Chemoprevention of Urothelial Cell Carcinoma Growth and Invasion by the Dual COX–LOX Inhibitor Licofelone in UPII-SV40T Transgenic Mice

Venkateshwar Madka1, Altaf Mohammed1, Qian Li1, Yuting Zhang1, Jagan M.R. Patlolla1, Laura Biddick1, Stan Lightfoot1, Xue-Ru Wu2, Vernon Steele3, Levy Kopelovich3, and Chinthalapally V. Rao1

Abstract

Epidemiologic and clinical data suggest that use of anti-inflammatory agents is associated with reduced risk for bladder cancer. We determined the chemopreventive efficacy of licofelone, a dual COX–lipoxigenase (LOX) inhibitor, in a transgenic UPII-SV40T mouse model of urothelial transitional cell carcinoma (TCC). After genotyping, six-week-old UPII-SV40T mice (n = 30/group) were fed control (AIN-76A) or experimental diets containing 150 or 300 ppm licofelone for 34 weeks. At 40 weeks of age, all mice were euthanized, and urinary bladders were collected to determine urothelial tumor weights and to evaluate histopathology. Results showed that bladders of the transgenic mice fed control diet weighed 3 to 5-fold more than did those of the wild-type mice due to urothelial tumor growth. However, treatment of transgenic mice with licofelone led to a significant, dose-dependent inhibition of the urothelial tumor growth (by 68.6%–80.2%, P < 0.0001 in males; by 36.9%–55.3%, P < 0.0001 in females) compared with the control group. The licofelone diet led to the development of significantly fewer invasive tumors in these transgenic mice. Urothelial tumor progression to invasive TCC was inhibited in both male (up to 50%; P < 0.01) and female mice (41%–44%; P < 0.003). Urothelial tumors of the licofelone-fed mice showed an increase in apoptosis (p53, p21, Bax, and caspase3) with a decrease in proliferation, inflammation, and angiogenesis markers (proliferating cell nuclear antigen, COX-2, 5-LOX, prostaglandin E synthase 1, FLAP, and VEGF). These results suggest that licofelone can serve as potential chemopreventive for bladder TCC. Cancer Prev Res; 7(7); 1–9. ©2014 AACR.

Introduction

Urinary bladder cancer is one of the common cancers worldwide, with the highest incidence rates in industrialized countries. In United States, it is the second most frequently diagnosed genitourinary cancer, with an estimated 563,640 people currently living with bladder cancer. In 2014, about 74,690 new cases of bladder cancer were anticipated (about 56,390 in men and 18,300 in women), and approximately 15,580 are expected to die of this cancer (1). The majority (90%–95%) of bladder cancers are transitional cell carcinomas (TCC). Of these, 70% to 75% are non-invasive, low-grade, papillary TCC, whereas up to 30% of all diagnosed tumors are classified as invasive TCCs, which pose a very high risk for distant metastases (2). Chemoprevention has been applied to many different tumor types, including TCC of the bladder, and has been proposed as a management strategy for patients with cancer at risk of developing second primary tumors (3). Inflammation is a known risk factor for many cancers, particularly epithelial cancers such as those of the urothelium. It plays important roles at different stages of tumor development, including initiation, promotion, invasion, and metastasis (4). Chronic inflammation is associated directly with muscle-invasive bladder cancer, particularly that due to urinary tract infection in invasive squamous cell carcinoma (5).

The metabolism of arachidonic acid by COX-2 and 5-lipoxygenase (5-LOX) generates prostaglandins and hydroxyeicosatetraenoic acids, respectively, that mediate a wide variety of physiologic processes, including cell proliferation, immune surveillance, cell differentiation, and inflammation. Clinically, aberrant expression of the COX-2 and 5-LOX enzymes has been observed in urothelial tumors and found to be associated with poor prognosis (6, 7). Concentrations of a urinary PGE metabolite (PGE-M) and leukotriene E4 (LTE4), biomarkers of the COX-2 and 5-LOX pathways, are elevated in smokers (8). In view of the recognized link between smoking, inflammation, and bladder cancer, targeting the inflammatory pathways seems to...
be an ideal approach to reduce the risk of urothelial cancer development. Several preclinical studies have suggested that the combination of a selective COX-2 inhibitor and a 5-LOX inhibitor is more effective than either agent alone in preventing cancer cell growth or tumor formation (9, 10). Dual inhibitors that block both COX and 5-Lox may provide synergistic anti-inflammatory effects and also decrease gastrointestinal side effects associated with NSAIDs (11) and select COX-2 inhibitors.

