Am J Cancer Res 2015;5(10):3030-3041
www.ajcr.us /ISSN:2156-6976/ajcr0014031

Original Article

TP53 modulating agent, CP-31398 enhances antitumor effects of ODC inhibitor in mouse model of urinary bladder transitional cell carcinoma

Venkateshwar Madka[1], Altaf Mohammed[1], Qian Li[1], Yuting Zhang[1], Gaurav Kumar[1], Stan Lightfoot[1], Xueru Wu[2], Vernon Steele[3], Levy Kopelovich[4], Chinthalapally V Rao[1]

[1]Center for Cancer Prevention and Drug Development, Stephenson Cancer Center, Department of Medicine, Hematology-Oncology Section, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA; [2]Department of Urology, NYU Medical Center, NY, USA; [3]Division of Cancer Prevention, Chemoprevention Agent Development Research Group, National Cancer Institute, Bethesda, MD, USA; [4]Weill Cornell Medical College, Cornell University, USA

Received August 5, 2015; Accepted August 20, 2015; Epub September 15, 2015; Published October 1, 2015

Abstract: Mutations of the tumor suppressor p53 and elevated levels of polyamines are known to play key roles in urothelial tumorigenesis. We investigated the inhibition of polyamines biosynthesis and the restoration of p53 signaling as a possible means of preventing muscle invasive urothelial tumors using DFMO, an ODC-inhibiting agent, and CP-31398 (CP), a p53 stabilizing agent. Transgenic UPII-SV40T male mice at 6weeks age (*n*=15/group) were fed control diet (AIN-76A) or experimental diets containing DFMO (1000 and 2000 ppm) or 150 ppm CP or both. At 40 weeks of age, all mice were euthanized and urinary bladders were evaluated to determine tumor weight and histopathology. Low-dose DFMO had a moderate significant inhibitory effect on tumor growth (38%, $P<0.02$) and tumor invasion (23%). High-dose DFMO had a 47% tumor inhibition ($P<0.0001$) and 40% inhibition tumor invasion. There was no significant difference between 1000 and 2000 ppm doses of DFMO ($P>0.05$). CP at 150 ppm alone had a strong inhibitory effect on tumor growth by 80% ($P<0.0001$); however, no effect on tumor invasion was observed. Interestingly, the combination of DFMO (1000 ppm) and CP (150 ppm) led to significant decrease in tumor weight (70%, $P<0.0001$) and tumor invasion (62.5%; $P<0.005$). Molecular analysis of the urothelial tumors suggested a modulation of polyamine biosynthesis, proliferation, cell cycle regulators resulting from the use of these agents. These results suggest that targeting two or more pathways could be an effective approach for chemoprevention. A combination of CP and DFMO appears to be a promising strategy for urothelial TCC prevention.

Keywords: Transitional cell carcinoma, UPII-SV40T, DFMO, CP-31398, ornithine decarboxylase, polyamines, chemoprevention

Introduction

Urinary bladder cancer is the second most frequently diagnosed genitourinary cancer worldwide. In the United States, 74,000 new cases & 16,000 deaths are expected in 2015 [1]. Almost 95% of all diagnosed bladder cancers are transitional cell carcinomas (TCC); one-third of these are classified as invasive TCCs, which pose a very high risk for distant metastases [2]. After surgery, 10% to 15% of the noninvasive TCCs also progress to the invasive form with recurrence, having acquired additional genetic mutations. Invasive TCC is difficult to treat and has a 5-year survival rate of only 6% in patients with distant metastases [1].

Several studies have elucidated the significance of p53 mutations and polyamines in urothelial tumors. More than half of the cases of invasive TCC have mutations in the tumor suppressor gene p53 [3, 4]. Increased polyamine concentrations in the blood and urine of patients with cancer reflect the enhanced levels of polyamine synthesis in cancer tissues. Urinary components functioning as tumor promoters have been shown to elevate ornithine decarboxylase (ODC) and the other polyamine-related enzymes [5]. Interestingly, tobacco smokers, who are at high risk of developing bladder cancers, were found to have increased levels of Arginase1 (Arg1) and ODC compared with non-smokers [6]. Further data suggest

