Synergistic Antitumor Activity of Vitamins C and K3 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 K3 (VK3) alone, or with a VC:VK3 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 G0/G1-S-phase interface, while cell death was due to autoschizis. Catalase treatment abrogated both cell cycle arrest and cell death which implicated hydrogen peroxide (H2O2) in these processes. The H2O2 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:VK3 combination, but decreased ATP levels following VK3 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 K3

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 than current agents [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, B6, C, E, and K3 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 me-
gadose vitamins daily vs the recommended daily allow-
ance 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 K3 for the treatment of:
acute lymphoblastic leukemia [13], acute myelogenous
leukemia [14-16], bladder [17-28], breast [29], glioblas-
toma [30], glioma [31], kidney [32], liver [33-37], lung
[38], ovarian [39-42] and prostate cancers [43-55]. Un-
like the majority of chemotherapeutic agents which target
rapidly dividing cells, VC:VK3 appears to target tumor
cells by inflammation [50]. Tumor cells possess a greater
need for glucose than normal cells and express facilita-
tive glucose transporters (GLUTs) to achieve this task.
Because of the structural resemblance of dehydroascor-
bic acid (DHA, the oxidized form of vitamin C) to glu-
cose, DHA can also enter the tumor cells and bio-accu-
ulate. Epithelial tumors appear to rely on superoxide
(inflammation) which is produced constitutively via NA
DPH oxidase of non-neoplastic stromal cells to oxidize
the ascorbic acid [56,57]. Once dehydroascorbic acid en-
ters 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, VK1 and the VC:VK3 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:VK3 combination.

2. Materials and Methods

2.1. Culture Conditions

Human bladder cancer cell lines (T24 and RT4) were
obtained from the American Type Culture Collection
(ATTC, Rockville, MD, USA) and were grown in cul-
ture medium according to ATTC instructions. All media
was supplemented with 10% fetal bovine serum (FBS,
Gibco, Grand Island, NY) and 50 μg/mL gentamicin sul-
fate (Sigma, St Louis, MO). All incubations were per-
formed at 37°C and with 5% CO2 unless other conditions
are stated. Vitamin C (VC) and menadione bisulfite (VK3)
were purchased from Sigma Chemical Company (St
Louis, MO, USA) and were dissolved in phosphate-buff-
ered saline (PBS) to create 8000 μM VC, 500 μM VK3
and 8000 μM VK3. To prevent photodegradation of the vitamins, all the vitamin solu-
tions were prepared and experiments were performed in a
darkened laminar flow hood.

2.2. Protein Concentration Assay

In all experiments total protein concentration was deter-
mined 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 de-
scribed previously [23]. Corning 96-well titer plates were
seeded with tumor cells (5 × 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 CD50 was calcu-
lated. The fractional inhibitory concentration index (FIC)
was employed to evaluate synergism.

2.4. Flow Cytometry

Determination of cell DNA content and ploidy were per-
formed according to our previously published procedure
[45]. Briefly, titer dishes were seeded with 1.0 × 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% RNase A. Following a 30-minutes incubation, DNA
ploidy and cell cycle analysis was performed on a Ortho
Cytoron flow cytometer. The data from 2 × 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 non-
toxic concentrations of catalase and the vitamin test solu-
tions were added to the wells and the titer plates were
incubated at 37°C in 5% CO2 for 1 h. The cells were
subsequently washed with PBS, overlaid with culture
media and incubated for 5 days. Cytotoxicity was evalu-
ated 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 × 10⁶) were seeded and then incubated at 37°C and 5% CO₂. After 24 h, the culture medium was removed and the cells were exposed for 1 h to culture media containing the vitamins at their CD₉₀ 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 CD₉₀ 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, VK₃ and the VC:VK₃ combination with a VC:VK₃ 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:VK₃ treatment of the RT-4 cells resulted in a 22-fold decrease of the CD₅₀ of VC (2430 to 110 µM) and a 12-fold decrease in the CD₅₀ of the VK₃ (12.8 to 1.10 µM) and a 12-fold decrease in the CD₅₀ of the VK₃ (12.8 to 1.10 µM) with an FIC value of 0.136 indicating that the combination was synergistic. T24 cells treated continuously for 5 days with VC:VK₃ resulted in a 41-fold decrease in VC (1,490 µM to 13.1 µM) and a 6-fold decrease in VK₃ (212 µM to 2.13 µM) with an FIC value of 0.158 also demonstrating a significant synergism after only 1 hr of VC:VK₃ treatment.