Licofelone is one such compound that is currently in phase III clinical trials for treatment of pain and inflammation associated with osteoarthritis (12, 13) and also has been shown to prevent colon (14, 15), lung (16), and prostate cancers (17). However, no studies have been reported on the chemopreventive effects of licofelone in urothelial cancer. Among the preclinical models for urothelial cancer, UPII-SV40T transgenic mice develop invasive urothelial tumors due to the expression of Simian virus large T antigen (Tag) driven by the urothelium-specific uropodlin II (UPII) promoter (18). These mice have been used as a model to elucidate the mechanism underlying urothelial tumorigenesis (19, 20) as well as in chemoprevention studies (21, 22). In the present study, we determined the chemopreventive efficacy of licofelone using the UPII-SV40T transgenic mouse model for invasive urothelial TCC.

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. 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 (18) were used. The required numbers of UPII-SV40T mice per group and at least in replicate tumor samples and at least in triplicate.

Breeding and genotyping

All mice were bred and genotyped as described earlier (22). 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 PCR. PCR for the SV40T gene was done using the primer 5'-CTTTGGAGCCTCCTTG-GATGCCA-3' (sense) and 5'-GCATGCATCAAAAACGTAGCAAATTCTGG-3' (antisense) and amplifying 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 seconds, and 72°C for 45 seconds. The PCR products, when separated on a 2% agarose gel, showed a 550-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 = 30 UPII-SV40T mice per group and n = 12 wild-type mice per group) and were fed a modified AIN-76A diet for 1 week. At 6 weeks of age, mice were fed control or experimental diets containing 0, 150, or 300 ppm licofelone (Fig. 1B) until termination of the study. Mice were checked routinely for signs of weight loss, toxicity, or any abnormalities. Previously, we have established maximal tolerated doses/optimal dose of licofelone in mice and found to be ≥500 ppm fed in the modified AIN-76A diet (15). The food intake and body weight of each animal were measured once weekly for the first 6 weeks and then once a month until termination. After 34 weeks on experimental diets (i.e., 40 weeks age), all mice were euthanized by CO2 asphyxiation and necropsied, and urinary bladders were collected and weighed to determine the tumor weight. A portion of the urinary bladder was fixed in 10% neutral-buffered formalin for histopathologic evaluation, and the rest was snap frozen in liquid nitrogen for further analysis.

Tissue processing and histologic analysis

Formalin-fixed, paraffin-embedded tissues were sectioned (4-μm) and stained with hematoxylin and eosin (H&E). Sections of each urothelial tumor were evaluated histologically by a pathologist blinded to the experimental groups. Carcinomas were classified into noninvasive CIS, invasive carcinomas (lamina propria invasive and muscularis propria invasive) types according to histopathologic criteria as previously described (22).

RT-PCR

Total RNA from urothelial tumor samples of male mice was extracted using the Totally RNA Kit as per the manufacturer’s instructions. Equal quantities of DNA-free RNA were used in reverse transcription reactions for making cDNA using SuperScript reverse transcriptase (Invitrogen). RT-PCR reactions were done for proliferating cell nuclear antigen (PCNA), p53, Bax, caspase 3, Prostaglandin E Synthase 1 (mPGES1), FLAP, VEGF, p16, and actin using SYBR green and specific primers (Table 1). Relative gene expression was calculated using the 2^{-ΔΔCT} formula (23). All experiments were performed using replicated tumor samples and at least in triplicate.
Immunohistochemistry

Modulatory effects of licofelone on the expression of COX-2, 5-LOX, and p21 were evaluated by immunohistochemistry as described previously (22). Briefly, sections of paraffin-embedded tissues were deparaffinized in xylene, rehydrated through graded ethanol solutions, and washed in 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 mAbs against COX-2, 5-LOX, and p21 (Santa Cruz Biotechnology). After several washes with PBS, they were incubated with appropriate secondary antibodies for 2 hours, 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, then counter stained with hematoxylin. Nonimmune rabbit immunoglobulins were substituted for primary antibodies as negative controls. Specimens were observed using an Olympus microscope IX71, and digital computer images were recorded with an Olympus DP70 camera.