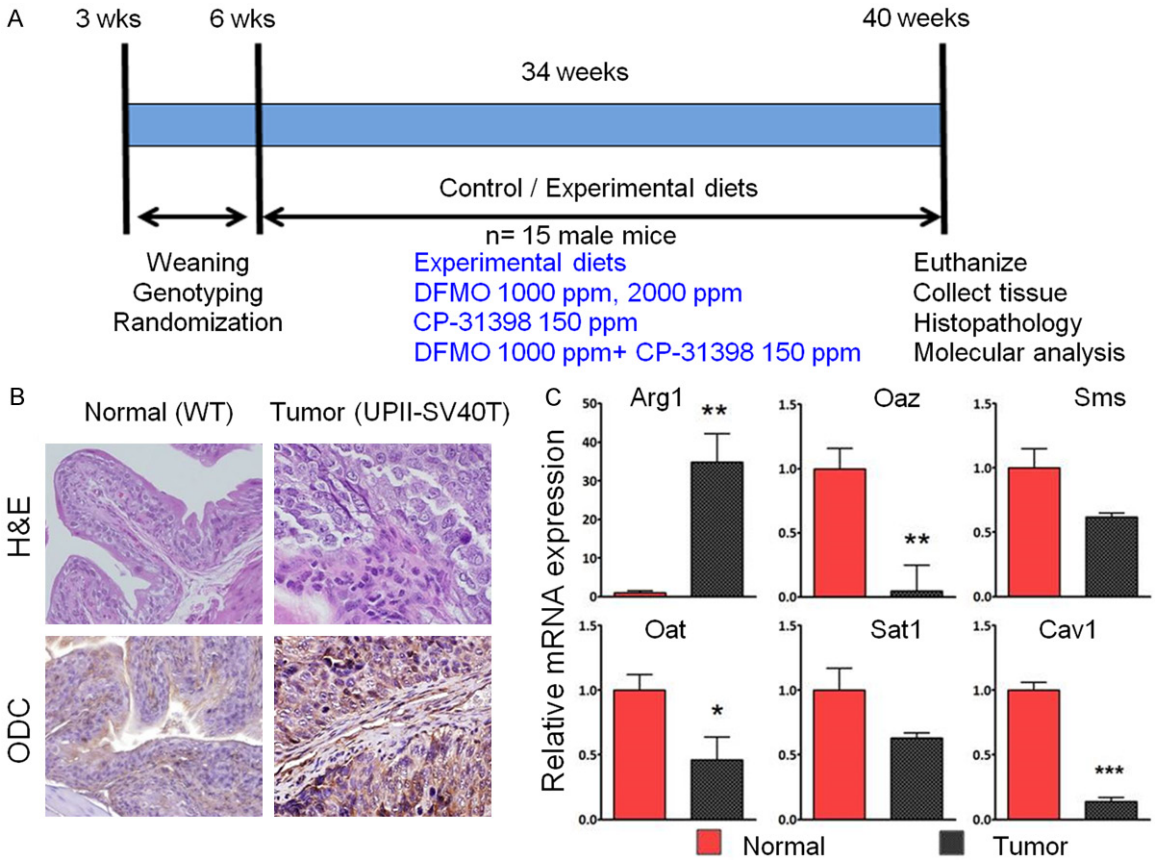

Figure 1. A. Experimental design for the study of the chemopreventive efficacy of DFMO, CP, and a combination in UPII-SV40T transgenic mice. Beginning at six weeks of age, male mice (15/group UPII-SV40T or 6/group WT) were fed experimental diets containing DFMO (1000 ppm or 2000 ppm) and/or CP (150 ppm) continuously for 34 weeks. We collected bladders from each mouse during necropsy, and weighed and analyzed the bladders for histopathology and expression of various markers, as described in the text. B. H&E staining showing representative normal urothelium in the wild-type mice and transitional cell carcinoma in UPII-SV40T transgenic mice. Immunohistochemical staining showing Ornithine decarboxylase over-expression in tumor compared to normal. C. Relative mRNA expression of Arginase (Arg1), Antizyme (Oaz), Ornithine amino transferase (Oat), and Spermidine/spermine N (1)-acetyltransferase 1 (Sat1), and Caveolin 1 (cav1) as analyzed by real-time PCR normalized to b-Actin.

that p53 mutations in tobacco-related cancers can be attributed to direct DNA damage from cigarette smoke carcinogens [7-9]. In view of the important role of ODC and p53 pathways in regulating cell growth and apoptosis, these pathways are potential targets for cancer prevention.

DFMO inhibits polyamine biosynthesis by irreversible binding to ODC. It has been shown to prevent several cancers [10-14]. Similarly, the synthetic styrylquinazoline (N'-(2-((E)-2-(4-methoxyphenyl)ethenyl)-quinazolin-4-yl)-N,N-dimethylpropane-1,3-diaminehydrochloride) CP-31398 (CP), is known to restore the DNA-binding activity of mutant p53 protein by stabilizing the conformation of the DNA-binding domain [15, 16]. Several *in vitro* and *in vivo* studies have successfully demonstrated the chemopreventive potential of this agent [17-21]. In the present study, we used a UPII-SV40T transgenic mouse model [22] to evaluate the chemopreventive effects of these two agents, either individually or in combination. These mice develop muscle invasive urothelial tumors and have been excellent tool in understanding the molecular mechanism underlying urothelial tumorigenesis [23, 24] and anticancer drug development [25-27].

Experimental section: 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). We used UPII-SV40T transgenic mice

specifically expressing a Simian Virus 40 large T antigen (SV40T) in urothelial cells under the control of the Uroplakin II (UPII) promoter and reproducibly developing high-grade carcinoma in situ (CIS) and invasive tumors urothelial tumors [27]. The required number of transgenic mice is generated by breeding as described earlier [27]. Animals were housed in ventilated cages under standardized conditions (21°C, 60% humidity, 12 h-light/12 h-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. DFMO and CP were procured from the National Cancer Institute chemoprevention drug repository. DFMO (1000 and 2000 ppm) or CP (150 ppm) were premixed with small quantities of casein and 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 [27]. 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 amplifying under the following PCR conditions: denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, 58°C for 45 sec, and 72°C for 45 sec. The PCR products, when separated on a 2% agarose gel, showed a 550 bp band.

Bioassay

Genotyped UPII-SV40T transgenic male mice were used in the efficacy study. The experimental protocol is summarized in **Figure 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, 1000, or 2000 ppm DFMO, 150 ppm CP, or a combination of 1000 ppm DFMO and 150 ppm CP until termination of the study (**Figure 1A**). 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 termination. After 34 weeks on experimental diets (i.e., at 40 weeks of age), all mice were euthanized by CO_2 asphyxiation and necropsied. We collected urinary bladders and weighed them to determine the tumor weight. A portion of the urinary bladders with tumor was fixed in 10% neutral-buffered formalin for histopathological evaluation. The remainders were snap frozen in liquid nitrogen for further analysis.

Serum profiling

Liver enzymes in serum such as Alkaline Phosphatase (ALP), Alanine transaminase (ALT), Aspartate transaminase (AST) along with Albumin, Globulin and total protein were quantified using chemistry analyzer (Catalyst, IDEXX laboratories), as per the manufacturer's instructions.

Tissue processing and histological analysis

Formalin-fixed, paraffin-embedded tissues were sectioned (4-μm) and stained with hematoxylin and eosin (H&E). Multiple sections of each urothelial tumor were evaluated histologically by a pathologist blinded to the experimental groups. Carcinomas were classified into non-invasive carcinoma in situ (CIS) and invasive carcinoma (lamina propria invasive and muscularis propria invasive) types according to histopathological criteria, as previously described [26, 27].

Real-time PCR

The total RNA from urothelial tumor samples was extracted using the Totally RNA Kit (Ambion) per the manufacturer's instructions. Equal quantities of DNA-free RNA were used in reverse transcription reactions to make cDNA using SuperScript reverse transcriptase (Invitrogen). Real-time PCR reactions were performed for arginase1 (Arg1), Ornithine amino transferase (Oat), antizyme (Oaz), Spermidine/spermine N (1)-acetyltransferase 1 (Sat1), p53, p21, p27, Caveolin-1 (Cav1), IL10, and Actin using SYBR green and specific primers (Supplementary Table 1). Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ formula [28]. All

Table 1. Effect of chemopreventive drug administration on body and organ weights

	Con	DL	CP	DL + CP
Body Weight (g)	32.4±0.7	32.9±0.5	31.0±0.5	32.8±0.5
Liver/BW (mg/g)	41.5±1.5	38.9±0.9	38.5±1.9	39.0±0.6
Kidney/BW (mg/g)	10.0±0.5	9.6±0.4	9.9±0.3	9.5±0.5
Spleen/BW (mg/g)	2.8±0.1	3.1±0.1	2.5±0.1	2.9±0.1
Testis/BW (mg/g)	2.6±0.1	2.5±0.1	2.7±0.1	2.7±0.1

The data shown are means ± SEM of 15 independent mice per group. No significant difference (P>0.05) was found between control and experimental groups. (Con: Control; DL: DFMO 1000 ppm; CP: CP-31398 150 ppm; DL+CP: DFMO 1000 ppm + CP-31398 150 ppm).

