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# Synergistic Antitumor Activity of Vitamins C and K<sub>3</sub> on Human Bladder Cancer Cell Lines

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## ABSTRACT

Exponentially growing cultures of human bladder tumor cells (RT4 and T24) were treated with Vitamin C (VC) alone, Vitamin K<sub>3</sub> (VK<sub>3</sub>) alone, or with a VC:VK<sub>3</sub> combination continuously for 5 days or treated with vitamins for 1 h, washed with PBS and then incubated in culture medium for 5 days. Co-administration of the vitamins enhanced the antitumor activity 12- to 24-fold for the RT-4 cells and 6- to 41-fold for the T24 cells. Flow cytometry of RT4 cells exposed to the vitamins revealed a growth arrested population and a population undergoing cell death. Growth arrested cells were blocked near the G<sub>0</sub>/G<sub>1</sub>-S-phase interface, while cell death was due to autschizis. Catalase treatment abrogated both cell cycle arrest and cell death which implicated hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in these processes. The H<sub>2</sub>O<sub>2</sub> production resulted in a moderate increase in lipid peroxidation and depletion of cell thiol levels. Analysis of cellular ATP levels revealed a transient increase in ATP production for VC and the VC:VK<sub>3</sub> combination, but decreased ATP levels following VK<sub>3</sub> treatment. Lipid peroxidation, thiol depletion and ATP modulation occurred at a 17-fold lower concentration in the vitamin combination than with either vitamin alone. These results suggested that the increased cytotoxicity of the vitamin combination was due to redox cycling and increased oxidative stress.

**Keywords:** Bladder; Carcinoma; Vitamin C; Vitamin K<sub>3</sub>

## 1. Introduction

Bladder cancer is the second most common urological malignancy in the United States of America with an estimated 72,570 new cases and 15,210 deaths in 2013 [1]. Unlike most epithelial tumors, divergent pathways of tumorigenesis are involved in urothelial carcinoma [2]. These separate mechanisms produce at least two distinct types of neoplasms: non-invasive, low-grade tumor and high-grade, often invasive, carcinoma [3]. Patients with low grade tumors usually undergo transurethral tumor resection (TUR). While the prognosis for these patients is usually good, they exhibit a lifelong risk of recurrence (50% - 70%) with occasional progression to invasion. Given the relatively high rates of recurrence and progression, it is necessary to consider adjuvant intravesical therapy in most patients [4,5]. Since the mid 1980s, the standard treatment for metastatic bladder cancer has been methotrexate, vinblastine, doxorubicin and cisplatin [6]. However, even with this regimen, the prognosis for patients with metastatic disease is poor with a median survival

being approximately 12 - 14 months [7]. Furthermore, addition of new drugs, such as gemcitabine, to the standard cisplatin-based regimens has not improved clinical outcomes [8,9]. In addition, the use of several targeted agents, such as, antiangiogenics, anti-epidermal growth factor receptor agents, and immunomodulatory agents did not result in a major breakthrough [7]. Finally, because of the lifelong need for monitoring for recurrence, the typical cost incurred by a bladder tumor patient from diagnosis to death has been reported to be the highest among all cancers [4,10]. Taken together, these facts demonstrate the need for the development of agents that are more effective, less toxic, and more cost effective agents that are more effective, less toxic and affordable [7].

Due to their low systemic toxicity, several vitamins have been evaluated for their chemopreventive and therapeutic potential abilities against bladder cancer [11]. Vitamins A, B<sub>6</sub>, C, E, and K<sub>3</sub> have all demonstrated activity in the prevention or treatment of bladder cancer [12]. In addition, Lamm *et al.* [12] performed a double blind, randomized trial in patients with bladder cancer

who were treated with transurethral resection plus megadose vitamins daily vs the recommended daily allowance of multivitamins. The overall recurrence rate was 80% in the recommended daily allowance arm and 41% in the megadose vitamin arm ( $p = 0.0011$ ). This vitamin treatment not only was nontoxic, but also produced a greater reduction in the rate of tumor recurrence than BCG immunotherapy which is the gold standard for the treatment of superficial bladder cancer. In addition, there is a growing body of evidence demonstrating the benefit of combining vitamins C and K<sub>3</sub> for the treatment of: acute lymphoblastic leukemia [13], acute myelogenous leukemia [14-16], bladder [17-28], breast [29], glioblastoma [30], glioma [31], kidney [32], liver [33-37], lung [38], ovarian [39-42] and prostate cancers [43-55]. Unlike the majority of chemotherapeutic agents which target rapidly dividing cells, VC:VK<sub>3</sub> appears to target tumor cells by inflammation [50]. Tumor cells possess a greater need for glucose than normal cells and express facilitative glucose transporters (GLUTs) to achieve this task. Because of the structural resemblance of dehydroascorbic acid (DHA, the oxidized form of vitamin C) to glucose, DHA can also enter the tumor cells and bio-accumulate. Epithelial tumors appear to rely on superoxide (inflammation) which is produced constitutively via NADPH oxidase of non-neoplastic stromal cells to oxidize the ascorbic acid [56,57]. Once dehydroascorbic acid enters the cells, it is reduced and retained as ascorbic acid (AA) which is not transportable through the bidirectional GLUTs [58,59]. The purpose of the current study is to evaluate VC, VK<sub>3</sub> and the VC:VK<sub>3</sub> combination for their antitumor activity against two human bladder cancer cell lines and to make an initial attempt to elucidate the mechanism(s) of action of the VC:VK<sub>3</sub> combination.

