial tumor growth (Fig 2A). The urinary bladders from experimental group mice weighed significantly less than those from the control group, suggesting the suppression of tumor growth by the administered agents. Doses of 8 and 16 ppm of rapamycin led to 63% (41.7 ± 9.4 mg,

$p<0.0001$) and 67% (37.1 ± 9.6 mg, $p<0.0001$) inhibition of tumor weight, respectively, compared with that of the control group (112.9 ± 9.79 mg). There were no significant differences between these two doses, indicating a lack of dose dependency. Treatment with 150 ppm of CP alone led to 77.5% tumor weight inhibition (25.45 ± 0.5 mg, $p<0.0001$). Finally, when 150 ppm CP was given in combination with the low 8 ppm dose of rapamycin, there was 83.6% significant inhibition of the tumor growth compared with tumors from control animals (16.47 ± 5.4 mg, $p<0.0001$). This reduction was also significant compared with the inhibition produced by low-dose rapamycin treatment alone ($p<0.05$; Fig 2A).

Synergistic effect of rapamycin and CP31398 on urothelial tumor invasion

To evaluate the effects of rapamycin and CP on urothelial tumor invasion, we performed histopathology on H&E stained sections of bladder tumors. All tumors were identified as high grade TCCs. In the mice fed control diet, the urothelial tumors were observed to be highly invasive, with the TCC growth into both the lamina propria and muscularis. At both doses tested, rapamycin alone significantly inhibited tumor invasion. In the rapamycin-treated groups, urothelial tumors were found to be non-invasive in 53% (8/15, 8 ppm rapamycin; $p<0.005$) and 66% (10/15, 16 ppm rapamycin; $p<0.0005$) of animals, with tumors being restricted to the urothelium (Fig 2B). Administration of 150 ppm CP alone moderately suppressed tumor invasion from the lamina propria to muscularis. However, CP did not prevent tumor invasion to the lamina propria. Interestingly, the combination of rapamycin and CP led to inhibition of tumor invasion in almost 73% (11/15; $p<0.0001$) of the mice. This inhibition was highly significant compared with that seen in the control and CP-alone groups. Although this inhibition was higher than that observed with both doses of rapamycin, this difference was not statistically significant (Fig 2B).

Proliferation is suppressed in drug-treated mouse tumors

We evaluated tumor cell proliferation in the urothelial tumors from control and treatment groups, and analyzed the relative expression of proliferation and cell cycle markers, such as pcna, cyclin D1, and cyclin A. mRNA and protein expression analysis completed using real-time pcr, western blot, and IHC analysis suggested that the urothelial tumor cells were highly proliferative in the control group, but were significantly inhibited in the treatment groups (Fig 3 and 4). Cells from control group tumors highly expressed pcna mRNA and protein, with a very high pcna index (Fig 3A, 3B, and 4A). Oral administration of rapamycin, CP, or their combination led to a significant inhibition of pcna mRNA (Fig 3A) and protein expression (Fig 3B, 4A). Tumor suppressor p53 was found to be significantly induced by both CP and the combination treatments (Fig 3A). Expression of cyclin A and cyclin D1 was also inhibited in the treated tumors (Fig 3A and 3B). IHC analysis of the treated tumors for Annexin-V expression showed an increase in apoptosis (Fig 3B). These results clearly suggest that inhibition of tumor growth was due to significant suppression of tumor cell proliferation with an induction of apoptosis produced by administration of rapamycin and CP, as well as their combination.

mTOR signaling is suppressed

The modulatory effect of treatments on the mTOR pathway was determined by comparative analysis of several mTOR-related biomarkers, such as raptor, rictor, Akt, sgk1, and 4E-BP1

(Fig 4) in the urothelial tumors from all groups. Administration of rapamycin and CP, either alone or in combination, inhibited expression of mTOR (Fig 4B) and p-mTOR (Fig 4A) and its components, raptor and rictor (Fig 4C). Protein levels of Akt and p-Akt were inhibited (Fig 4A). Several other molecules of the pathway, including Sgk1 and 4E-BP1, were also significantly downregulated by the treatments (Fig 4C). Although both agents produced inhibitory effects when used alone, the effect was more pronounced when the agents were administered in combination.