Table 2. Serum analysis of the control and treated mice

	Con	DL	CP	DL + CP
Alt (U/dL)	44.5±8.5	51.0±11.0	40.0±13.0	41.4±3.5
Alkp (U/dL)	31.5±8.5	27.0±12.0	37.0±8.0	24.0±9.0
AST (U/dL)	150±56.8	137±22.4	162±34.8	142±44.0
Alb (mg/dL)	2.5±0.4	2.3±0.2	2.4±0.1	2.3±0.1
Glob (mg/dL)	2.4±0.1	2.5±0.1	2.6±0.2	2.6±0.3
TP (mg/dL)	4.7+0.5	4.8+0.2	5.1+0.3	5.0+0.4

The data shown are means ± SEM of six independent mice per group. No significant difference (P>0.05) was found between control and experimental groups. (Con: Control; DL: DFMO 1000 ppm; CP: CP-31398 150 ppm; DL+CP: DFMO 1000 ppm + CP-31398 150 ppm).

experiments were performed at least in triplicate using replicated tumor samples.

Immunohistochemistry (IHC) and immunofluorescence (IF)

We evaluated the expression of ODC, Ki-67, Cyclin A, Cyclin E, p21, p53, and MMP2 using IHC and IF in control and DFMO treated tumor tissue. Briefly, sections of paraffin-embedded tissues were deparaffinized in xylene, rehydrated through 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 min in a boiling water bath. Endogenous peroxidase activity was quenched by incubation in 3% H_2O_2 in PBS for 5 min. Nonspecific binding sites were blocked using Protein Block for 20 min. Then, sections were incubated overnight at 4°C with 1:300 dilutions of monoclonal antibodies against ODC, Ki-67 (12202P), Cyclin A (sc-596), Cyclin E (sc-481), p53 (sc-6243), p21 (sc-817), and MMP-2 (bs-0412R) (Santa Cruz Biotechnology). Following primary antibody, sections were incubated for 2 hrs with appropriate secondary antibody, then visualized with 3,3'-diaminobenzidine (DAB) and counterstained with H&E for IHC or with DAPI for immunohistofluorescence (IHF). We observed specimens using an Olympus microscope IX71, and recorded digital computer images with an Olympus DP70 camera.

Western blotting

Proteins (60 ug) in lysates from bladders of control and treated mice were separated by 10% 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), p53 (sc-6243), p21 (sc-817), Bax (sc-493), Cyclin A (sc-596), Cav1 (sc-894), MMP-2 (bs-0412R), MMP-7 (bs-0423), and tubulin (sc-5286) 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).

Statistical analysis

The data are presented as means ± standard errors (*SE*). Differences in body weights were analyzed by Analysis of Variance (ANOVA). 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 with 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 observation

We initially performed molecular analysis of the normal and tumor-bearing urothelium from both groups of mice to compare the expression of enzymes that play important roles in polyamine biosynthesis and regulation. Immunohistochemical and qPCR analysis of the urothelial tumors showed an increase in ODC expression (**Figure 1B**), while arg1 was found to be

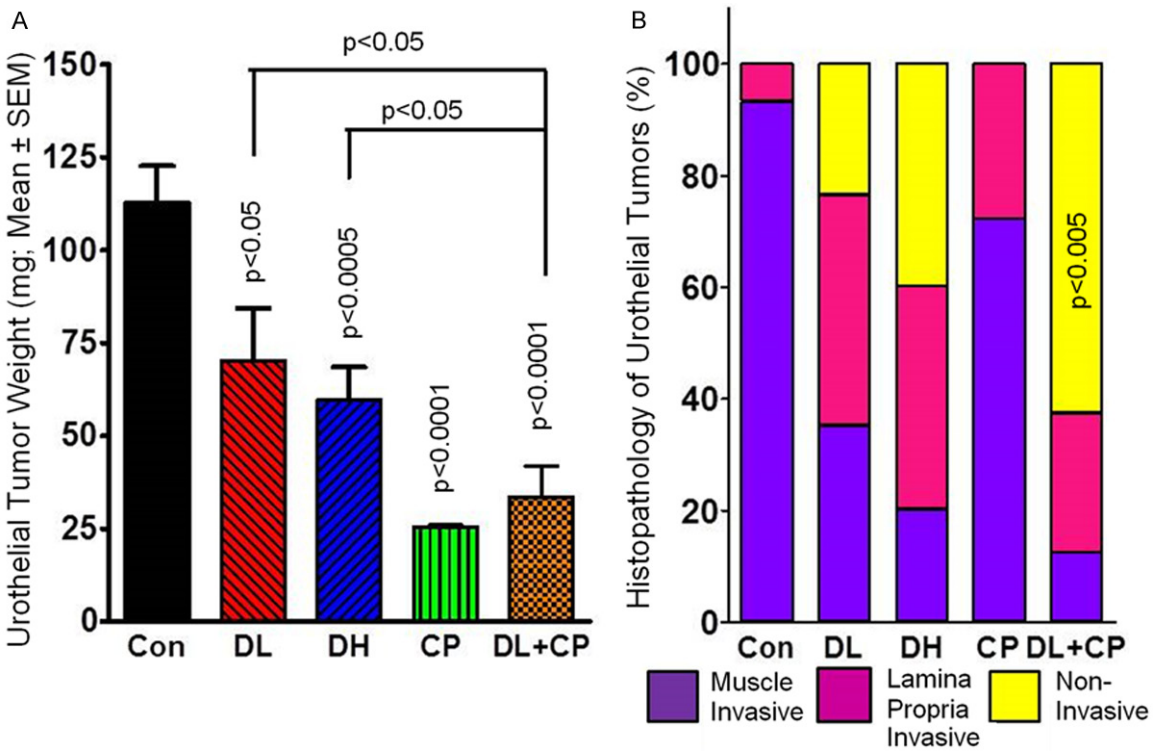

Figure 2. A. Effect of chemopreventive agents (DFMO/CP) on urothelial tumor growth was determined by comparing the weights of urothelial tumors from the control and treatment group transgenic mice. Administration of the chemopreventive agents alone or in combination led to a significant decrease in urothelial tumor weights when compared with control-diet-fed mice suggesting tumor inhibiting effect of the test agents. B. Effect of chemopreventive agents (DFMO/CP) on tumor progression was studied by comparing the incidence of invasive TCC in transgenic mice. There was a significant reduction in the percentage of mice with muscle-invasive urothelial tumors in the treatment groups compared with the controls suggesting the synergistic suppressive effect of DFMO and CP on tumor progression. (Con: Control; DL: DFMO 1000 ppm; DH: DFMO 2000 ppm; CP: CP-31398 150 ppm; DL+CP: DFMO 1000 ppm + CP-31398 150 ppm).

amplified by about 35 fold in the tumors compared with normal urothelium (**Figure 1C**). On the other hand, Oaz, Oat, and Sat1, which regulate polyamine concentrations, were significantly downregulated in the tumors compared with normal urothelium (**Figure 1C**) suggesting a significant disregulation of polyamine pathway. Effect of drug administration on mice growth was analyzed by comparing the body weights of control and experimental group. At the end of study there was no significant difference in mean body weights and organ weights between control and drug-treated animals (**Table 1**). The gross anatomy of control and experimental diet fed wild-type and transgenic mice revealed no evidence of any abnormality in size, appearance and weights of liver, spleen, testis, and other major organs such as pancreas, heart, lung, intestines. Also there was no significant difference in the liver enzyme profile in the serum of the animals fed control and experimental diets suggesting lack of overt-toxicity (**Table 2**).