## 2. Materials and Methods

### 2.1. Culture Conditions

Human bladder cancer cell lines (T24 and RT4) were purchased from the American Type Culture Collection (ATTC, Rockville, MD, USA) and were grown in culture medium according to ATTC instructions. All media was supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY) and 50 µg/mL gentamicin sulfate (Sigma, St Louis, MO). All incubations were performed at 37°C and with 5% CO<sub>2</sub> unless other conditions are stated. Vitamin C (VC) and menadione bisulfite (VK<sub>3</sub>) were purchased from Sigma Chemical Company (St Louis, MO, USA) and were dissolved in phosphate-buffered saline (PBS) to create 8000 µM VC, 500 µM VK<sub>3</sub> and 8000 µM VC:80 µM VK<sub>3</sub> test solutions. To prevent photodegradation of the vitamins, all the vitamin solutions were prepared and experiments were performed in a darkened laminar flow hood.

### 2.2. Protein Concentration Assay

In all experiments total protein concentration was determined using the method of Bradford [60]. Sham-treated cells served as controls in all experiments.

### 2.3. Cytotoxicity Assay

The cytotoxicity assay was performed using the micro-tetrazolium assay [MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-diphenyltetrazolium bromide] assay as described previously [23]. Corning 96-well titer plates were seeded with tumor cells ( $5 \times 10^3$  per well) and incubated for 24 hr. Vitamin test solutions were serially diluted with media in twelve 2-fold dilutions. Each dilution was added to seven wells of the titer plates and co-incubated with the tumor cells for 5 days. After vitamin treatment and the incubation period, cytotoxicity was evaluated using the MTT assay. Following linear regression, the line of best fit was determined and the CD<sub>50</sub> was calculated. The fractional inhibitory concentration index (FIC) was employed to evaluate synergism.

### 2.4. Flow Cytometry

Determination of cell DNA content and ploidy were performed according to our previously published procedure [45]. Briefly, titer dishes were seeded with  $1.0 \times 10^6$  RT4 cells suspended in MEM (10% FCS). Following 24 hours of incubation, the MEM was removed and the cells were washed twice with 3 ml of PBS. The cells in each titer dish were then overlaid with 2 ml of MEM containing the vitamins. Human foreskin fibroblast cells served as diploid internal standard cells in flow cytometric studies. After a one hour incubation period with vitamins, the cultures were washed free of vitamin and overlaid fresh MEM. Following a 24-hour of incubation period, the cells were harvested from the titer dishes and suspended in 0.1% NP-40 in a Tris-citrate solubilization buffer which contained propidium iodide (PI, 5 mg/ml) and 0.1% RNase A. Following a 30-minute incubation, DNA ploidy and cell cycle analysis was performed on a Ortho Cytoron flow cytometer. The data from  $2 \times 10^4$  cells were collected (when possible), stored, and analyzed using ModFit Cell Cycle Analysis.

### 2.5. Addition of Catalase

Titer plates were seeded and incubated as described in the cytotoxicity assay. After 24 h, the appropriate nontoxic concentrations of catalase and the vitamin test solutions were added to the wells and the titer plates were incubated at 37°C in 5% CO<sub>2</sub> for 1 h. The cells were subsequently washed with PBS, overlain with culture media and incubated for 5 days. Cytotoxicity was evaluated using the MTT assay.

## 2.6. Analysis of Lipid Peroxidation

Lipid peroxidation was evaluated using the thiobarbituric acid (TBA) method [61]. RT4 cells were treated and harvested as described in the thiol assay. After centrifugation, the cell pellets were resuspended in 6.0% TCA (trichloroacetic acid), mixed with 1 ml of 0.25 N HCl containing 0.375% TBA and 15% TCA, heated in a water bath for 15 min at 95°C and then allowed to cool. Following centrifugation the supernatant was monitored fluorimetrically for malondialdehyde (MDA) production using an excitation wavelength of 532 nm and an emission wavelength of 555 nm. Data was expressed as nM MDA per mg of protein, calculated on the basis of an MDA standard curve generated using 1,1,3,3-tetramethoxypropane.

## 2.7. Analysis of ATP

RT4 cells ( $1.0 \times 10^6$ ) were seeded and then incubated at 37°C and 5% CO<sub>2</sub>. After 24 h, the culture medium was removed and the cells were exposed for 1 h to culture media containing the vitamins at their CD<sub>90</sub> concentrations. The cells were then washed with PBS, overlaid with vitamin-free culture media and solubilized in somatic cell ATP releasing reagent (Sigma Chemical Co, St Louis, USA) at 1-h intervals for 6 h and intracellular ATP content was assayed using an ATP bioluminescent assay kit (Sigma, St Louis, U.S.A.) [62]. Bioluminescence was measured using a Beckman LS 9000 scintillation counter set for single photon counting. Data was expressed as nM ATP per mg of protein, calculated on the basis of an ATP standard curve.

## 2.8. Analysis of Protein Thiols

Thiols were assayed using the method of Nagelkerke and co-workers [63]. RT4 cells were exposed for 1 h to culture media containing the vitamins at their CD<sub>90</sub> concentrations. The cells were then washed with PBS, overlaid with vitamin-free culture media, trypsinized at 1-h intervals for 6 h and centrifuged for 5 min at 1000 rpm. The cell pellets were washed twice with 6.5% TCA and resuspended in 1 ml of 0.5 M Tris-HCl (pH 7.6). Subsequently, 50 µl of 10 mM methanolic Ellman's Reagent was added and the solution was incubated at room temperature. After 20 min, the solution was centrifuged for 5 min at 1000 rpm and the absorbance of the supernatant was measured at 412 nm. Data was expressed as µM thiols per mg of protein, calculated on the basis of a reduced glutathione (GSH) standard curve.

