Intravesical N-(4-hydroxyphenyl) retinamide and Adriamycin induces apoptosis in bladder cancer

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TABLE OF CONTENTS

1. ABSTRACT

The objective of this study was to evaluate the intravesical application of N-(4-hydroxyphenyl) retinamide (4-HPR) and adriamycin (ADM), as a treatment modality in a an animal model of chemically-induced bladder cancer. Bladder cancer developed in 50.0% of female Wistar rats, 4-6 weeks after intravesical application of the chemical carcinogen, N-methyl-N-nitrosourea (MNU). There was no significant difference in side effects induced by local versus systemic 4-HPR. Although tumor growth was inhibited by 4-HPR and ADM alone, tumor size was lower when both agents were used together. Apoptosis occurred at a higher rate in the combination group than when 4-HPR or ADM was used alone. The results suggest that intravesical use of 4-HPR and ADM may increase their efficacy in treatment of bladder cancer.

2. INTRODUCTION

Bladder cancer is a common health problem around the world. Approximately 260,000 of new cases of bladder cancer are diagnosed each year, and bladder cancer accounts for approximately 115,000 cancer deaths per year worldwide (1). It is the fourth most commonly diagnosed cancer in men and the eighth most common cancer in women in the United States and it has the sixth highest incidence of all cancers in the developed world (1, 2). The incidence of bladder cancer continues to increase, which may be partially due to increase in bladder carcinogens in the environment. A number of etiological factors are associated with the development of bladder cancer with nearly 50% of bladder cancers caused by aryl amine exposure from cigarette smoke, and approximately 20%–25% related to occupational exposure to chemicals, such as benz[a]pyrene, benzidine, and beta-napthylamine (3). The incidence of bladder cancer in occupationally exposed people is up to 50 times that of the non-exposed population (3).
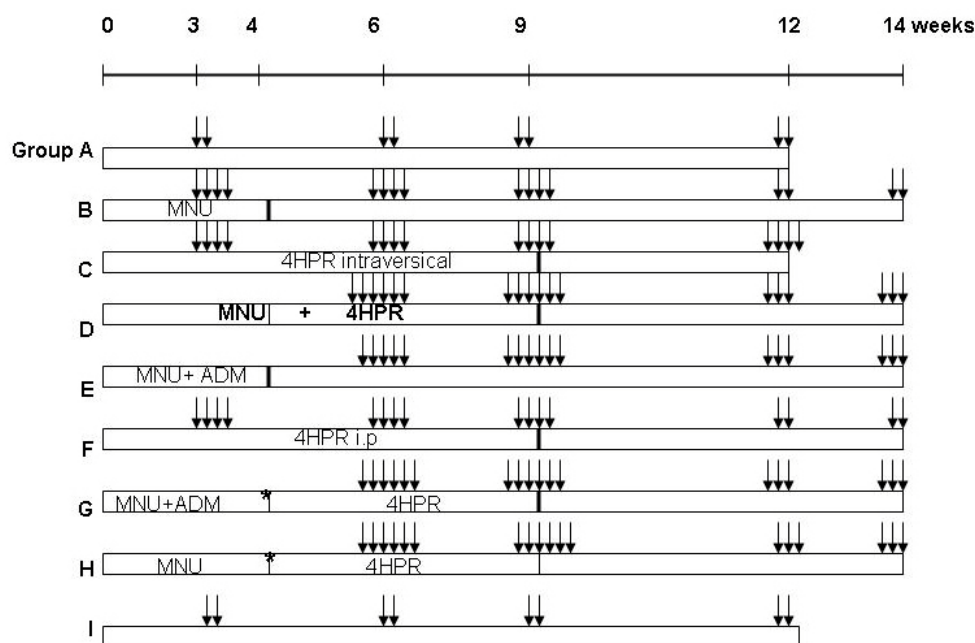
Figure 1. Experimental design for animal sacrificing schedule. MUN (0.5 mg/week) was applied intravescially for 2 weeks. Then, the animals were sacrificed at different time points. Number of animal is represented by the number of arrows. The treatment groups are shown in table 1.