Western blotting

Proteins (60 μg) in lysates from bladders of control and licofelone-treated mice were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked with 5% nonfat milk (Biorad) in TBS and incubated with antibodies for PCNA, p53 cyclin E, and caspase 3 (Santa Cruz Biotechnology). After several washes with TBS, the membranes were incubated with secondary antibody (either goat anti-mouse or goat anti-rabbit conjugated with horseradish peroxidase; 1:10,000 dilution) for 1 hour. Protein was detected on BioMax MR film (Kodak) using chemiluminescence (Super Signal; Pierce Biotechnology). Equal protein loading was confirmed by detection of tubulin. Selected blots were quantified using Image J software.

Statistical analysis

The data are presented as mean ± SE. Differences in body weights were analyzed by ANOVA. Statistical differences between urothelial tumor weights in the control and treated groups were evaluated using unpaired t test with Welch correction. Tumor incidences (percentage of mice with urothelial tumors) were analyzed by the Fisher 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 of the transgenic and wild-type mice fed control and licofelone-containing modified AIN76A diets were weighed weekly and monitored throughout the study. Gross anatomy of wild-type and transgenic mice revealed no evidence of any abnormality in organ size, or changes in appearance of

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Figure 1. A, experimental design for study of the chemopreventive efficacy of licofelone in UPII-SV40T transgenic mice. At 6 weeks of age, groups (30/group UPII-SV40T or 12/group WT) of mice were fed experimental diets containing 0, 150, or 300 ppm licofelone continuously for 34 weeks, and bladders from each mouse were evaluated histopathologically and analyzed for expression of various markers as described in the text. B, structure of licofelone, a dual COX–LOX inhibitor. C, H&E staining showing representative normal urothelium in the wild-type mice and invasive high-grade transitional cell carcinoma in UPII-SV40T transgenic mice. Inset, representative urinary bladders from wild-type and transgenic mice. D, expression of SV40T, PCNA, COX-2, 5-LOX, VEGF, and β-actin as analyzed by RT-PCR.
liver, spleen, heart, lung, seminal vesicles, testis, ovaries, or prostate. Thus, doses applied in the efficacy studies were expected to be nontoxic.

Urothelial tumor growth is inhibited by licofelone in transgenic mice

UPII-SV40T mice spontaneously develop urothelial tumors, as result of which there is a significant increase in urinary bladder weights compared with wild type. At 40 weeks age, these tumors are histopathologically classified as high-grade tumors invading both lamina propria and muscularis while wild-type bladders show normal urothelium (Fig. 1C). These tumors showed an overexpression of the PCNA, COX-2, 5-LOX, and VEGF compared with that of the normal urothelium from wild-type mice (Fig. 1D).

At the end of the experiment, no significant differences in body weights were observed (Fig. 2A and B). A chemopreventive effect of dietary licofelone administered at 150 or 300 ppm was found on urothelial tumor growth. Male and female UPII-SV40T mice fed control diet had urothelial tumors that weighed an average of 112.9 mg; 35% to 49% less at both doses, respectively (12.6 \( < \) 0.0001) compared with tumors of control mice due to significant inhibition of tumor growth (Fig. 2C). A similar significant inhibition of tumor growth (Fig. 2D) was observed in female UPII-SV40T mice fed control diet weighed an average of 9.8 mg and 19.3 \( < \) 0.0001; Fig. 2F).

Antiproliferative and antiangiogenic effects of licofelone

Urothelial tumors of the transgenic mice were found to have significant overexpression of the proliferation and angiogenesis markers PCNA and VEGF (Fig. 1D) compared with those in the normal urothelium of bladders from wild-type mice. As a result of licofelone administration, 12% to 50% \( P < 0.01 \) of the male mice had tumors that were classified as high-grade, noninvasive CIS (Fig. 2E). High-dose licofelone was found to have better effect on tumor invasion compared with the lower dose. In the female group, both doses led to prevention of tumor invasion in 41% to 44% of the mice \( P < 0.003 \); Fig. 2F).