## 2.9. Statistics

Three-way ANOVA was performed using BMDP statistical software. In the three-way ANOVA, the two-way

interactions were tested at the 0.005 level of significance, while all other effects were tested at the 0.0022 level of significance. A summary of the experimental design is given in **Figure 1**.

## 3. Results

### 3.1. Cytotoxicity

VC, VK<sub>3</sub> and the VC:VK<sub>3</sub> combination with a VC:VK<sub>3</sub> ratio of 100:1 have been evaluated for their cytotoxicity against two human bladder carcinoma cell lines following continuous 5-day vitamin exposure or 1-h vitamin exposure followed by a 5-day incubation in media (**Table 1**). A continuous 5-day exposure to VC:VK<sub>3</sub> treatment of the RT-4 cells resulted in a 22-fold decrease of the CD<sub>50</sub> of VC (2430 to 110 µM) and a 12-fold decrease in the CD<sub>50</sub> of the VK<sub>3</sub> (12.8 to 1.10 µM) and a 12-fold decrease in the CD<sub>50</sub> of the VK<sub>3</sub> (12.8 to 1.10 µM) with an FIC value of 0.136 indicating that the combination was synergistic. T24 cells treated continuous for 5 days with VC:VK<sub>3</sub> resulted in a 41 fold decrease in VC (1,490 µM to 13.1 µM) and a 6-fold decrease in VK<sub>3</sub> (212 µM to 2.13 µM) with an FIC value of 0.158 also demonstrating a significant synergism after only 1hr of VC:VK<sub>3</sub> treatment.

Taper and his associates [34] have shown that the VC:VK<sub>3</sub> combination exhibited antitumor activity with exposure times as short as 1 h. We sought to determine if the vitamins would exert significant antitumor activity against RT-4 and T24 cells following a 1 h exposure (**Table 1**). A 1 h VC:VK<sub>3</sub> treatment of the RT-4 cells resulted in an 18-fold decrease in the CD<sub>50</sub> value of VC (4740 µM reduced to 267 µM) and a 22-fold decrease in VK<sub>3</sub> CD<sub>50</sub> values (60.7 µM reduced to 2.68 µM) with a FIC value of 0.100. The same VC:VK<sub>3</sub> 1hr treatment on T24 cells resulted in a 41-fold decrease in the CD<sub>50</sub> of VC (4970 µM reduced to 120 µM) and a 59-fold decrease in the CD<sub>50</sub> values of VK<sub>3</sub> (73.2 µM reduced to 1.21 µM) with an FIC of 0.093.

### 3.2. Flow Cytometry

Flow cytometry was also employed to determine whether vitamin treatment effects the cell cycle of RT4 cells. Human foreskin fibroblasts were mixed with RT4 cells and then analyzed by flow cytometry in an effort to determine the channel number of the true diploid G<sub>0</sub> - G<sub>1</sub> peak. The mean channel of the fibroblast G<sub>0</sub> - G<sub>1</sub> peak is 59 and the mean channel of the G<sub>2</sub> - M peak is 118. The mean channel of the RT4 cell G<sub>0</sub> - G<sub>1</sub> is 108, and the mean channel of the G<sub>2</sub> - M peak is 216. The DNA index (mean channel of RT4 G<sub>0</sub> - G<sub>1</sub>/mean channel of fibroblast G<sub>0</sub> - G<sub>1</sub>) is 1.83. This reading indicates that RT4 is an aneuploid cell line. In fact it is hypotetraploid. The