Dual modulation of ODC and p53 pathways inhibits growth of muscle invasive urothelial tumors

Administration of DFMO or CP, or their combination had an inhibitory effect on urothelial tumor growth (**Figure 2A**). Urinary bladders from the control group transgenic mice developed large urothelial tumors (mean tumor wt. 112.9±9.79 mg) resulting in a drastic increase in bladder weights. However, treatment with DFMO alone at 1000 ppm or 2000 ppm led to a dose-dependent ~37-47% decrease in tumor weight (70.3±14.14 mg, $P<0.05$; 59.6±8.9, $P<0.005$ respectively), suggesting a significant inhibition of tumor growth. Similarly, treatment with 150 ppm CP led to ~80% tumor weight inhibition (22.45±6.7, $P<0.0001$). Treatment with a com-

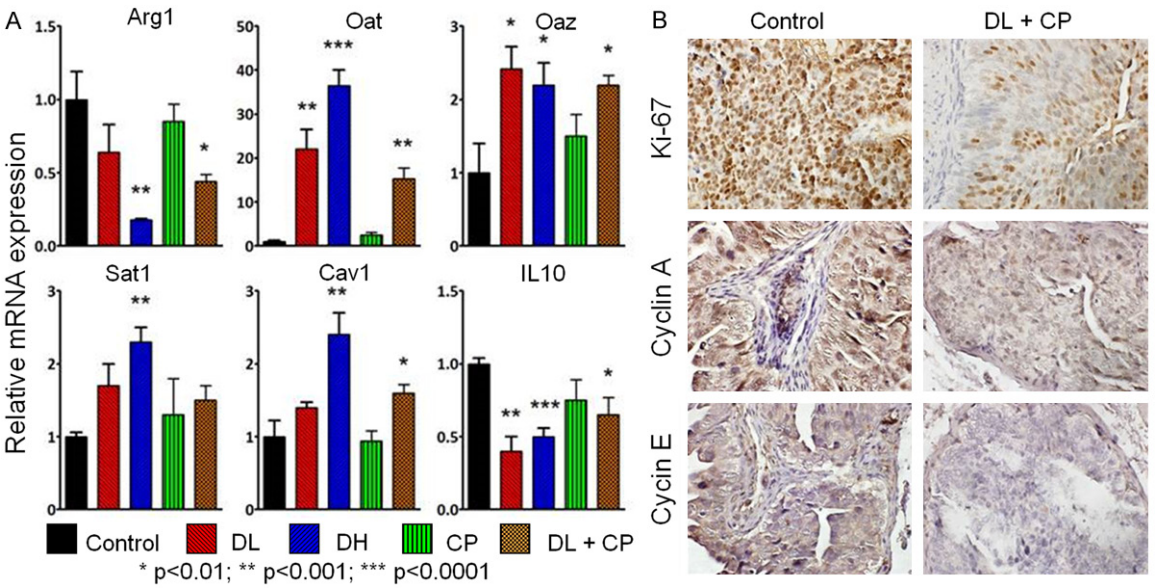

Figure 3. Co-administration of the chemopreventive agents (DFMO/CP) led to significant modulation effect on ODC pathway and Proliferation. A. Real-time PCR analysis of the ODC pathway genes in the tumors of control and treatments group mice shows altered expression of Arg1, Oat, Oaz, Sat1, Cav1, and IL10. Treatment led to a decrease in Arg1 and IL10 along with an increase in Oat, Oaz, and Sat1 mRNAs. B. Immunohistochemical analysis of the urothelial tumors from control and treatment groups showed a significant decrease in the expression of proliferation marker proteins Ki-67, Cyclin A, and Cyclin E. (Con: Control; DL: DFMO 1000 ppm; DH: DFMO 2000 ppm; CP: CP-31398 150 ppm; DL+CP: DFMO 1000 ppm + CP-31398 150 ppm).

bination of the two agents produced a 70% tumor weight inhibition (33.59±8.2, $P<0.0001$). In spite of the dose dependent increase in tumor inhibition there was no significant difference ($P>0.05$) between the inhibitory effect of the 1000 and 2000 ppm doses of DFMO when given alone. Although the tumor inhibition in the combination group was slightly less than CP alone, there was no significant difference between the two. However, there was a significant difference ($P<0.05$) in the tumor weight inhibition of the combined treatment group when compared with animals treated with either doses of DFMO (**Figure 2A**).

DFMO and CP-31398 synergize to prevent urothelial tumor invasion

Histopathology was performed on the H&E stained tumor section to evaluate the effect of DFMO and CP on tumor invasion. Urinary bladders from control had highly invasive urothelial tumors with very few invading lamina propria but most of the tumors penetrating into the muscularis propria. CP at 150 ppm led to a slight inhibition of tumors invading the muscularis however the effect was not significant to prevent the invasion to lamina propria as well. Transgenic mice receiving 1000 ppm and 2000 ppm DFMO showed strong suppression of tumor invasion in 23% and 40% of the mice. Importantly the muscle invasive tumors were significantly prevented in 63% (6/17; P<0.005) and 78% (3/15; P<0.0005) of the mice respectively (**Figure 2B**). Interestingly, treatment with the combination of 1000 ppm DFMO and 150 ppm CP an increased inhibition of tumor invasion in 62.5% of the mice (10/16, $P<0.0005$) also there was significant suppression of muscle invasive in 87% (2/16; P<0.0001) of these mice (**Figure 2B**).