Retinoids are a group of compounds that regulate cell growth and differentiation (4-8). However, little is known about their mechanism of action against bladder cancer cells. Natural and synthetic retinoids have been shown to reduce bladder cancer recurrence in clinical trials (4-5).There is a high rate of treatment discontinuation due to side effects (6-8). The synthetic retinoid 4-HPR has been used as an antiproliferative and differentiation-inducing agent (9) and has been shown to inhibit carcinogenesis in the mammary gland, head and neck, lung, prostate, and bladder. The synthetic form has been less toxic than other efficacious natural retinoids (10-15). 4-HPR is currently being used in clinical trials conducted by the Southwestern Oncology Group and the University of Texas, M. D. Anderson Cancer Center to prevent and treat recurrent superficial bladder cancer.

Adriamycin (ADM) is a chemotherapeutic agent which has been used for the intravesical treatment of bladder cancer. It inhibits protein synthesis and acts by binding DNA base pairs and inhibiting topoisomerase II. ADM has been shown to decrease tumor recurrence as much as 17% in comparison to surgery alone, but did not affect tumor progression (16).

Here, we show that the combined therapeutic efficiacy of 4-HPR and ADM was higher when both were used together in an animal model of bladder cancer.

3. MATERIALS AND METHODS

3.1. Animals
A total of 136 female Wistar rats aged 6 - 7 weeks, 180 – 220 grams, were purchased from Experimental Animals Center, National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China (License No. SCXK11-00-0010). The animals were randomly distributed in polypropylene cages, and supplied with filtered water plus Sulfamethoxazole Chemitrim (20mg/5L), and fed with standard ground commercial diet. They were housed in special pathogen free-grade animal laboratory, at 22 ^{o}C, with relative humidity between 50- 60 % and 12/12 hour light-dark cycle.

3.2. Chemical agents
N-methyl-N-nitrosourea (MNU) was purchased from Sigma Chemical Co. (St. Louis, MO). MNU dissolved in 0.1M citric acid at final concentration of 20mg/ml (pH 6.0), right before use. 4-(N-hydroxyphenyl) retinamide (4-HPR) was purchased from Sigma Chemical Co. (St. Louis, MO). 4-HPR dissolved in 100% alcohol as stock solutions of 25mg/ml and stored in an atmosphere of N_2 at -80° C. Adriamycin (ADM) was purchased from Wan Le Pharmaceutical Factory, ShenZhen, China. ADM dissolved in 5% glucose at a final concentration of 1mg/ml, immediately before use.

3.3. Experimental design
The experimental design is presented in table 1. Rats were randomly divided into a total of 9 groups as shown in Table1. The animals were sacrificed at 3, 6, 9, 12 and 14 weeks (Figure 1). Two groups of control animal were treated with H_2O or alcohol, respectively. Three groups were treated with both MUN and 4-HPR, and two additional groups were treated with either MUN plus ADM or MUN plus ADM and 4-HPR. Animal body weight was registered weekly during the whole experiment.

Table 1. Animal treatment group

Group	Number of rats	Treatment	Dose	Total treatment times
A	8	Control (H$_2$O)	-	-
B	16	MNU	2mg/2w, intravesical,	4 times (2/w for 2w)
C	16	4-HPR	0.5mg/w, intravesical	9 times
D	18	MNU+4-HPR	2mg/2w, intravesical,	4 times (2/wfor 2w)
			0.5mg/w, intravesical,	9 times
E	18	MNU+ADM	0.2mg/w, intravesical	4 times (2/w for 2w)
			2mg/2w, intravesical,	4 times (2/w for 2w)
F	16	4-HPR	0.5mg/w, i.p	
G	18	MNU	2mg/2w, intravesical	4 times (2/w for 2w)
		+ADM	0.2mg/w, intravesical	4 times
		the 4th week+4-HPR	0.5mg/w, intravesical	6 times
H	18	MNU the 4th week + 4-HPR	2mg/2w, intravesical,	4 times (2/w for 2w)
			0.5mg/w, intravesical,	9 times
I	8	alcohol	0.2ml/w, intravesical,	9 times

Water and food consumption were measured weekly at the beginning of the experiment and every week from weeks 4 to 14.

3.4. Tumor induction by MNU

Bladder tumors were induced according to Steinberg *et al* (17-19). Twenty milligram of MNU was dissolved in 0.1 M citric acid buffer solution (pH = 6.0). MNU solution was prepared freshly and was used within 50 min after preparation.