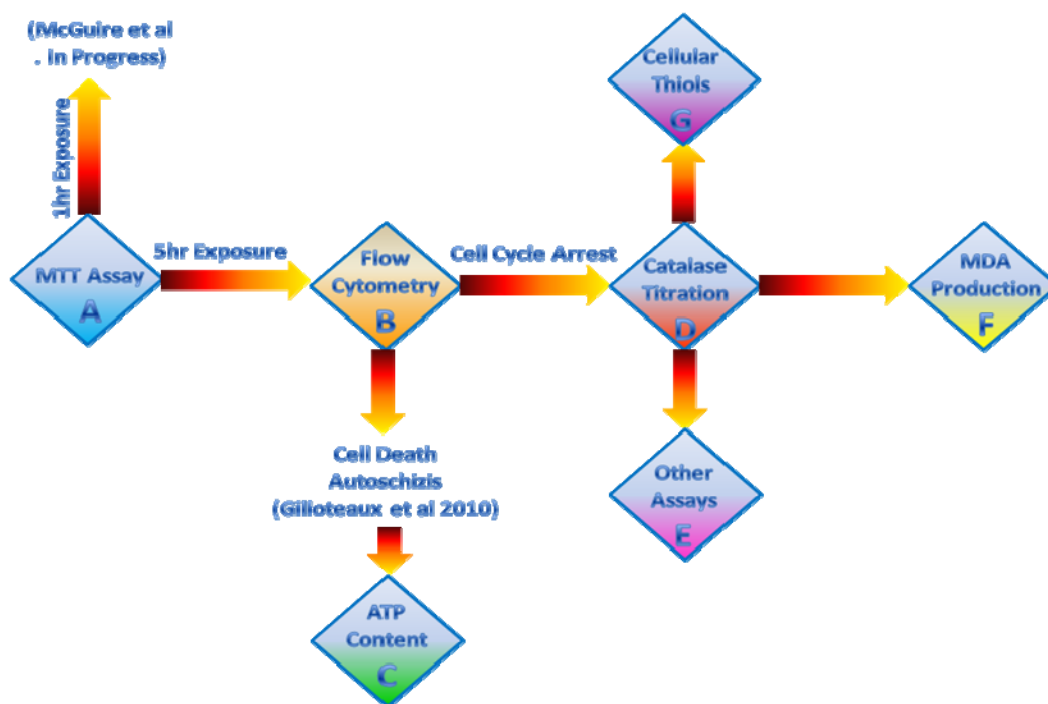


Figure 1. (A) An MTT assay was employed to measure VC:VK<sub>3</sub> induced cell cytotoxicity. Since VC:VK<sub>3</sub> was equally potent following a 1hr or 5 day exposure both values were reported. (B) Since the MTT value may be a composite measure of metabolic arrest, cell death and cell cycle arrest, cell cycle arrest was measured by flow cytometry and cell death by autoschizis was reported earlier. (C) Electron microscopy (Gilloteaux *et al.* 2010) showed that mitochondrial architecture was damaged by the VC:VK<sub>3</sub> combination, therefore changes in ATP levels were used to evaluate cellular energy content and to determine if cell death was ATP-dependant or ATP-independent. (D) The role of hydrogen peroxide in the activity of VC:VK<sub>3</sub> was determined by exogenous catalase titration. (E) If H<sub>2</sub>O<sub>2</sub> was not responsible for the antitumor activity other mechanistic activities were performed (not shown). (F) If H<sub>2</sub>O<sub>2</sub> were involved in the mechanism of action lipid peroxidation should occur. Therefore, malondialdehyde production was monitored. (G) VC:VK<sub>3</sub> have been reported to form a redox pair. If redox was involved there should be a concomitant decrease in cellular thiol content.

Table 1. Antitumor activity of vitamins against bladder carcinoma cells.

Cell Line	Incubation Time	Vitamins Alone		Vitamin Combination		FIC
		VC CD <sub>50</sub> (μM)	VK <sub>3</sub> CD <sub>50</sub> (μM)	VC CD <sub>50</sub> (μM)	VK <sub>3</sub> CD <sub>50</sub> (μM)	
RT4	1 h	4740 ± 27.2	60.7 ± 4.01	267 ± 4.04	2.68 ± 0.05	0.100
	5 days	2430 ± 28.3	12.8 ± 0.03	110 ± 9.73	1.10 ± 0.10	0.136
T24	1 h	4970 ± 27.4	73.2 ± 5.91	120 ± 7.0	1.21 ± 0.07	0.093
	5 days	1490 ± 141	13.1 ± 0.01	212 ± 7.6	2.13 ± 0.06	0.158

FIC =  $\frac{CD_{50}^{A \text{ comb}}}{CD_{50}^{A \text{ alone}}} + \frac{CD_{50}^{B \text{ comb}}}{CD_{50}^{B \text{ alone}}}$ , where  $CD_{50}^{A \text{ alone}}$  and  $CD_{50}^{B \text{ alone}}$  are 50% cytopathic doses of each vitamin alone;  $CD_{50}^{A \text{ comb}}$  and  $CD_{50}^{B \text{ comb}}$  are the 50% cytopathic doses of the vitamins administered together. Antitumor activity was measured by a MTT assay following a 1 h exposure and 5 day incubation or a 5 day exposure to VC, VK<sub>3</sub> or a vitamin combination with a VC:VK<sub>3</sub> ratio of 100:1. Values are the mean ± the standard error of the mean of three experiments with six readings per experiment.

distribution of the cells within the phases of the cell cycle can be found in **Table 2**. Like untreated RT4 cells, VC-treated RT4 cells exhibited a G<sub>0</sub> - G<sub>1</sub> peak in channel 115 and a G<sub>2</sub> - M peak in channel 231. An aneuploid shoulder was visible on the G<sub>0</sub> - G<sub>1</sub> peak in channel 130 and the trace also showed a small amount of sub-G<sub>0</sub> - G<sub>1</sub> "multi-cut debris". When compared to control cells, the VC-treated cells exhibited 34% of the cells in G<sub>0</sub> - G<sub>1</sub>

phase and 60% of cells in the S phase as opposed to 76% of the cells in G<sub>0</sub> - G<sub>1</sub> phase and 18% of the cells in S phase in control cells. VK<sub>3</sub>-treated cells showed a G<sub>0</sub> - G<sub>1</sub> peak in channel 121 and a G<sub>2</sub> - M peak in channel 241. Sub-G<sub>0</sub> - G<sub>1</sub> multi-cut debris was also evident. As a consequence of this treatment, the proportion of RT4 cells in the G<sub>0</sub> - G<sub>1</sub> phase decreased to 30% while the number of cells in S phase increased to 64%. VC:VK<sub>3</sub>-treated cells

**Table 2. Cell cycle distribution of the RT4 cells.**

Treatment	G <sub>0</sub> - G <sub>1</sub> phase	S-phase	G <sub>2</sub> -M phase
Vitamin C	34	60	6
Vitamin K <sub>3</sub>	30	64	6
Vitamin C and K <sub>3</sub>	53	37	10
RT4 Control	76	18	6
Fibroblast Control	89	1	10

RT4 cells were exposed to the vitamins at their 90% cytotoxic doses for 24 h and then harvested. DNA ploidy and cell cycle analysis was performed on a FACScan flow cytometer and analyzed using ModFit Cell Cycle Analysis. Sham-treated RT4 cells served as the negative control. Human foreskin fibroblasts served as diploid controls.

showed a G<sub>0</sub> - G<sub>1</sub> peak in channel 107 and a G<sub>2</sub> - M peak in channel 214. Sub-G<sub>0</sub> - G<sub>1</sub> multi-cut debris was also present. As a consequence of this treatment, the proportion of RT4 cells in the S phase and G<sub>2</sub>-M phase were 37 and 10% respectively, compared with 18% and 6% for control cells.