Suppression of tumor cell survival and angiogenesis markers

Urothelial tumors from control group mice were observed to have a high expression of cell survival and angiogenesis-regulating molecules, such as hif1 α , vegf, and AR (Fig 4A and 5A). Administration of rapamycin or CP led to significant suppression of these pro-survival markers. Hif1 α and vegf were observed to be significantly suppressed in the treated tumors compared with the control tumors (Fig 4A, 5A, and 5B). The mRNA and protein levels of the androgen receptor that is known to play a key role in urothelial tumor growth were suppressed upon the administration of the two drugs (Fig 4A, 5A, and 5B).

Discussion

Bladder cancer poses a serious public health challenge, given the high rate of incidence and the cost for managing this disease. Muscle-invasive tumors are the most difficult to treat and pose a significant threat to life, due to their metastatic nature. Approximately 80% of human bladder cancers (BC) are non-muscle invasive when first diagnosed and are usually treated by transurethral tumor resection. But 50–80% of patients experience cancer recurrence. To prevent or delay tumor recurrence, intravesical therapy with Bacillus Calmette-Guerin (BCG), is frequently used as an adjunctive after TUR. Other agents such as mitomycin, gemcitabine, and pirarubicin are being investigated clinically (27–29). However, BCG may not be effective in all patients; have significant side effects such as infection by BCG and require urethral catheterization. Therefore there is need to identify agents for primary and secondary prevention of BC. There has been significant progress in the molecular understanding of this disease, and several key biomarkers associated with the disease progression have been identified (30). Considering the high incidence, recurrence, poor prognosis, and high mortality of muscle-invasive BC, there is a pressing need to identify and develop agents that can prevent urothelial cancer incidence, tumor growth, progression, and possible recurrence.

Given the number of cases diagnosed at non-invasive stages and the availability of molecular data, there is ample opportunity to intervene and prevent tumor progression to the lethal invasive form. Tumor suppressor TP53 and mTOR are recognized as important targets for bladder cancer. Therefore, several molecules that inhibit the dysregulated mTOR pathway or restore the inactivated TP53 pathway are in development and under various preclinical and clinical stages of investigation (18, 31–33). Rapamycin and CP have been shown to exert antitumor effects in various cancer models *in vitro* and *in vivo* (15, 19, 24). Since cancer cells survive by altering multiple pathways, it will be important to use a combination of agents that can modulate multiple pathways to produce better antitumor effects that can also

lower the risks of side effects associated with a high-dose single agent approach (34–35). Here, we demonstrate that dual targeting of the mTOR and p53 pathways using rapamycin in combination with CP results in inhibition of BC growth and invasion using a transgenic mice model (Fig 5C).

Altered expression/inactivation of TP53 protein is an important predictor of progression in bladder cancer. *In vivo* studies have clearly demonstrated that p53 deficiency induced higher incidence and aggressive growth of BC (36). Further, we have previously reported that oral administration of the p53 modulator CP-31398 alone can have significant effects on urothelial tumor growth and invasion (24). However, the effects were profound only when CP was used at a higher dose (300 ppm). There was also a significant difference between the sexes, suggesting a particular lack of inhibition of tumor invasion in males, in which the tumor growth was more aggressive. These results suggest a need for either a significantly higher single dose of CP or the inhibition of additional pro-tumorigenic pathways, since targeting a single pathway was insufficient to suppress BC invasion. Therefore, to avoid the risk of unwanted side effects associated with high doses and to develop an effective combination, we used the low dose (150 ppm) of CP combined with an mTOR inhibitor and compared the effects. Interestingly, we found that the combination of CP and rapamycin had a profound effect on tumor growth and progression, compared with the effects of these agents when administered individually.