After being anesthetized with 100 mg/kg i.p. Ketamine, and aseptic treatment of local skin with 0.1% Benzalkonium Bromide, 0.1 ml of MNU solution was injected through a 23-gauge Teflon angiocatheter intravesically twice/week for two weeks (a total of 4 doses).

3.5. Intravesical and intragastric treatment with 4-HPR and ADM

A concentration of 0.5 mg/ml 4-HPR was used for intravesical treatment and for intragastric treatment (orally by gavage). Animals received 0.5 mg of 4-HPR (1 ml of 0.5 mg/ml) once a week for a total of 9 weeks. The concentration of 4-HPR was equivalent to human 200mg dose, calculated by the equation Y+K (W$^{0.75}$) where Y is the resting animal's energy output in kcal/24 h (also termed the minimum energy cost), K is a constant based on core temperature, which is specific for each species, and W is mass in kilograms. A concentration of 0.2 mg ADM (0.2 ml of stock solution) was used for intravesical treatment. once a week for 9 weeks (Table 1).

3.6. Histological analysis and tumor evaluation

Rats were sacrificed by continuous flow of CO$_2$, and necropsy was performed. Bladders from all groups were resected, removed quickly and washed in distilled water. After being weighed, bladders were opened longitudinally and the number and size of tumors were determined. The tumor size was measured with callipers and calculated according to the equation: v = (lxw^2)/2, which V is tumor volume, the long dimensions (mm) is L, and short dimensions (mm) is W. The 1/3 was fixed with 10% formalin and embedded in paraffin for hematoxylin and eosin staining and immunostaining, 1/3 was placed in phosphate balanced saline (PBS) for analysis of apoptosis, and 1/3 was stored at -80 ^{o}C. Liver, lung, brain, kidney, ovary, and uterus was examined for presence of tumor metastasis.

Bladders were fixed in formalin for 24 hrs and then embedded in paraffin. Paraffin embedded sections were stained with hematoxylin and eosin (H&E) and used in immunohistochemcial staining.

3.7. Acridine orange (AO) staining of urine

Urine was collected for 24 hrs before sacrificing the animals and 3-5 ml blood from each animal was collected and stored at -80 ^{o}C. Urine was centrifuged at 300 g for 15 min, and pellets were air dried. The pelleted cells were placed slides by cytospin. Slides were fixed in 95% alcohol for 10 min, washed in 0.1 M phosphate (pH 6.0) buffer solution, and then soaked in 0.01 g/dl acridine orange phosphate buffer for 3 min. Slides were stained by 0.10 M calcium-chloride for 30 sec to 1 min, and washed by 0.15 M phosphate buffer.

3.8. Flow cytometry

1/3 of each bladder tissue was washed in PBS 2 to 3 times, cut into 1 mm^3 fragments and centrifuged at speed 1,000 g for 5 min. The supernatant was discarded and the tissue was digested in 0.25% trypsin (Sigma-Aldrich) for 20 min at 37 °C. A single cell suspension was obtained by vortexing and filtration of the sample through a 100 µm mesh nylon filter. The cell suspension was then centrifuged at 300 g for 5 min and cell pellet was resuspended in 1ml BSA/PBS buffer (Sigma). Samples were analyzed using a FACS Calibar flow cytometer (Beckton Dickson Corp.USA). Fluorescence was measured at 488 nm air cooled argon ion laser. The samples were rinsed in PBS twice and finally DNA was stained using 1.0 µg /ml propidium iodide (in PBS containing 0.1 mg/ml RNAse (Serva, Heidelberg, Germany). The samples were allowed to stand for 40 min in the dark at 4°C before flow cytometric analysis. The analysis was performed by using the FloMax Software (Partec GmbH, Münster, Germany). Cell cycle analysis was performed using Modfit LT 2.0 (Verity Software House Inc, Topsham, Maine, USA). The following data were collected: (1) the percentage of apoptosis cells and (2) the DNA index of the rat bladder cell populations.