### 3.3. Hydrogen Peroxide

In our previous studies, catalase administration to DU145 and T24 cells was shown to abrogate the antitumor activity of the vitamins at catalase doses as low as 100 µg/ml [17,45]. Therefore, administration of exogenous catalase has been employed to elucidate the role of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in the antitumor activity of vitamins. Catalase administration to RT4 cells abrogated the antitumor activity of VC at catalase doses as low as 100 µg/ml (**Table 3**). The majority of the antitumor activity of the vitamin combination was lost at a catalase concentration of 100 µg/ml. However, the antitumor activity of VK<sub>3</sub> could not be completely neutralized by the administration of catalase even at concentrations as high as 1000 µg/ml. Conversely, the antitumor activity of VC:VK<sub>3</sub> was lost following administration of as little as 300 µg/ml of catalase. These results demonstrated that H<sub>2</sub>O<sub>2</sub> production was necessary for the antitumor activity of the vitamins.

### 3.4. Lipid Peroxidation

Exposure of tumor cells to VC, VK<sub>3</sub> or the VC:VK<sub>3</sub> combination has been shown to generate hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and other reactive oxygen species (ROS) that may initiate membrane lipid peroxidation [17,45]. Therefore the effect of vitamin treatment on cellular lipid peroxidation (**Table 4**) was examined using the thiobarbituric acid method. The lipid peroxidation of sham-treated RT-4 cells displayed an average value of 3.17 nM(MDA)/mg of protein. However, this is only a measure of the lipid peroxidation that occurs during the heating

**Table 3. Effect of catalase treatment of RT-4 cells antitumor activity.**

Catalase Concentration (µg/ml)	Antitumor Activity of Vitamin C (% of control)	Antitumor Activity of Vitamin K <sub>3</sub> (% of control)	Antitumor Activity of VC:VK <sub>3</sub> (% of control)
0	100	100	100
100	0	63	13
200	0	22	9
300	0	15	0
400	0	11	0
500	0	11	0
1000	0	0	0

RT4 cells were incubated with the vitamins and increasing doses of catalase for 1 hour. The tumor cells were subsequently washed with PBS and overlain with culture media. Cytotoxicity was evaluated after 5 days using a MTT assay. Values are the mean of three experiments with three readings per experiment.

**Table 4. Vitamin-induced lipid peroxidation in RT-4 cells.**

Time in Hours	Control	VC	VK <sub>3</sub>	VC:VK <sub>3</sub>
1	3.11 ± 0.23 <sup>†</sup>	3.44 ± 0.40	4.34 ± 0.49	6.40 ± 0.21
2	2.94 ± 0.45	3.21 ± 0.07	4.70 ± 0.40	5.70 ± 0.21
3	3.01 ± 0.64	3.98 ± 0.20	5.84 ± 0.21	5.27 ± 0.21
4	3.42 ± 0.46	4.27 ± 0.60	5.77 ± 0.50	5.55 ± 0.61
5	3.39 ± 0.72	3.48 ± 0.30	4.27 ± 0.21	4.98 ± 0.41

<sup>†</sup>nM of MDA/mg of protein. RT-4 cells were treated for with the vitamins at their CD<sub>90</sub> doses, harvested at one hour intervals for 5 h and assayed for lipid peroxidation using the thiobarbituric acid method. Malondialdehyde (MDA) production was monitored fluorimetrically and data was expressed as nM MDA per mg of protein, calculated on the basis of a MDA standard curve. Values are the mean ± standard error of the mean of three experiments with three readings per experiment and were compared to the control.

of samples to 95°C during the assay and can, therefore, be considered as a baseline for MDA production. Lipid peroxidation values following VC treatment peaked at 4.27 nM/mg with an average value of 3.67 nM/mg, while lipid peroxidation of VK<sub>3</sub>-treated cells was significantly higher at 5.84 nM/mg with an average of 4.98 nM/mg. Lipid peroxidation values for VC:VK<sub>3</sub> peaked at 6.7 nM/mg with an average value of 5.58 nM/mg of protein. The treatment of the cells with the vitamins resulted in a statistically significant alteration in lipid peroxidation (p < 0.005). This lipid peroxidation was vitamin related because lipid peroxidation values rapidly returned to control levels when the vitamins were removed (data not shown).

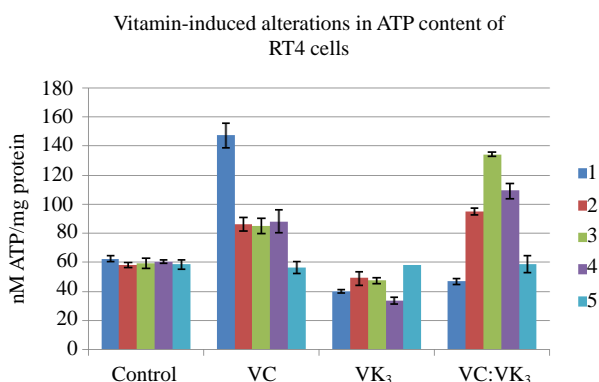
### 3.5. ATP Production

Transmission electron microscopy has shown that mito-

chondrial architecture was altered by vitamin treatment [36,63]. In the following experiments, intracellular levels of ATP synthesis was measured to determine if vitamin-induced cell death was related to mitochondrial damage and subsequent “ATP-less” cell death (Figure 2). The ATP content of sham treated RT4 cells displayed averaged of 59.64 nM ATP/mg of protein. VC exposure resulted in an increase in ATP levels to  $147 \pm 8.64$  nM during the first hour. Subsequently, the ATP levels decreased to  $86.0 \pm 4.73$  nM during the second hour and remained relatively constant during the third and fourth hours and then fell to  $56.1 \pm 4.09$  nM during the final hour. VK<sub>3</sub> treatment lowered ATP levels to  $39.9 \pm 0.99$  nM during the first hour. ATP levels rose slightly to  $48.8 \pm 4.52$  nM during the second hour, remained relatively constant for the next 3 hours and increased to near control levels during the final hour. The VC:VK<sub>3</sub> combination produced a slight decrease in ATP concentration to  $46.7 \pm 2.13$  nM during the first hour. ATP levels increased during the second and third hours to  $134 \pm 1.46$  nM and decreased gradually to near control levels during the final two hours. These results demonstrate that pulse treatment of RT4 cells with VC alone or with the VC:VK<sub>3</sub> combination resulted in a transient increase in intracellular ATP levels following vitamin treatment. The treatment of the cells with the vitamins resulted in a significant alteration in ATP levels ( $p < 0.005$ ).