Licofelone induces proapoptotic molecules in urothelium

Molecular effects of licofelone were determined by analyzing expression of mRNA and protein for various proapoptotic/antitumor molecules. RT-PCR analysis of the mRNA showed a significant increase in the levels of the tumor suppressor protein p53 in tumors from TCC to high-grade invasive TCC. To determine the effect of licofelone on this progression, formalin-fixed bladders from the control and licofelone-fed transgenic mice were sectioned and stained with H&E and their histopathology was compared. Bladders from the transgenic mice fed control diet had high-grade, muscle-invasive TCC at 40 weeks of age. However, licofelone-fed mice showed significant inhibition of invasive tumors in both male and female groups. As a result of licofelone administration, 12% to 50% \( P < 0.01 \) of the male mice had tumors that were classified as high-grade, noninvasive CIS (Fig. 2E). High-dose licofelone was found to have better effect on tumor invasion compared with the lower dose. In the female group, both doses led to prevention of tumor invasion in 41% to 44% of the mice \( P < 0.003 \); Fig. 2F).

Table 1. List of primers used for RT-PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCNA</td>
<td>5′-TAAGAAGAGGAGGCGCTTAA-3′</td>
<td>5′-TAAGTGTCCCATGCAGCAA-3′</td>
</tr>
<tr>
<td>p53</td>
<td>5′-TGAAACGCGCCTATCTTAA-3′</td>
<td>5′-GGCAAAACAGCACCTCAA-3′</td>
</tr>
<tr>
<td>Bax</td>
<td>5′-CAGGATGCGCTCACCAAGAA-3′</td>
<td>5′-GCAAAGTAGAAGGGCAGCAA-3′</td>
</tr>
<tr>
<td>p16</td>
<td>5′-AGTCGCCGTCGACAGACCTG-3′</td>
<td>5′-CGGGGAAAGTGTGGTGGTC-3′</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>5′-AGACCTTTTGTGTTGTAGTTA-3′</td>
<td>5′-AGCTTGCGCTTCCAGTCAGAC-3′</td>
</tr>
<tr>
<td>Cox-2</td>
<td>5′-GAACCTTTGCACAGCCTTAC-3′</td>
<td>5′-GCCGCGGCAAAAGGTTA-3′</td>
</tr>
<tr>
<td>5-LOX</td>
<td>5′-GGGAACGATGAGGACAGCTACTAC-3′</td>
<td>5′-TCCAAGGGCACAAGGTTA-3′</td>
</tr>
<tr>
<td>mPGES1</td>
<td>5′-GCCGGAATGATGTAGACTCTGTT-3′</td>
<td>5′-GTTGAGCCTCTCAGCAGTTC-3′</td>
</tr>
<tr>
<td>FLAP</td>
<td>5′-GGAGATTCCTCGAGGAGCATCTT-3′</td>
<td>5′-GCGGATTAGCACAGATAAGAA-3′</td>
</tr>
<tr>
<td>VEGF</td>
<td>5′-GCAGCTTTGCGGACAGGGCTTTAC-3′</td>
<td>5′-GCAAATCTCGAGAAGGCTTCT-3′</td>
</tr>
<tr>
<td>SV40T</td>
<td>5′-GCAGCTTAACTGGACCTTCTAGG-3′</td>
<td>5′-GGGGGTGGTGAAGGCTTCAA-3′</td>
</tr>
<tr>
<td>Actin</td>
<td>5′-AGATCTGGCACACACCCCTTC-3′</td>
<td>5′-AGGAGGAGGAGAAGGCTTCAA-3′</td>
</tr>
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licofelone-fed mice (Fig. 3A). Similarly, p53 protein was induced by licofelone in the treated group as determined by immunoblotting (Fig. 3B). The proapoptotic molecules Bax (Fig. 3A) and caspase 3 (Fig. 3A and B) were also induced by the licofelone treatment, suggesting that licofelone may lead to inhibition of tumor growth by inducing apoptosis. Licofelone treatment also led to an increase in p21 expression (Fig. 4A).

Licofelone inhibits expression of proinflammatory COX-2 and 5-LOX molecules

The effect of licofelone on expression of the proinflammatory molecules COX-2, 5-LOX, mPGES-1, and FLAP was determined. Urothelial tumors from the transgenic mice fed control diet showed a significant overexpression of COX-2 and 5-LOX (Figs. 1D and 4A) compared with normal urothelium. Licofelone had an inhibitory effect on both of these proinflammatory molecules (Fig. 4A). Similarly, there was significant downregulation of mPGES1 (Fig. 4B) and FLAP (Fig. 4B) that can contribute to proinflammatory effects.