3.9. *In-situ* Terminal deoxynucleotidyl transferase (TdT)-mediated fluorescein-deoxyuridine-triphosphate (dUTP) nick-end labeling (TUNEL) assay

Paraffin sections were washed in distilled water with 0.5% triton X-100 for 10 min for 2-3 times, and incubated with 20 µg/ml Proteinase K for 15 min

Table 2. Pathologic finding in animal treatment groups

Group	Treatment	#Rat	Normal	TCC	CIS	Inflamm	Necrosis	Hyper	[2] Death
A	Control-H$_2$O	7	6	0	0	0	1	1	0
B	MNU	16	0	8	1	1	0	4	2
C	4-HPR	16	12	0	0	2	1	1	0
D	MNU+ 4-HPR	18	5	4	3	0	0	3	3
E	MNU+ADM	18	4	3	3	0	0	4	4
F	4-HPR	18	13	0	0	0	0	3	0
G	MNU+ ADM+ [1]4-HPR	18	6	2	3	0	0	3	4
H	MNU+ [1]4-HPR	18	5	3	4	0	1	3	2
I	Control- Alcohol	7	5	0	0	2	0	1	1
	Total	136	56	20	14	5	3	23	15

[1] 4-HPR: 4-HPR were treated at the fourth week. [2]Death referred as accidental death.

Table 3. Tumor number and size

Group	Treatment	No. Rat	Tumors	Tumor size	Ratio	P value
B	MNU	10	9	31±10.2	90.0%	-
D	MNU+4-HPR	15	7	0.9± 0.6	46.7%	0.040 compared with B group
E	MNU+ADM	14	6	3.7±1.8	42.9%	0.033 compared with B group
G	MNU+ADM+4-HPR[1]	14	5	1.5±0.9	35.7%	0.013 compared with B group
H	MNU+4-HPR[1]	16	7	1.0±0.7	43.8%	0.037 compared with B group

4-HPR[1]: 4-HPR was treated at the 4th week. MNU: N-methyl-N-nitrosourea; 4-HPR: N-(4-hydroxyphenyl) retinamide; ADM: adriamycin

at 37 ^{o}C. Slides were washed again and incubated with 1 × TDT (Terminal Deoxynucleotidyl Transferase) buffer (Gibco/BRL) for 15 min at room temperature, then incubated with 0.3 μg/μl TDT plus biotinylated dUTP 20mM in TDT buffer (Gibco/BRL) for 1 hr at 37 ^{o}C. Slides were washed and stained with Avidin-Biotin-peroxidase Complex (Vectastain ABC kit).

3.10. Statistical analysis

Frequency tabulation and summary statistics were prepared. Pearson's chi-square test (X2) was performed to assess the tumor incidence between two binary variables such as the control and the treatment groups or different treatment groups. Two-sided P values will be determined in all analysis.

4. RESULTS

4.1. Tumor incidence

The total number of experimental rats was 136. There were 15 (11.0%) accidental deaths due to inflammation and other causes before or at the end of these experiments. 121 rats (89.0%) survived until the end of this experiment. The causes of death include urethral injury of 3 rats, urinary tract infection of 9 rats, pyonephrosis of 2 rats, and gastrointestinal infection of 1 rat (Table 2).

Of the 88 animals treated with MNU, 34 developed urinary bladder cancer confirmed by histopathologic examination (46.6%), 17 were presented as simple or atypical hyperplasic lesions (19.3%) (Table 3). The morphologic changes in the urinary bladders were categorized as normal, simple or atypical hyerplasia, carcinoma *in situ* (CIS), and transitional cell carcinoma (TCC) (data not shown). In 5 groups of MNU-treated rats, the bladder pathologic changes is summarized as follows: bladder mucous membranes became thicker by 5 - 7 layers with normal epithelium at the second week, by the end of the fourth week, the mucous membranes of bladder walls showed changes. There were points of hemorrhage and apoptosis which was detected under microscope. At week six, the simple hyperplasia and/or papilloma were formed, but the epithelium around the papilloma was normal. At end of the eighth week, cancerous

tumors started to develop and atypical hyperplasic changes were significantly shown in bladder epithelium. By the end of twelfth week, carcinoma were developed and accompanied with ulceration, hyperemia and hemorrhage. The tumor number, size, and statistical analysis were shown in table 3.

By performing X^2 Test for the accurate probabilistic method and comparing the groups two by two, we found that there are significant differences between D,E,G, H and B group (P<0.05) (table 3) and there is no significant difference between E,G, H and D (P >0.05), G, H and E (P >0.05), G and H (P >0.05).