### 3.6. Thiols

Administration of VK<sub>3</sub> to hepatocytes is known to induce a variety of effects including: depletion of GSH and oxidation of protein sulphhydryl groups in cytoskeletal



**Figure 2.** Cultures of exponentially growing RT4 cells were treated for 1 hour with the vitamins at their CD<sub>90</sub> doses and then harvested at one hour intervals for 5 h. ATP content was assayed using a bioluminescence assay. Data has been expressed as nM ATP per mg of protein, calculated on the basis of an ATP standard curve. Values are the mean  $\pm$  standard error of the mean of three experiments with three readings per experiment and were compared to the control ( $p < 0.05$  between all groups relative to control).

proteins [35,65]. Therefore, the effect of vitamin treatment on cellular thiols has been examined (Table 5). The thiol content of sham-treated RT4 cells averaged  $1.39 \mu\text{M/mg}$  of protein. VC treatment resulted in a decrease in cellular thiol levels to  $0.92 \pm 0.31 \mu\text{M/mg}$  during the first hour. Subsequently, the thiol level remained constant during the second hour, dropped precipitously to  $0.47 \pm 0.03 \mu\text{M/mg}$  during the third hour, rebounded to  $0.73 \pm 0.12 \mu\text{M/mg}$  of during the fourth hour and then returned to  $0.45 \pm 0.03 \mu\text{M/mg}$  during the final hour. VK<sub>3</sub> treatment lowered thiol levels to  $0.62 \pm 0.5 \mu\text{M/mg}$  during the first hour. Thiol levels remained constant during the next three hours and then dropped slightly to  $0.54 \pm 0.1 \mu\text{M/mg}$  during the final hour. The VC:VK<sub>3</sub> combination produced a decrease in thiol concentration to  $0.63 \pm 0.05 \mu\text{M/mg}$  during the first hour. Thiol levels gradually decreased to  $0.45 \pm 0.03 \mu\text{M/mg}$  during the second hour and then remained constant. VC:VK<sub>3</sub> treated cells induced significant depletion of cellular thiols, The treatment of the cells with the vitamins resulted in a significant alteration in thiol levels ( $p < 0.005$ ).

## 4. Discussion

VC exhibits selective toxicity against a plethora of tumor cell lines as well as experimental tumors [64,65]. In addition, VC is a chemosensitizing agent [66,67] and radiosensitizing agent [68]. The mechanism(s) responsible for the antitumor activity of VC appears to be related to the prooxidant properties of ascorbate and dehydroascorbate, the oxidative product of ascorbate, which generate intracellular H<sub>2</sub>O<sub>2</sub> and other reactive oxygen species (ROS) which may deplete cellular thiol levels and initiate membrane lipid peroxidation [45,66].

Likewise, VK<sub>3</sub> is a synthetic derivative of phylloquinone (VK<sub>1</sub>) that exhibits *in vitro* cytotoxic activity against a variety of tumor cell lines [69] as well as *in*

**Table 5.** Vitamin-induced alterations in the thiol content of RT4 cells.

Time in Hours	Control	VC	VK <sub>3</sub>	VC:VK <sub>3</sub>
1	$1.37 \pm 0.28$	$0.92 \pm 0.31$	$0.62 \pm 0.05$	$0.63 \pm 0.05$
2	$1.42 \pm 0.38$	$0.94 \pm 0.02$	$0.65 \pm 0.12$	$0.45 \pm 0.03$
3	$1.38 \pm 0.72$	$0.47 \pm 0.03$	$0.67 \pm 0.04$	$0.47 \pm 0.04$
4	$1.46 \pm 0.45$	$0.73 \pm 0.12$	$0.59 \pm 0.03$	$0.52 \pm 0.03$
5	$1.33 \pm 0.27$	$0.45 \pm 0.03$	$0.54 \pm 0.01$	$0.47 \pm 0.02$

$\mu\text{M}$  of Thiol/mg of protein. RT4 cells were treated for 1 hour with the vitamins at their CD<sub>90</sub> doses, harvested at one hour intervals for 5 h and assayed for cellular thiol content by monitoring absorbance following reaction with Ellman's Reagent. Data has been expressed as  $\mu\text{M}$  thiol per mg of protein, calculated on the basis of a GSH standard curve. Values are the mean  $\pm$  standard error of the mean of three experiments with three readings per experiment and were compared to the control ( $p < 0.005$ ).

*in vivo* antitumor activity [70]. VK<sub>3</sub> is also a chemosensitizer for most of traditional chemotherapeutic agents [70]. Administration of VK<sub>3</sub> to tumor cells induces depletion of glutathione, reduction of nicotinamide adenine dinucleotide phosphate and adenosine triphosphate pools, oxidation of sulfhydryl groups in cytoskeletal proteins, and induction of single-stranded DNA breaks [71-73].