The incidence of bladder cancer is approximately three fold higher in men than in women. One reason for this difference could be the high prevalence of smoking among men; smoking is linked to cancer, causing p53 mutations (37). In addition, sex hormone signals could also play a role in bladder tumorigenesis, as suggested by several preclinical studies (21, 38–39). The importance of androgen signaling in regulating BC development was elucidated using castrated and AR knockout (ARKO) mouse models (21, 38–39). Therefore in the present study we see a decrease in AR expression as a result of p53 induction that negatively regulates AR (40) as well as a result of rapamycin-induced inhibition of protein synthesis. However, the effect was even more profound in combination. Increasing the transcriptionally active p53, by either enhancing the stability of wild-type p53 or by reverting mutant p53 to its wild-type conformation, with its ability to block cell cycle progression and induce apoptosis, has been considered as an important approach to cancer treatment. CP not only restores p53 functions in mutant p53-expressing cells, but can also significantly increase the protein level by blocking ubiquitination and degradation of p53, leading to cell cycle arrest (16). Consistent with previous observations, we observed increased p53 levels and a decrease in cell cycle (cyclin D1, cyclin A) and proliferation biomarkers (PCNA) in treated tumors. *Invitro* and *invivo* studies have clearly shown that CP can restore the function of mutant p53 and also induce p53 signalling in wild type p53 expressing cells (18, 41–42). Our results clearly demonstrate that administration of CP suppresses urothelial tumorigenesis that occurs through inactivation of p53. While mutation of the p53 is a frequent event, half of the tumors still have normal p53 or that might have been inactivated by alternate mechanisms such as mdm2 overexpression (43), CP with its potential to function in both p53 mutant and wildtype tumors has a broader application (18, 41–42). Although the molecular mechanism of p53 inactivation is different in SV40T mice compare to human tumors our results provide clear evidence that targeting p53 using cp can

result in significant advantage in preventing urothelial tumor growth. The combination of CP and rapamycin will have promising application clinically after validation using other BC mouse models carrying p53 mutations.

mTOR plays a central role as a regulator of growth, integrating diverse signals of growth factors, nutrients, and energy sufficiency. Components of the PI3K/Akt/mTOR pathway are frequently mutated or altered in BC, leading to hyperactive mTOR signaling. Such alterations contribute to dysregulated cell proliferation and angiogenesis, in part through enhanced protein synthesis rates. Therefore, the mTOR pathway has become an important target for BC, and several components of this pathway are being explored as novel therapeutic targets (6, 8, 10, 44). Clinically, mTOR inhibitor rapamycin is already part of an anticancer regimen for renal cell carcinoma, and has been reported to reduce the incidence of post-transplantation malignancies, including urothelial carcinoma (45). Further, rapamycin was shown to have better anticancer effects when administered early (46), supporting its use in intervention studies. In the present study, we found that rapamycin administration, individually or in combination with CP, led to significant inhibition of mTOR signaling, as demonstrated by the downregulation of several key pathway components. The downregulation of rictor and 4E-BP1 are particularly significant in view of their association with BC invasion and prognosis. 4E-BP1 was found to correlate with the prognosis of MIBC, and its inactivation using specific inhibitors could be a novel approach for treating MIBC (44). Rictor has been shown to play a vital role in invasive tumor growth and is considered a critical determinant of BC invasion (47). While mTORC1 is considered to be a direct target for rapamycin, other studies have found that prolonged administration of rapamycin will also lead to mTORC2 inhibition (48). More importantly, the downstream effectors of the mTOR pathway, such as Vegf and hif1 α , which contribute to angiogenesis and tumor cell survival were also suppressed, leading to significant tumor inhibition following the combination treatment.

Urothelial tumors, with their corresponding multiple altered genes and pathways can negate a drug's effect or escape from treatment by bypassing the factor that is targeted by a specific inhibitor. Therefore, simultaneous inhibition of multiple tumor cell pathways may result in more effective inhibition (34). Overall, our study clearly demonstrates for the first time that the combination of mTOR and p53 modulators has better inhibitory effects on urothelial tumor growth and invasion than the individual treatments, and supports the use of a combinational approach for better efficacy and management of cancers (34, 49–50). Importantly simultaneous targeting of mTOR and p53 pathways could be novel choice of treatment option for patients with non-muscle-invasive bladder cancer in the post-operative prevention setting with high risk for disease recurrence and progression to MIBC. However the results need to be validated using other preclinical models.

Conclusion

In summary, muscle invasive urothelial carcinomas can be prevented if intervention occurs early by targeting appropriate molecules that play key roles in this process. Our results suggest that the combination of targeting p53 signaling along with the mTOR pathway may serve as a potential approach for inhibiting invasive urothelial cancers. It would be important