Discussion

Clinically, 75% to 85% of patients with bladder cancer present with nonmuscle-invasive bladder cancer (NMIBC) that is confined to the mucosa (stage Ta, CIS) or submucosa (stage T1). These patients can be identified based on hematuria, the most common finding in NMIBC and cytology of urine. If untreated, approximately 54% of patients with CIS progress to muscle-invasive disease; even in treated patients, these tumors often recur and some progress to invasive or metastatic carcinoma, for which the treatment options become limited. Hence, there is a need to develop targeted regimens that will prevent disease progression, improve response to treatment, and also prevent recurrence. Inflammation of the bladder due to infectious as well as noninfectious etiologies such as medication, radiation, foreign bodies, chemicals, and autoimmune responses affects the bladder function directly and also is a potential risk factor for urothelial cancer development. Inflammatory pathways thus have been considered important targets for bladder cancer prevention as well as to increase the response to chemotherapy (24).
Prominent expression of COX-2 and 5-LOX enzymes has been described in bladder cancers, and expression of these proteins correlates with tumor grade and invasion (6, 7, 25–27). Overexpression of COX-2 and 5-LOX enzyme metabolites has been reported in smokers, who represent a high-risk population for bladder cancer (8). We found the urothelial tumors of the UPII-SV40T transgenic mice to have elevated levels of these enzymes (COX-2, 5-LOX) compared with normal tissue (Figs. 1D and 4A). Hence, this transgenic mouse was an appropriate model to test the effect of anti-inflammatory agents targeting these enzymes.

COX-2 inhibitors such as nimesulide (28) and celecoxib (29), also traditional NSAIDs such as indomethacin (30) and ibuprofen (31), have been demonstrated to prevent bladder cancer in vitro and in vivo models. Sabichi and colleagues (32) did not observe a clinical benefit of celecoxib in preventing recurrence of NMIBC in a randomized controlled trial, although, celecoxib had a marginally significant effect on reducing metastatic recurrences compared with placebo. However, nimesulide was capable of preventing bladder tumor recurrences in a dose-dependent manner (28). Similarly, targeting 5-LOX using the specific inhibitor AA861 caused a dose-dependent inhibition of bladder cancer cell growth (25). While targeting either of these enzymes has shown inhibitory effects, it has been found that COX-2 inhibition includes shunting of arachidonic acid into the 5-LOX pathway leading to increased risk for cardiovascular disease (8). The striking interrelationship of COX-2 and 5-LOX biologic functions suggests that drugs that are able to block both COX-2 and 5-LOX pathways may provide a better approach to bladder cancer prevention.

Licofelone is a promising novel dual COX–LOX inhibitor with proven chemopreventive efficacy in several cancer models both in vitro and in vivo. Here, we have shown that licofelone has a significant inhibitory effect on urothelial tumour growth and invasion in both male and female mice in a dose-dependant manner. Licofelone was able to significantly prevent tumor invasion in the female mice even at a lower dose compared with that male. This is particularly interesting in view of the differences in the risk for urothelial cancer incidence between these two sexes. Also, hormonal factors are known to play an important role in bladder...
in vivo. Similar modulation of genes that affect cell proliferation and apoptosis of urothelial tumors to invasive disease and pathways by licofelone is associated with prevention of this disease. Here, we demonstrate that suppression of inflammatory genes associated with the cell cycle and proliferation (34) increased expression of p21 was seen in the treatment groups. B, effect of licofelone on expression of mPGES1 and FLAP analyzed by RT-PCR.