When VC and VK<sub>3</sub> were combined in a ratio of 100:1 and administered to three human tumor cell lines, the vitamin combination exhibited a synergistic inhibition of cell growth at concentrations that were 10 to 50 times lower than for the individual vitamins [74]. The vitamin combination also potentiated the *in vitro* growth inhibitory activities of several chemotherapeutic agents 3- to 14-fold [75]. The VC:VK<sub>3</sub> combination also inhibited tumor growth, increased lifespan and decreased metastasis of C3H mice implanted with a murine transplantable liver tumor (TLT) cells [34-37,49]. Additional studies indicated that the VC:VK<sub>3</sub> combination was an effective chemosensitizer [34,35] and radiosensitizer that induced little systemic or major organ pathology [35,36]. The potentiation and specificity of the antitumor activity was attributed to the possible generation of hydrogen peroxide followed by membrane lipid alteration, DNase activation and DNA destruction by the VC:VK<sub>3</sub> combination in the catalase-deficient cancer cells [35]. When VC and VK<sub>3</sub> were combined in a 100:1 ratio, the VC:VK<sub>3</sub> interaction not only fostered single-electron reduction to produce the long-lived semiquinone and ascorbyl radicals and increased the rate of redox cycling of the quinone to form H<sub>2</sub>O<sub>2</sub> and other ROS, but also fostered two-electron transfer, which ensured that ascorbic acid (AA) and dehydroascorbic acid (DHA) were present at pharmacologic levels for a protracted period of time [24,50,76,77]. Single electron redox cycling generated a moderate increase in oxidative stress [24,50,76] and induced a complex stress response that resulted in structural damage to the catalase-deficient cancer cells. A number of cellular processes were effected by the presence of AA and especially DHA, including: modulation of signal transduction, cell cycle arrest and inhibition of glycolytic respiration, inhibition of metastasis [15,23,24,26,35,49,50,78,79]. Together the two redox cycles reactivated DNase I and II and induced autoschizic cell death [20,23,46]. A number of other laboratories have subsequently have modified or added more components to the VC:VK<sub>3</sub> combination. Modification of one of the constituents (*i.e.* brominated VK<sub>3</sub>) or replacement of the VK<sub>3</sub> with any number of constituents (arsenic trioxide, benzoquinones, coenzyme Q10, doxorubicin, lipoic acid, resveratrol, vitamin B12, Vitamin E and others) [67,80-85].

In the current study, VC, VK<sub>3</sub> and the VC:VK<sub>3</sub> combination were evaluated for their antitumor activity against two human bladder cancer cell lines (RT4 and

T24). An MTT assay was employed to demonstrate that a VC:VK<sub>3</sub> combination with a VC:VK<sub>3</sub> ratio of 100:1 exhibited synergistic antitumor activity against both the RT4 and the T24 cell lines following continuous 5-day vitamin exposure or a 1-h vitamin exposure followed by a 5-day incubation in culture media. The fact that there was a close agreement between the CD<sub>50</sub> values of the VC:VK<sub>3</sub> combination for the 1-h and 5-day vitamin exposures indicated that the antitumor activity generated during a 1-h vitamin treatment was almost as effective as the antitumor activity generated during a continuous 5-day vitamin treatment. These results were consistent with those found in both prostate cancer (DU145) and bladder cancer (T24) cell lines and suggested some of the events responsible for triggering tumor cell death occurred during the first hour of vitamin treatment [17,45]. Characterization of the 5 day vitamin treatment is presented in this report while characterization of the 1-h vitamin treatment will be presented in a future report. In a previous study with T24 cells, flow cytometry revealed that VC:VK<sub>3</sub> treatment resulted in a growth arrested population of cells and a population of cells undergoing autoschizic cell death [24].

Likewise, in the current study, a cell cycle block was observed at G<sub>0</sub> - G<sub>1</sub>/S. From previous studies, it is known that enhanced population of cells in early S phase represent both growth arrested and autoschizic cells [24]. The disposition of these autoschizic cells was described in a previous publication [22]. Similar results were seen in an *in vivo* study when UMUC-14 tumorigenic urothelial carcinoma cells were implanted into the subcutis of nude mice and the mice were treated with gemcitabine (GEM), VC:VK<sub>3</sub> or a GEM-VC:VK<sub>3</sub> combination. *In vivo* analysis suggested that synergistic antitumor activity was due to antiproliferative effects rather than to enhanced apoptosis. However, there was significant necrosis in combination group tumors which was most likely autoschizis [28]. From our experience with VC:VK<sub>3</sub>, these effects can be attributed to the combined effects of H<sub>2</sub>O<sub>2</sub> and the vitamins [26].

When RT4 cells were co-incubated with catalase and VC, VK<sub>3</sub> or the VC:VK<sub>3</sub> combination, the antitumor activity of the vitamins was negated. The fact that a greater amount of catalase was required to destroy the antitumor activity of VK<sub>3</sub> than was required to destroy the antitumor activity of the vitamin combination, suggested that while H<sub>2</sub>O<sub>2</sub> was involved in the mechanism of action of these vitamins, the enhanced antitumor activity of the vitamin combination was not simply due to an excessive increase in H<sub>2</sub>O<sub>2</sub> production. Furthermore, the fact that the majority of VC is known to enter tumor cells through Glut channels as DHA coupled with the observation that antitumor activity of the vitamins was abrogated by exogenous catalase suggested that at least a portion of lost



antitumor activity of VC and VC:VK<sub>3</sub> in the presence of catalase may be a function of diminished extracellular conversion of VC to DHA and a decreased uptake of the vitamin by the tumor cells [86].