Enzymes of polyamine biosynthesis are modulated

Following the administration of chemopreventive agents, we analyzed the urothelial tumors to compare the modulatory effects of the treatments on the expression of deregulated polyamine biosynthesis enzymes. Arg1 plays a critical role in polyamine biosynthesis by converting arginine to ornitine, and significantly over expressed in tumors, was found to be strongly inhibited by DFMO, as well as the treatment combination (**Figure 3A**). Oat diverts Ornitine from the polyamine biosynthesis pathway by converting it to proline, and was also found to be induced (**Figure 3A**). Antizyme (Oaz), Sat and

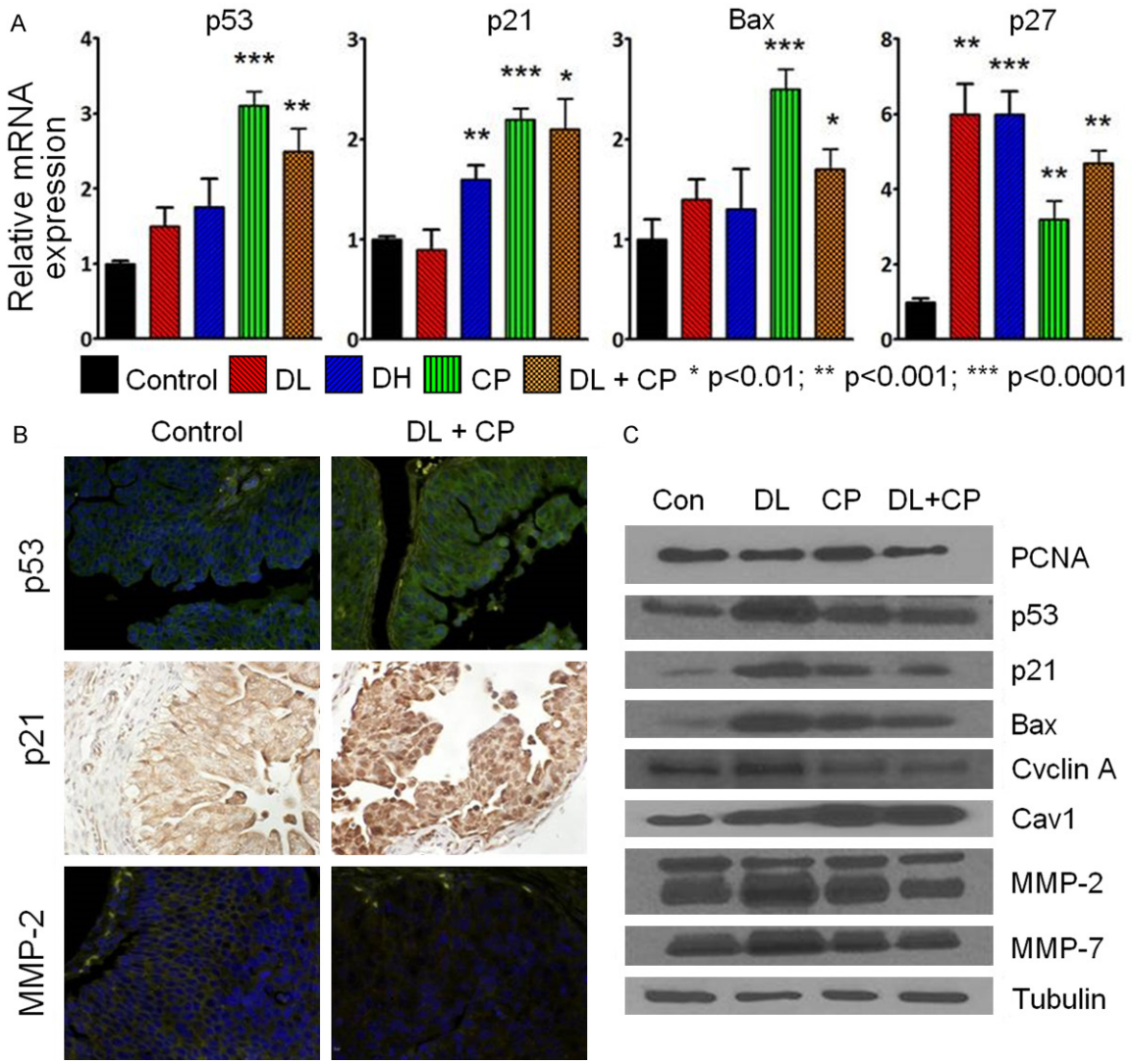

Figure 4. Co-administration of DFMO and CP had an inducing effect on p53 signaling. A. An increase in the expression of p53, p21, Bax, and p27 mRNA was observed in the treated tumor tissues compared to control. B. Similar IHC/IFC analysis showed an increase in p53, p21 and decrease in MMP-2. C. Western blot analysis revealed a decrease in protein levels of PCNA, Cyclin A, MMP-2, MMP-7 with an increase in p53, p21, Bax, and Cav1 in the chemopreventive group tumors compared to the control. (Con: Control; DL: DFMO 1000 ppm; DH: DFMO 2000 ppm; CP: CP-31398 150 ppm; DL+CP: DFMO 1000 ppm + CP-31398 150 ppm).

Cav1 that regulate polyamine concentration were induced in the groups receiving both doses of DFMO and in those mice receiving the combined treatment (**Figure 3A**). IL10 was found to be inhibited in the DFMO-treated groups (**Figure 3A**).

Proliferation is inhibited

We evaluated mRNA and protein levels of the proliferation markers in control and treated urothelial tumors (**Figures 3B**, **4A-C**). Tumors from the control group had high Ki67-positive cells; treatments with chemopreventive agents led to a significant reduction in the number of these cells (**Figure 3B**, Supplmentary Figure 1). Similarly, cell cycle regulators Cyclin A and Cyclin E were highly expressed in the control group tumors, but this expression was inhibited in the treated tumors (**Figure 3B**, Supplmentary Figure 1). Protein levels of the PCNA and Cyclin A were also suppressed in the treated tumors compared to control (**Figure 4C**). These results clearly suggest a significant inhibition of tumor cell proliferation by DFMO, CP, and the combination.

Tumor suppressor p53 was found to be significantly induced by both CP and the combination

treatment (**Figure 4A-C**, Supplmentary Figure 2). Further, there was an increase in the expression of p21, p27, and Bax mRNA (**Figure 4A** and **4B**), with significant nuclear accumulation of the p21 protein (**Figure 4B**). Expression of matrix-metalloproteinases (MMP-2 and MMP-7) that contribute to tumor invasion was an inhibited in the treated tumors (**Figure 4B**, **4C** and Supplmentary Figure 3).