4.2. Fluorescent acridine orange (AO) staining

The experiment was performed in order to detect the tumor cells in the urine.Animal urine was collected on the day before sacrifice and stained with AO dye. The tumor cells were observed under photoelectric microscope. The nuclei of tumor cells were detected by green-yellow or yellow fluorescence, and the cytoplasm was detected by orange fluorescenc (data not shown). In MNU-treated five groups (B, D, E, G and H group), the positive rate for abnormal cell nucleus was 50.0% (44 out of 88 rats).

4.3. Cell Cycle and Apoptosis induced by 4-HPR and ADM

Apoptosis induction by 4-HPR and ADM were detected by TUNEL assay. Animal tissue was cut by small pieces and cells were washed and collected. The cell cycle was analyzed by flow cytometry (Figure 2), 4-HPR, ADM and combination of ADM and 4-HPR increased subG1 and G1 phases. The strongest effect on cell cycle change was found in ADM and combination of ADM and 4-HPR (Figure 2). The cells taken from animal in both 4-HPR- and ADM- treated group were detected apoptosis and the apoptotic cells population were increased in treatment group, especially ADM and combination of ADM and 4-HPR had strong effects. The percentage of apoptosis population was 3%, 5%, 18%, 38%, and 43% for groups of A, B, D, E, and G, respectively. Both 4-HPR- and ADM- treated animal sections were detected positive apoptosis staining (Figure 3). ADM had stronger apoptosis induction than that of 4-HPR used alone, and the
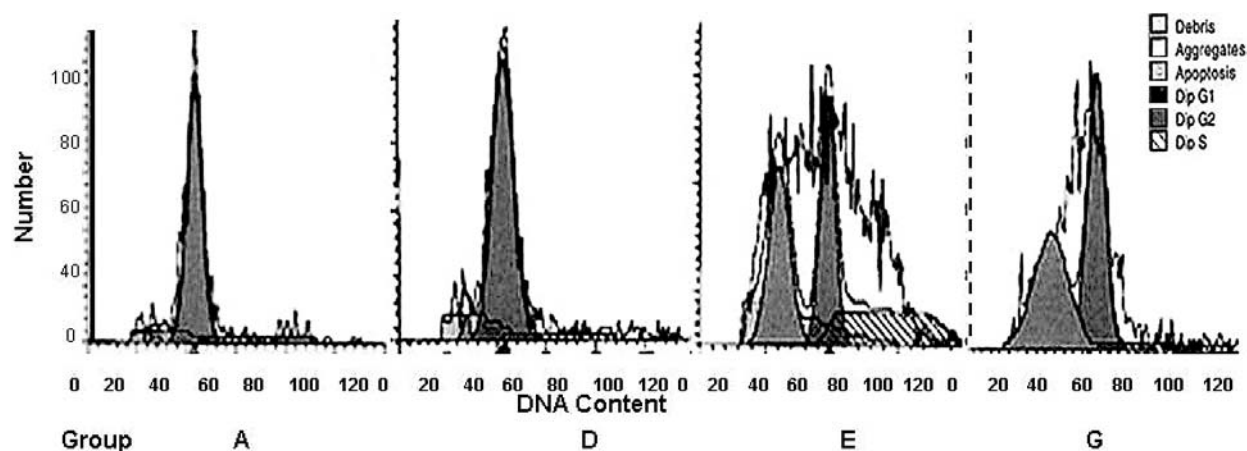
Figure 2. Representative result of flow cytometric analysis of cell cycle and apoptosis in various groups. B (MNU) group (Data not show) was similar with A (control) group. The subG1 and G1 phases increased in E (MNU+ADM) and G (Combination of ADM and 4-HPR) group.
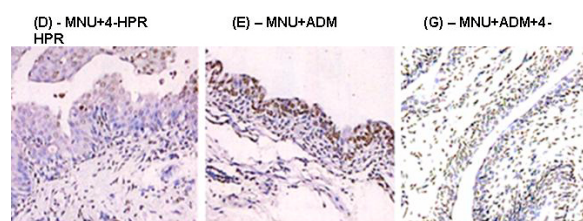
Figure. 3. Representative *in-situ* apoptosis staining in treated animal group. Apoptosis induction could not detected in normal urothelium. Apoptosis could not be detected in D, E and G groups, which small amount of cells labeled by *in-situ* for apoptosis (dark brown) in 4-HPR only treatment group (D), a large amount of cells labeled by *in-situ* for apoptosis (dark brown) in ADM and combination of ADM and 4-HPR treatment groups (E) and (G).

combination of ADM and 4-HPR was the strongest positive apoptosis staining than either agent use alone (Figure 3).