to investigate rapamycin in combination with other molecules that restore p53 signaling by inhibiting the alternative p53-inactivating mechanisms. Similarly a combination of p53 modulators with other key pathways, such as inflammation or polyamine biosynthesis, may also be worth investigating for better treatment efficacy and disease management.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
A. Experimental design for study of the tumor efficacy of rapamycin, CP, and a combination in UPII-SV40T transgenic mice. At 6 weeks of age, groups (15/group UPII-SV40T or 6/group WT) of male mice were fed experimental diets containing rapamycin (8ppm or 16 ppm) and/or CP (150 ppm) continuously for 34 weeks. Bladders were collected during necropsy and weighed and analyzed for histopathology and expression of various markers. B. Body weights (g; Mean ± SEM), Liver to BW ratio (mg/g; Mean ± SEM) and Spleen to W ratios (mg/g; Mean ± SEM) of the forty-week-old male transgenic mice fed control or

experimental diets. (Con: Control; RL: Rapamycin 8ppm; RH: Rapamycin 16ppm; CP: CP-31398 150ppm; RL+CP: Rapamycin 8 ppm + CP-31398 150 ppm)

C. Comparison of the bladder weights of wild-type and UPII-SV40T transgenic mice.

Weights of the urinary bladders in transgenic mice increased by several folds due to incidence of TCC. MRI images showing the presence of large tumor inside the UPII-SV40T mice UB (inset showing UB with tumor). H&E staining showing representative normal urothelium in the wild type mice and TCC in UPII-SV40T transgenic mice.

Figure 2.

A. Tumor growth inhibitory effects of orally administrated rapamycin, CP singly or their combination in male transgenic mice. There was a significant inhibition of urothelial tumor growth in the treatment groups compared with the control-diet-fed mice.

B. Effect of rapamycin, CP and their combination with CP) on incidence of invasive TCC in male transgenic mice. A significant reduction of percentage of mice with invasive urothelial tumors was observed in the treatment groups compared with the controls. (Con: Control; RL: Rapamycin 8ppm; RH: Rapamycin 16ppm; CP: CP-31398 150ppm; RL+CP: Rapamycin 8 ppm + CP-31398 150 ppm)

Figure 3.

A. Tumor cell proliferation was significantly suppressed by the administered drugs. Relative mRNA expression of proliferation and cell cycle markers such as pcna and cyclin-D1 was analyzed using real-time PCR and found to be inhibited while p53 was increased. (*= $p<0.05$; **= $p<0.005$; ***= $p<0.0005$). (Con: Control; RL: Rapamycin 8ppm; CP: CP-31398 150ppm; RL+CP: Rapamycin 8 ppm + CP-31398 150 ppm)

B. Immuno-histochemical analysis performed on microsectioned urothelial tumor tissues also showed a significant decrease in the expression of pcna, cyclin A and Annexin-V in the treatment groups compared with control.

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Figure 4.

A. mTOR signaling was suppressed in the treated tumors. Administration of rapamycin, CP, or their combination led to significant down regulation of several proteins (pcna, Akt, p-Akt, p-mTOR, hif1 α , AR) in urothelial tumors from the treatment group. Protein levels were analyzed using western blotting.

B. Immuno-histochemical detection of mTOR expression in the urothelial tumors of mice fed control and experimental diets (rapamycin, CP, and their combination). A significant inhibition of the protein expression was observed upon treatments.

C. Urothelial tumors of the transgenic mice treated with rapamycin, CP, or their combination showed a significant suppression in the expression of mTOR pathway components: rictor, raptor, Sgk1, and 4E-BP1. Gene expression analysis was performed using real-time PCR and was normalized to Gapdh. (*= $p<0.05$; **= $p<0.005$; ***= $p<0.0005$) (Con: Control; RL: Rapamycin 8ppm; CP: CP-31398 150ppm; RL+CP: Rapamycin 8 ppm + CP-31398 150 ppm)

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Figure 5.

A. Tumor cell survival and invasion related proteins were suppressed upon drug treatment. Immuno-histochemical analysis performed on microsectioned urothelial tumor tissues showed a significant decrease in the expression of hif1α and AR in the treatment groups compared with control.

B. Administration of rapamycin, CP, and their combination led to a significant decrease in expression levels of hif1α, vegf, and AR. Relative mRNA expression was analyzed using

real-time PCR. (*= $p<0.05$; **= $p<0.005$; ***= $p<0.0005$) (Con: Control; RL: Rapamycin 8ppm; CP: CP-31398 150ppm; RL+CP: Rapamycin 8 ppm + CP-31398 150 ppm
C. Schematic representation of the molecular mechanism leading to the inhibition of urothelial tumor growth upon treatment with rapamycin and CP.