Discussion

Bladder cancer (BC) is a leading global cause of cancer mortality in men. The risk of developing this cancer is three times greater in men than in women [1]. Most patients (~80%) with urinary bladder cancer are found to have non-muscle-invasive BC at the time of diagnosis; this can be effectively treated by transurethral resection (TUR). However, there is frequent recurrence (up to 70%) and this diagnosis requires lifelong, costly follow-up procedures to test and treat for tumor recurrence and progression. Currently, the standard treatment to prevent intravesical recurrence and muscle-invasive progression is intravesical instillation of bacille Calmette-Guérin (BCG), to which all patients may not respond and is associated with high rate complications from lower urinary tract symptoms to systemic reaction, or infection by BCG. As a result, human urinary bladder cancer is the most expensive cancer to treat on a per-patient basis. Therefore, our long-term goal is to develop agents that can prevent bladder cancer. In the present study we report that a combination of DFMO (ODC inhibitor) and CP (p53 stabilizing agent) had a synergistic inhibitory effect on urothelial tumor growth and progression *in vivo* in the UPII-SV40T transgenic mouse model. Epidemiological data suggest that the progression of bladder cancer is a result of a complex interplay of genetic and environmental factors [29]. Recognizing that carcinogenesis is a multistep process, prevention strategies aimed at reducing cancer deaths are being developed using compounds that can interfere with the various stages of cancer development. In view of the high incidence, tumor recurrence and mortality there is a need to identify effect regime for prevention of this cancer at every possible stage.

Polyamines are ubiquitous, low molecular weight aliphatic amines with multiple intracellular functions. They can influence gene expression, interact with membrane phospholipids, and influence ion channels. In a normal cell, polyamine levels are tightly regulated through feedback mechanisms affecting synthesis, intracellular conversion, and transport however during cancer this regulation is lost. The model in present study was found to have dysregulated ODC pathway (**Figure 1C**) similar to the dysregulation of polyamine metabolism during carcinogenesis that often leads to an increase in tissue levels of polyamines in cancer [30]. Further activated oncogenes have also been shown to increase polyamine uptake in transformed cells resulting in higher intracellular polyamine concentrations [31, 32]. Cancer cells with a greater capability to synthesize polyamines are associated with increased production of proteinases, such as serine proteinase, MMPs, cathepsins, and plasminogen activator, which can degrade surrounding tissues and contribute to invasion [33]. Recent advances in our understanding of polyamine function, metabolic regulation, and differences between normal cells and cancer cells have reinforced the interest in targeting this pathway for drug development. ODC being an important enzyme in polyamine biosynthesis inhibition of polyamine biosynthesis by ODC inhibitor, DFMO has been studied in several cancers. While DFMO is clinically approved for hirsutism and African typanosomiasis, several of the p53 restoring agents are at various stages of development. Availability of enormous information on the safety and side effects of DFMO supported by anti-tumor pre-clinical data makes these agents excellent candidates for developing into cancer prevention singly as well as a combination drug.

Previous studies have shown that inhibition of ODC activity is effective in inhibiting urothelial cancers. Using MBT-2 mouse bladder tumor cell lines, researchers found that ODC inhibition has antitumor effects *in vitro* and *in vivo* [34]. 1, 3 diaminopropane (DAP), another ODC inhibitor, was shown to inhibit OHBBN-induced urothelial tumors in rats [35]. Our results also indicated that inhibition of polyamine biosynthesis by DFMO inhibits tumor growth and invasion. We have previously demonstrated the chemopreventive effect of CP where it was found to have a moderate tumor invasion in the male mice when administered at 300 ppm [27]. It is now clear that the risk for BC development in men is higher compared to women due to smoking prevalence and hormonal factors. Although our data indicate that CP alone may provide inhibition of tumor invasion it appears

that to achieve significant effects there may be a need for much higher doses that increases the risk of unwanted toxicity. Alternatively it also appears that targeting p53 alone may not be sufficient indicating the possibility of additional mechanisms involved in tumor spread. Therefore using a low dose combination of molecular targeted agents might provide better tumor inhibitory effects with less side-effects. Interestingly here we found that the combination of CP and DFMO had a profound effect on tumor growth and progression, compared with the effects of these agents when administered individually. These findings are in accordance with similar synergistic effects of DFMO combined with other agents that have been reported in preclinical mouse models of urinary bladder [11], colon [14], and skin [12, 36] cancers. It is important to note that targeting polyamine biosynthesis along with inflammatory pathways, using a DFMO and Sulindac combination, was shown to clinically effective in preventing colon adenomas, effectively reducing both total and advanced adenomas [37].

Intracellular polyamine contents are regulated by biosynthesis, catabolism, uptake, and efflux mechanisms [38]. We found that treatment with DFMO led to a modulation of the deregulated polyamine biosynthesis enzymes involved in these processes. Inhibition of arginase expression with an increased expression of Oaz, Oat, and Sat1 has been demonstrated. Antizyme negatively regulates polyamine uptake and biosynthesis [39]. Caveolin-1 (Cav1) that potentially regulates polyamine uptake by cells is known to be downregulated in cancer cells leading to polyamine uptake [32, 40]. Similarly we found correlation with cav1 expression and tumor growth.

Tumor cells are highly proliferative [41] and increase concentrations of cyclins [42] that are positive regulators of cell cycle progression. In the present study, we found that treated tumors had decreased proliferation markers Ki67 and cyclins and increased in apoptosis markers p53, p21, Bax, and p27. Polyamines are known to play a role in the transition of the cell cycle from G2/M to the G1 phase; this process is delayed by a reduction in polyamine levels [43]. Transcription of cyclin A is indirectly regulated by the tumor suppressor protein TP53. Activated TP53 turns on several downstream pathways, including p21, leading to cell cycle arrest [44]. In TCC of the bladder, lower p27 expression has been observed in metastatic or invasive tissue than in primary tumors [45]. Loss of p27 upregulates MnSOD in an STAT3-dependent manner, disrupts intracellular redox activity, and enhances cell migration [46]. Therefore, the induction of p27 in the treatment group could be partly responsible for the inhibition of tumor invasion.

During tumor development, there is a constant interaction between the tumor cells and the immune cells. Their interaction determines whether tumor regression or growth occurs. Endogenous polyamines are known to play a major function in the regulation of macrophage activation [47, 48]. Several immunosuppressive genes are found to be induced during bladder tumor growth [49]. Similar to tumor cells, arginase 1 (Arg1) is upregulated in tumor-infiltrating myeloid cells by tumor-secreted cytokines, including interleukin IL-4 and IL-10. Overexpression of Arginase in the tumor leads to arginine depletion, which further leads to suppressed T-cell responses and favors tumor-associated immune suppression [50]. We found a significant inhibition of Arg1 and IL10 in the tumors of the DFMO and combination treatment groups. We therefore assume that the synergistic effect of DFMO could possibly be due to its partial contribution to suppression of anti-tumor immunity.

Conclusion

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 with other prominent pathways like polyamine biosynthesis can serve as a potential chemopreventive strategy for invasive urothelial cancers. Further evaluation is warranted using other bladder cancer models to translate our find for primary secondary and post-operative prevention of this deadly disease. A similar combinational approach with other selective inhibitors may also be investigated for better preventive efficacy and better management of the disease.