Figure 4. A, effect of licofelone on p21, COX-2, and 5-LOX expression in urothelial tumors. Immunohistochemical analysis was performed with paraffin-embedded and microsectioned bladder tissues as described in Materials and Methods. A significant decrease in COX-2 and 5-LOX expression with an increased expression of p21 was observed in the treatment groups. B, effect of licofelone on expression of mPGES1 and FLAP analyzed by RT-PCR.

cancer, with androgen (20, 33) having significant influence on tumor growth. The differences in sex hormonal levels between the sexes may explain some of the excess risk observed in males, kinetics of tumor growth, and also their response to drugs. From the present study as well as the etiological observations, it seems that strong interventions or combinational strategies may result in effective prevention of the disease in the males due to their association with higher risk. We found that licofelone suppressed proliferation markers such as PCNA while inducing proapoptotic markers such as p53, Bax, and caspase 3. Licofelone also showed inhibitory effects on expression of the inflammation-related enzymes COX-2 and 5-LOX in the urothelial tumors. Previous studies have shown that transgenic mice overexpressing COX-2 specifically in the urothelium exhibit a high incidence of transitional cell hyperplasia in the bladder with a proportion of lesions progressing to invasive carcinoma in conjunction with significant amplification of genes associated with the cell cycle and proliferation (34). Here, we demonstrate that suppression of inflammatory pathways by licofelone is associated with prevention of this progression of urothelial tumors to invasive disease and modulation of genes that affect cell proliferation and apoptosis. Similar in vivo effects of licofelone have been reported in colon and lung tumor studies. Licofelone inhibited colonic tumors in a mouse colon cancer model by 72% to 100%, caused a significant reduction in small intestinal polyps (15), and provided better efficacy that helped celecoxib in suppressing tumor growth. In a benzo(a)pyrene-induced lung cancer model, licofelone inhibited tumor multiplicity by 26% with a significant decrease in tumor incidence (16).

Induction of apoptosis by licofelone has been reported in various cancer cell lines, including colon (35) and prostate (17) cancer cells. A dose-dependent inhibition of PCNA expression with an increase in apoptosis also was observed in licofelone-treated lung tumors (16) as well as colon tumors (15, 36). Similarly, dose-dependent inhibition of COX-2 and 5-LOX was observed with licofelone treatment in lung tumors (16). We found that licofelone had a modulatory effect on cell-cycle regulatory proteins like Cyclin E and p16. Although expression of oncogenic Cyclin E was inhibited in licofelone-treated tumors, expression of the tumor suppressor p16 was induced. Cyclin E is an important regulator of entry into the S phase in the mammalian cell cycle and may play an oncogenic role because it has been observed to undergo amplification in bladder and many other cancers (37). The tumor suppressor protein p16 is most frequently deleted (38) or inactivated by hypermethylation (39) in bladder cancers. Inactivation of the p53 pathway is observed in bladder cancers and in the SV40T in vitro, in vivo models. Here, licofelone-treated urothelial tissues were found to have higher levels of p53 and p21 protein compared with the untreated groups. NSAIDs are known to protect p53 tumor suppressor function by inhibition of electrophilic prostaglandin formation (40) and also exert antitumor effects in COX-independent manner by effect transcriptional factors, cell-cycle regulators like cyclins and p21 (41).

Angiogenesis plays a key role in tumor growth and metastasis. VEGF, one of the major angiogenic factors, was found to be greater in bladder cancer tissue compared with normal urothelium (Fig. 1D). It correlated with the histologic grade and the presence of carcinoma in situ. Patients with higher VEGF had a significantly shorter progression-free survival compared with those with lower levels (42). Elevated levels of VEGF and its receptors were found in bladder cancer specimens and expression correlated with...
the invasiveness (43). We observed a significant downregulation of VEGF in the urothelial tumors of licofelone-treated mice (Fig. 3B). COX-2 and VEGF function coordinately in induction of angiogenesis (44). Suppression of COX-2 by aspirin led to inhibition of the VEGF pathway, and the inhibition of the COX-2/VEGF-dependent pathway was effective in reducing tumor-associated angiogenesis, tumor growth, and tumor metastasis (45). In the present study, we observed inhibition of both COX-2 and 5-LOX along with VEGF in the licofelone-treated urothelial tumors.

Conclusions
Targeting of inflammatory pathways is an attractive strategy for prevention of urothelial cancers. In the present study, we have shown that licofelone, a dual COX/LOX inhibitor, inhibits urothelial tumor growth and prevents invasion in vivo. The tumor-inhibitory effects of licofelone correlate with effects on inflammatory and tumor biomarkers. Given the proven safety profile of licofelone, it is a promising candidate for urothelial cancer prevention and is worth considering for further development for the future clinical trials.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
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