5. DISCUSSION

The goal for using an animal model for chemical-induced bladder cancer is to better understand how chemical interact with bladder epithelium as well as invasive carcinoma formation. The model system will allow us to further understand the relationship between bladder carcinogenesis and development of cancer and also enable us to investigate the effects of various biological agents and chemopreventive agents before they are used in clinical trials. There several chemical-induced bladder cancer model, tobacco carcinogenesis related animal models (20); occupational exposure related models (21,22). MNU-induced bladder cancer was chosen in our model, since it caused transitional cell carcinoma (TCC) (17).

The incidence of occupational bladder cancer was 50 times higher than that of the non-exposed population (3). People occupationally exposed to carcinogens with the higher risk factor for development of bladder cancer (3). Approximately, 60-80% of uroepithelial TCC are superficial

or superficial-invasive tumors, and most of them are low histological grade papillary tumors (TIS, Ta, and T1) (16). These types of tumors are considered as low invasive with 20% muscle infiltrating (T2-T4) and 5% are metastasis at the time of diagnosis (23). Treatment of bladder cancer is mostly dependent on the tumor stage, and has widely varied of clinical outcome. Most superficial diseases are treated conservatively by transurethral removal of the tumor without removal of the bladder. Muscle-infiltrating tumors are general treated by cystectomy plus systemic chemotherapy and radiotherapy. Of superficial tumors, about 20% are cured by surgical removal of the lesion, however, 50-70% of patients experience a recurrence within 12 months after resection (24-25), and 10-30% of patient progress to invasive disease and metastasis. Although recent advances treatment such as chemotherapy and radiotherapy improved bladder cancer mortality, the best strategies for prevention and treatment of recur or progress has not been identified.

Treatment of recurrent tumors with instillation of cytotoxic or other anticancer agents by bacillus Calmette-Guerin (BCG) has been shown to be most effective and delays subsequent recurrence compared with surgery alone, but could not complete prevent recurrence (23). Chemoprevention with natural and synthetic retinoid is a new approach in prevention of superficial or recurrent superficial tumors (27-33). However, the toxic side effects of retinoids have resulted in limited accrual of subjects into clinical trials, stopped clinical trial early, or resulting non-interpretable study results (27, 29, 30). The synthetic retinoid, 4-HPR has a low toxicity profile and is used in bladder cancer chemoprevention for superficial disease (33, 34). With regard to the side effect of retinoids, the suitable concentration of 4-HPR was not determined (34). As the report of 4-HPR trial, the total of 99 subjects with resected superficial bladder cancer (Ta, T1) were randomized to either 4-HPR at 200 mg day p.o. for 24 months, or no intervention. The activity of 4-HPR were measured as DNA flow cytometry as surrogate end-point biomarkers (34). The control and treatment groups had no significant change of DNA content and recurrence-free survivals were similar between the two groups (34).

The local treatment of chemopreventive agent has been initiated, we are the first report of intravesical using chemopreventive agent 4-HPR and combination of chemopreventive agent 4-HPR with cytotoxic agent ADM in animal model. Local treatment may avoid side effect and increase the efficacy. As we know, the metabolism and storage of retinoid are both through liver, using systemic retinoid could reduce the concentration in some target organs through liver detoxification. In our study, first, we studied bladder cancer development, bladder tumor induction was confirmed by both acridine orange (AO) staining of tumor cells and pathologic examination of bladder tissue. Scheduled sacrificing was used to observe the tumor initiation and promotion. Second, we compared both chemotherapeutic and chemopreventive agents' local effects. Both chemotherapeutic and chemopreventive agents had effects on carcinogenesis inhibition ($p<0.05$) and apoptosis induction. ADM had a stronger effect than 4-HPR when used alone in apoptosis induction, as we expected. Furthermore, the combination of chemotherapeutic and chemopreventive agents had the strongest effect as observed in Figure 3 for apoptosis induction in combination treatment group. However, there are several limitations in the study. First, we did not compare the effects of 4-HPR systemic and local treatment in this study. Second, 4-HPR combining with other chemotherapeutic agents should be tested in animal model. Follow up study will be carried out and will be provided more information.