The observation that H<sub>2</sub>O<sub>2</sub> generation was essential for the antitumor activity of the vitamin combination suggested that lipid peroxidation may have been responsible for the vitamin-induced cytotoxicity. When the RT4 cells were co-incubated with the vitamins for 1 - 5 h, the level of lipid peroxidation increased 1.1- to 1.3-fold for VC, 1.3- to 1.9-fold for VK<sub>3</sub> and 1.5- to 2-fold for VC:VK<sub>3</sub>. These results suggested that, while lipid peroxidation increased, the modest increase in lipid peroxidation was not the primary cause of the cytotoxicity of the vitamin combination. These results were consistent with those of McGuire and co-workers [87] who demonstrated that when the vitamins were removed from the RT4 cells following a 1-h exposure, lipid peroxidation values returned to near control levels while the cells continued to undergo autophagocytosis.

Since VC:VK<sub>3</sub> treatment of RT4 cells was shown to produce severely damaged mitochondria [26], intracellular levels of ATP were measured to determine if vitamin induced cell death was related to the depletion of ATP. Treatment of RT4 cells with VK<sub>3</sub> decreased intracellular ATP levels to approximately 60% to 80% of control levels, while following exposure to VC, intracellular ATP levels increased 2.4-fold during the first hour, decreased to about 1.5-fold greater than control during the second hour and remained constant for the next two hours. Following VC:VK<sub>3</sub> exposure, intracellular ATP levels decreased 25% during the first hour, rose to a maximum of about 2.3-fold greater than control by 3 hours and then began to decrease. Interestingly, the fact that the level of intracellular ATP levels for all treatments decreased to control levels by the fifth hour may be indicative of the duration of the pharmacological activity of the vitamins in RT4 cells. These results are consistent with those seen in prostate cancer cells (DU145) and another bladder cancer cell line (T24) and suggested that ATP depletion was not the proximal cause of VC:VK<sub>3</sub>-induced tumor cell death in RT4 cells [17,45]. While the cause of these ATP spikes has not yet been elucidated, they may reflect the ability of the VC:VK<sub>3</sub> to form a shunt around a defective region of complex III of the electron transport chain by having menadione accept electrons from coenzyme Q (ubiquinone), shuttle them to ascorbate and then to cytochrome c. Impaired oxidative phosphorylation has been observed in a variety of cancers including: prostate tumors, and bladder (infiltrating bladder urothelial) carcinomas due to alterations in the protein complexes, especially in complex III [88,89]. Such a shunt was observed in a patient with a defect in electron transport at complex III in skeletal muscle [91]. The shunt was able

to bypass the antimycin-a-sensitive site in both forward and reversed electron transport (had two intact phosphorylation sites) and produced a shift from glycolytic activity to increased mitochondrial oxidative phosphorylation and a diminution of lactic acidosis.

The effect of vitamin treatment on cellular thiols was examined because redox cycling was shown to draw down cell thiols [71-73,77,78,87]. Following a one hour VC treatment, the intracellular thiol levels of RT4 decreased to 67% of control levels. Thiol levels remained at this level for another hour and then decreased to 34% of control levels. Subsequently, thiol levels oscillated between 34% and 50% of control levels. Exposure of RT4 cells to VK<sub>3</sub> led to a drop in intracellular levels to 45% of that of control levels. Following a one hour VC:VK<sub>3</sub> treatment, the intracellular thiol levels of RT4 decreased to 46% of control levels. In the second hour, intracellular thiol levels decreased to approximately 35% of control levels and then remained constant for the remaining 3 hours. These results suggested that the cells were only affected by vitamin treatment when the oxidative stress of the vitamins surpassed the reducing ability of the cellular thiols and cellular or genetic damage occurred. Tumor cells appeared to be particularly susceptible because they have reduced levels of catalase, superoxide dismutase, and/or glutathione peroxidase as well as other ROS detoxifying enzymes. Therefore, they have difficulty in metabolizing hydrogen peroxide and other ROS that can accumulate, alter cellular processes, and induce cellular damage or cell death [74].

While the morphologic trends and defects observed following combined vitamin treatment were the composite of the biochemical and cytological damage induced by ascorbate or the menadione-treated alone, the damage induced by VC:VK<sub>3</sub> was seen at a VC concentration of 520  $\mu$ M and a VK<sub>3</sub> concentration of 5.2  $\mu$ M concentrations which were 17-fold less than the concentrations of 8750 and 90  $\mu$ M when VC and VK<sub>3</sub> were administered individually. The values indicated a synergistic interaction between VC and VK<sub>3</sub>. The vitamin combination was shown to be safe and effective against human prostate cancer cell lines in vitro [44,45], against androgen-independent prostate cancer in nude mice [47] and in two clinical trials with endstage prostate cancer patients [51,55]. Likewise, VC:VK<sub>3</sub> was shown to be safe and effective against human bladder cancer cell lines [17-27] and in a murine bladder cancer model when administered alone or in conjunction with gemcitabine [28].

## 5. Conclusion

While the results described in the current study provide information concerning the effect of VC:VK<sub>3</sub> administration on tumor cells death, cell cycle arrest, cellular ATP

levels, the effect of redox cycling on cellular thiol levels and confirm the role of H<sub>2</sub>O<sub>2</sub> in lipid peroxidation and tumor cell death, the major limitation of these techniques is that they provide no information concerning the genes and signal transduction mechanisms involved these processes. The authors concluded that, based on their results, a clinical trial was warranted to examine the efficacy and toxicity of VC:VK<sub>3</sub>. Because of these results, on July 31, 2007, the combination of sodium ascorbate and menadione sodium bisulfite (tradename Apatone<sup>®</sup>, designation request #06-2366) was granted orphan drug status for the treatment of metastatic or locally advanced, inoperable transitional cell carcinoma of the urothelium (stage III and IV bladder cancer). Efforts are underway to conduct a phase II clinical trial for this indication.

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