Acknowledgements

The authors thank the University of Oklahoma Health Sciences Center Rodent Barrier Facility and Ms. Kathy Kyler for valuable suggestions and editorial help. This study was funded by NIH/NCI (NCI-CN53300).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Chinthalapally V Rao, Center for Cancer Prevention and Drug Development, Stephenson Cancer Center, Department of Medicine, Hematology-Oncology Section, University of Oklahoma Health Sciences Center (OUHSC), 975 NE 10th Street, BRC Building II, Room 1203, Oklahoma 73104, OK, USA. E-mail: cv-rao@ouhsc.edu

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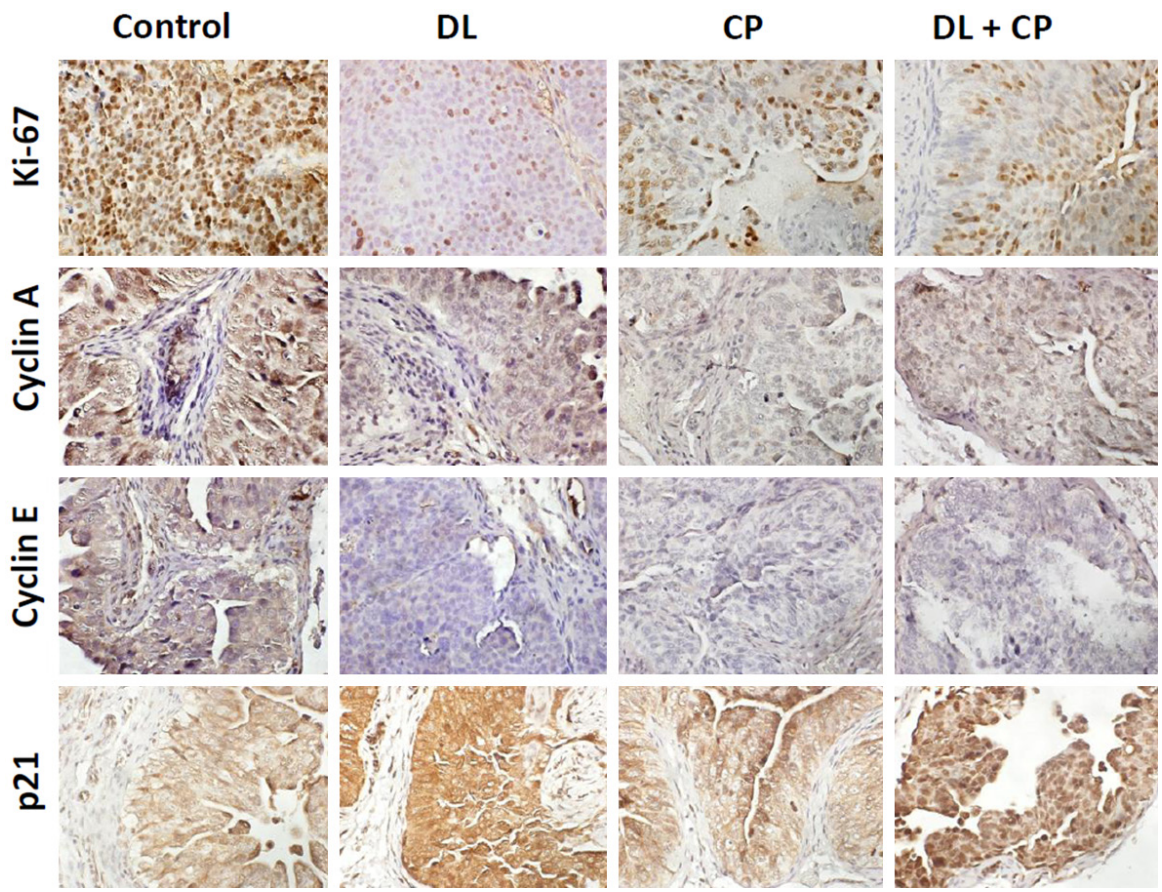
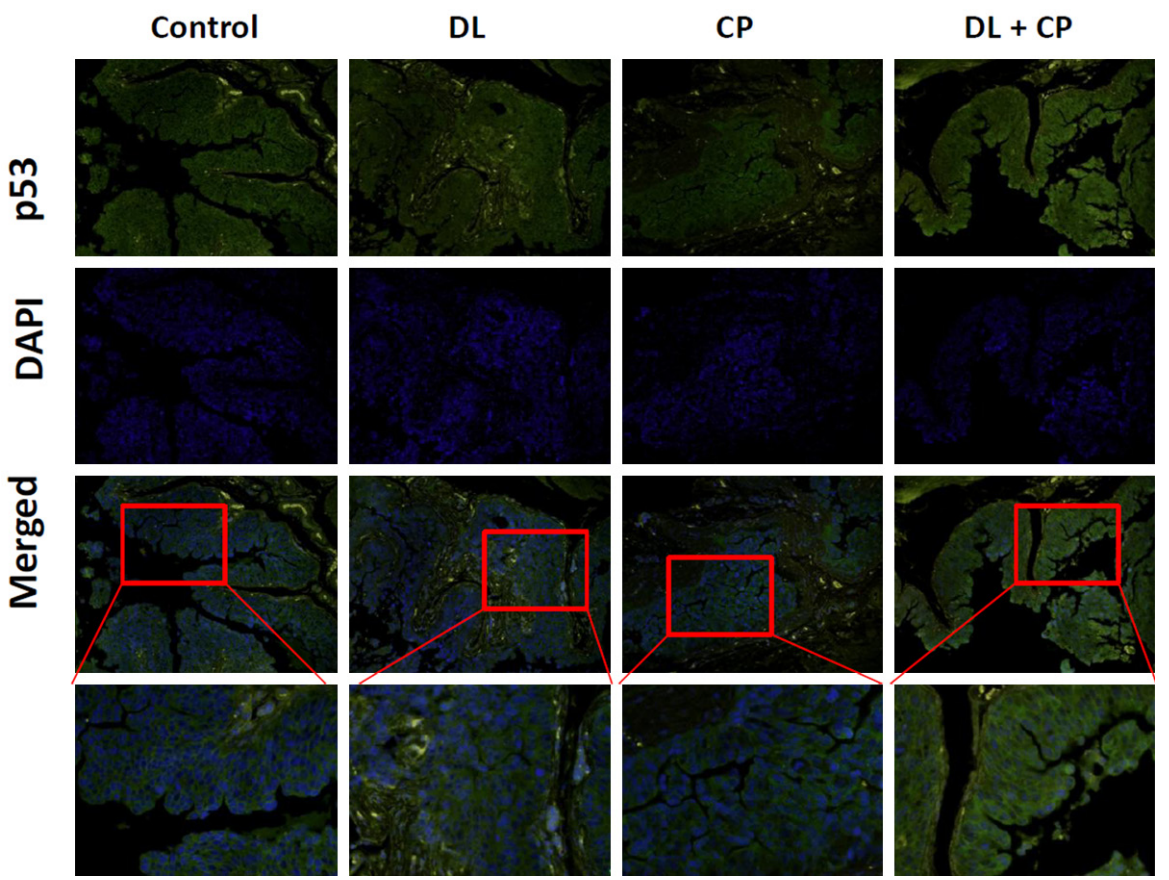
Supplementary Table 1. List of primers used for real-time PCR analysis