6. CONCLUSIONS

The present data highlight the local treatment of bladder cancer with in a chemopreventive agent in combination with a chemotherapeutic agent. Drug delivery is an important factor for treatment effects, especially retinoid. Local chemoprevention and treatment of bladder cancer warrant current clinical study.

7. ACKNOWLEDGEMENT

8. REFERENCES

1. Cohen, S.M., Shirai, T., and Steineck, G. Epidemiology and etiology of premalignant and malignant urothelial changes. Scand J Urol Nephrol Suppl. 205**,** 105-115.(2000

2. Atlanta, G.A. Cancer facts and figures. *American Cancer Societ.* 95, 234-240 (1995)

3. Jianjun, Zhang, Zhenbin, Fan, Yanning, Gao, Zejun, Xiao, Changling, Li, Qian, and Shujun, Cheng. Detecting Bladder Cancer in the Chinese by Microsatellite Analysis: Ethnic and Etiologic Considerations. *JNCI* 93, 45-51 (2001)

4. Verma AK. Retinoids in chemoprevention of cancer. *J Biol Regul Homeost Agents* 17, 92-97 (2003)

5. Camacho LH. Clinical applications of retinoids in cancer medicine. *J Biol Regul Homeost Agents* 17, 98-114 (2003)

6. Studer, U.E., Biedermann, C., Chollet, D., Karrer, P., Kraft, R., Toggenbutg, H., and Vonbank, F. Prevention of recurrent superficial bladder tumors by oral etretinate: preliminary results of a randomized, double blind multicenter trial in Switzerland. *J Urol* 131, 47-50 (1984)

7. Pedersen, H., Wolf, H., Kromann Jensen, S., lund, F., Hansen, E., Olsen, P. R., and Sorensen, B. L. Administration of retinoid as prophylasix of recurrent non-invasive bladder tumors. *Scand J Urol Nephrol* 18, 121-126 (1984)

8. Prout, G.R., and Barton, B.A. 13-cis-retinoic acid in chemoprevention of superficial bladder cancer. *J Cell Biochem* 161, 148-154 (1992)

9. Kelloff, G.J. N-(4-hydroxyphenyl)retinamide (4HPR): Clinical Development Plan. *J Cell Biochem. Suppl* 20,176-196 (1994)

10. Tradati, N., Chiesa, F., Rossi, N., Grigolato, R., Formeli, F., Costa, A., and De Palo, G. Successful topical treatment of oral lichen planus and leukoplakias with fenretinide (4-HPR). *Cancer Lett* 76,109-111 (1994)

11. Modiano, M.R., Dalton, W.S., Lippman, S.M., Joffe, L., Booth, A.R., and Meyskens, F.L. Phase II study of fenretinide N-(4-hydroxyphenyl)retinamide) in advanced breast cancer and melanoma. *Invest New Drugs* 8, 317-319 (1990)

12. Zou, C. P., Kurie, J.M., Lotan, D., Zou, C.C., Hong, W.K., and Lotan, R. N-(4-Hydroxyphenyl) Retinamide (4HPR) induces apoptosis in human non-small cell lung cancer (NSCLC) cell lines. *Clin Cancer Res* 4, 1345-1355 (1998)

13. Pollard, M., Luckert, P.H., and Sporn, M.B. Prevention of primary prostate cancer in Lobund-Wistar rats by N-(4-hydroxyphenyl)retinamide. *Cancer Res* 51, 3610-3611 (1991)

14. Decensi, A., Bruno, S., Giaretti, W., Torrisi, R., Curotto, A., Gatteschi, B., Geido, E., Polizzi, A., Costantini, M., Bruzzi, P., Nicolo, G., Costa, A., Boccardo, F., Giuliani, L., and Santi, L. Activity of 4-HPR in superficial bladder cancer using DNA flow cytometry as an intermediate endpoint. *J Xell Biochem* 161 (Suppl.), 139-147 (1992)

15. Zou, C.P., Liebert, M., Zou, C.C., Grossman, H.B., and Lotan, R. Identification of effective retinoids for inhibition growth and inducing apoptosis in bladder cancer cells. *J of Urol* 165, 986-992 (2001)

16. Kurth, K., and Tunn, U. Adjuvant chemotherapy for superficial transitional cell bladder carcinoma: Long term results of a EORTC randomized trial comparing doxorubicin, ethoglucid and TUR alone. *J of Urol* 158, 378-384 (1997)

17. Steinberg, G.D., Brendler, C.B., Ichikawa, T., Squire, R.A., and Isaacs, J.T. Characterization of an N-methyl-N-nitrosourea-induced autochthonous rat bladder cancer model. *Cancer Res* 50, 6668-6674 (1990)

18. Hasegawa, R., Futakuchi, M., Mizoguchi, Y., Yamaguchi, T., Shirai, T., Ito, N., Lijinsky, W. Studies of initiation and promotion of carcinogenesis by N-nitroso compounds. *Cancer Let* 123, 185-191 (1998)

19. Elsasser-Beile, U., Ruhnau, T., Freudenberg, N., Wetterauer, U., and Mengs, U. Antitumoral effect of recombinant mistletoe lectin on chemically induced urinary bladder carcinogenesis in a rat model. *Cancer* 91, 998-1004 (2001)

20. Landis, S. H., Murray, T., Bolden, S., and Wingo, P.A. Cancer Statistics. *CA Cancer J Clin* 49, 8-31 (1999)

21. Morgan, D.t., Bowsher, W., Griffiths, D.F.R., and Matthews, P.N. Rationalisation of follow-up in patients with non-invasive bladder tumors. A preliminary report. Br *J of Urol* 67, 158-161 (1991)

22. Heney, N.M., Ahmed, S., Flanagan, M.J., Frable, W., Corder, M.P., Hafermann, M.D., and Hawkins, I.R. National bladder collaborative group A: superficial bladder cancer: progression and recurrence. *J of Urol* 130, 1083-1086 (2002)

23. Kurth, K.H., Bouffioux, C., Sylvester, R., Van der Meijden, A.P.M., Oosterlinck, W., Braus, M. Treatment of superficial bladder tumors: achiements and needs. *Eur J Uro* 37,1-9 (2000)

24. Studer, U. E., Biedermann, C., Chollet, D., Karrer, P., Kraft, R., Toggenbutg, H., and Vonbank, F. Prevention of recurrent superficial bladder tumors by oral etretinate: preliminary results of a randomized, double blind multicenter trial in Switzerland. *J of Urol* 131, 47 (1984)

25. Studer, U. E., Jenzer, S., Biedermann, C., Chollet, D., Kraft, R., and Von Toggenburg, H. Adjuvant treatment with vitamin A analogue (etretinate) after transurethral resection of superficial bladder tumors. Final analysis of a prospective, randomized multi-center trial in Switzerland. *Eur J Urol* 28, 284-290 (1995)

26. Pedersen, H., Wolf, H., Kromann Jensen, S., lund, F., Hansen, E., Olsen, P. R., and Sorensen, B. L. Administration of retinoid as prophylasix of recurrent non-invasive bladder tumors. *Scand J Urol Nephrol* 18, 121 (1984)

27. Prout, G.R., and Barton, B.A. 13-cis-retinoic acid in chemoprevention of superficial bladder cancer. *J Cell Biochem* supp 161, 148-158 (1992)

28. Hill, D.L. Retinoids and cancer prevention. *Annu Rev Nutr* 12,161-181 (1992)

29. Kakizoe, T. Chemoprevention of cancer focusing on clinical trials. *Japan J of Clin Onco* 33, 421-442 (2003)

30. Lippman, S.M., and Lotan, R. Advances in the Development of Retinoids as Chemopreventive Agents. *J Nutrition* 130, 49-54 (2000)

31. Decensis, A., Torrisi, R., Bruno, R., Costantini, M., Curotto, A., Nicolò, A., Malcangi, B., Baglietto, L., Bruttini, G.P., Gatteschi, B., Rondanina, G., Varaldo, M., Perloff, M., Malone W.F., and Bruzzi, P. Randomized Trial of Fenretinide in Superficial Bladder Cancer Using DNA Flow Cytometry as an Intermediate End Point. *Cancer Epi Biomaker Prevention* 9, 1071-1078 (2000)

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