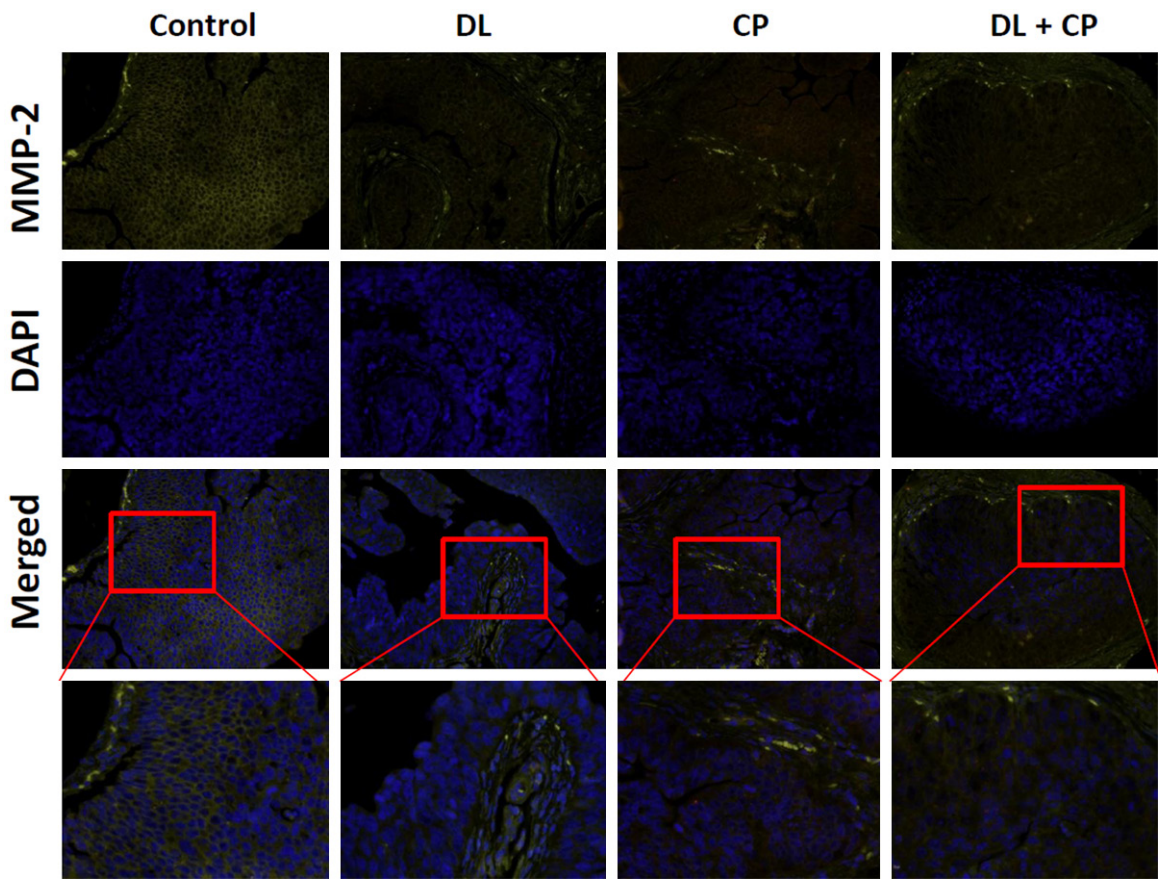
Gene	Forward primer	Reverse primer	Reference	Amplicon (bp)
Arg1	5'-GCTGTCTTCCCAAGAGTTGGG-3'	5'-ATGGAAGAGACCTTCAGCTAC-3'	NM_007482.3	224
Oat	5'-TGCCACCCAAAGATCATAGATG	5'-TGTACTCCTCGTATTCACCAAGG-3'	NM_016978.2	103
Oaz	5'-CTGAATGCTGTGTTTGTCAC-3'	5'-GGTCACCTGACCATCTTAAA-3'	NM_001301034.1	97
Sat1	5'-CCCTTGCTTTCTATCTTGTG-3'	5'-CATGACTGCCACTTTAAACA-3'	NM_009121.4	117
Sms	5'-TTCGGGTGACTCAGTTCCTGCTAA-3'	5'-AACGGAGACCCTCCTTCAGCAAAT-3'	NM_009214.3	199
p53	5'-TGAAACGCCGACCTATCCTTA-3'	5'-GGCACAAACACGAACCTCAAA-3'	NM_011640.3	92
p21	5'-CGAGAACGGTGGAACTTTGAC-3'	5'-TCCCAGACGAAGTTGCCCT-3'	NM_007669.4	62
p27	5'-AGTGTCCCTTTCGGTAAGAATG-3'	5'-TCAGAACCTCCAAGTGAGAATAAG-3'	NM_009875.4	118
Bax	5'-CAGGATGCGTCCACCAAGAA-3'	5'-GCAAAGTAGAAGAGGGCAACCA-3'	NM_007527.3	197
IL10	5'-CATCGATTTCTTCCCTGTGAA-3'	5'-TCTTGGAGCTTATTAAAGGCATTC-3'	NM_010548.2	77
Cav1	5'-GCGACCCCAAGCATCTCAA-3'	5'-ATGCCGTCGAAACTGTGTGT-3'	NM_007616.4	91
SV40T	5-CTTTGGAGGCTTCTGGGATGCAACT-3'	5'-GCATGACTCAAAAAACTTAGCAATTCTG-3'	NC_001669.1	574
Actin	5'-AGATCTGGCACCACACCTTC-3'	5'-GGGGTGTTGAAGGTCTCAAA-3'	NM_007393.4	139



Supplementary Figure 1. Expression of Ki67, Cyclin A, Cyclin E and p21 analyzed by IHC. DL, Control; CP, DL+CP.



Supplementary Figure 2. Expression of p53 analyzed by IF. DL, Control; CP, DL+CP.



Supplmentary Figure 3. Expression of MMP-2 analyzed by IF. Control, DL, CP, DL+CP.