Taper and his associates [34] have shown that the VC:VK₃ 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:VK₃ treatment of the RT-4 cells resulted in an 18-fold decrease in the CD₅₀ value of VC (4740 µM reduced to 267 µM) and a 22-fold decrease in VK₃ CD₅₀ values (60.7 µM reduced to 2.68 µM) with a FIC value of 0.100. The same VC:VK₃ 1hr treatment on T24 cells resulted in a 41-fold decrease in the CD₅₀ of VC (4970 µM reduced to 120 µM) and a 59-fold decrease in the CD₅₀ values of VK₃ (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 G₀ - G₁ peak. The mean channel of the fibroblast G₀ - G₁ peak is 59 and the mean channel of the G₂ - M peak is 118. The mean channel of the RT4 cell G₀ - G₁ is 108, and the mean channel of the G₂ - M peak is 246. The DNA index (mean channel of RT4 G₀ - G₁/mean channel of fibroblast G₀ - G₁) is 1.83. This reading indicates that RT4 is aneuploid cell line. In fact it is hypotetraploid. The
Figure 1. (A) An MTT assay was employed to measure VC:VK3 induced cell cytotoxicity. Since VC:VK3 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 autolysis was reported earlier. (C) Electron microscopy (Gilloteaux et al. 2010) showed that mitochondrial architecture was damaged by the VC:VK3 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:VK3 was determined by exogenous catalase titration. (E) If H2O2 was not responsible for the antitumor activity other mechanistic activities were performed (not shown). (F) If H2O2 were involved in the mechanism of action lipid peroxidation should occur. Therefore, malondialdehyde production was monitored. (G) VC:VK3 have been reported to form a redox pair. If redox was involved there should be a concomitant decrease in cellular thiol content.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Incubation Time</th>
<th>Vitamins Alone (CD50) µM</th>
<th>Vitamin Combination (CD50) µM</th>
<th>FIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT4</td>
<td>1 h</td>
<td>4740 ± 27.2</td>
<td>60.7 ± 4.01</td>
<td>2.68 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>5 days</td>
<td>2430 ± 28.3</td>
<td>12.8 ± 0.03</td>
<td>110 ± 9.73</td>
</tr>
<tr>
<td>T24</td>
<td>1 h</td>
<td>4970 ± 27.4</td>
<td>73.2 ± 5.91</td>
<td>120 ± 7.0</td>
</tr>
<tr>
<td></td>
<td>5 days</td>
<td>1490 ± 141</td>
<td>13.1 ± 0.01</td>
<td>212 ± 7.6</td>
</tr>
</tbody>
</table>

FIC = CD50A comb/CD50A alone + CD50B comb/CD50B alone, where CD50A alone and CD50B alone are 50% cytopathic doses of each vitamin alone; CD50A comb and CD50B 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, VK3 or a vitamin combination with a VC:VK3 ratio of 100:1. Values are the mean ± the standard error of the mean of three experiments with six readings per experiment.

Table 2. Antitumor activity of vitamins against bladder carcinoma cells.

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 G0 - G1 peak in channel 115 and a G2 - M peak in channel 231. An aneuploid shoulder was visible on the G0 - G1 peak in channel 130 and the trace also showed a small amount of sub-G0 - G1 “multi-cut debris”. When compared to control cells, the VC-treated cells exhibited 34% of the cells in G0 - G1 phase and 60% of cells in the S phase as opposed to 76% of the cells in G0 - G1 phase and 18% of the cells in S phase in control cells. VK2-treated cells showed a G0 - G1 peak in channel 121 and a G2 - M peak in channel 241. Sub-G0 - G1 multi-cut debris was also evident. As a consequence of this treatment, the proportion of RT4 cells in the G0 - G1 phase decreased to 30% while the number of cells in S phase increased to 64%. VC:VK2-treated cells
Table 2. Cell cycle distribution of the RT4 cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>G0 - G1 phase</th>
<th>S-phase</th>
<th>G2-M phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin C</td>
<td>34</td>
<td>60</td>
<td>6</td>
</tr>
<tr>
<td>Vitamin K3</td>
<td>30</td>
<td>64</td>
<td>6</td>
</tr>
<tr>
<td>Vitamin C and K3</td>
<td>53</td>
<td>37</td>
<td>10</td>
</tr>
<tr>
<td>RT4 Control</td>
<td>76</td>
<td>18</td>
<td>6</td>
</tr>
<tr>
<td>Fibroblast Control</td>
<td>89</td>
<td>1</td>
<td>10</td>
</tr>
</tbody>
</table>

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.

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<td>1</td>
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</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Catalase Concentration (µg/ml)</th>
<th>Antitumor Activity of Vitamin C (% of control)</th>
<th>Antitumor Activity of Vitamin K3 (% of control)</th>
<th>Antitumor Activity of VC:VK3 (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>63</td>
<td>13</td>
</tr>
<tr>
<td>200</td>
<td>0</td>
<td>22</td>
<td>9</td>
</tr>
<tr>
<td>300</td>
<td>0</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>400</td>
<td>0</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>500</td>
<td>0</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>1000</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

RT4 cells were incubated with the vitamins and increasing doses of catalase for 1 h. 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.

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<tr>
<td>0</td>
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<td>13</td>
</tr>
<tr>
<td>200</td>
<td>0</td>
<td>22</td>
<td>9</td>
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<tr>
<td>300</td>
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<td>500</td>
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<td>11</td>
<td>0</td>
</tr>
<tr>
<td>1000</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

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 (H2O2) 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 VK3 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:VK3 was lost following administration of as little as 300 µg/ml of catalase. These results demonstrated that H2O2 production was necessary for the antitumor activity of the vitamins.

3.4. Lipid Peroxidation

Exposure of tumor cells to VC, VK3 or the VC:VK3 combination has been shown to generate hydrogen peroxide (H2O2) 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. 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.

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

<table>
<thead>
<tr>
<th>Time in Hours</th>
<th>Control</th>
<th>VC</th>
<th>VK3</th>
<th>VC:VK3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.11 ± 0.23†</td>
<td>3.44 ± 0.40</td>
<td>4.34 ± 0.49</td>
<td>6.40 ± 0.21</td>
</tr>
<tr>
<td>2</td>
<td>3.01 ± 0.45</td>
<td>3.21 ± 0.07</td>
<td>4.70 ± 0.40</td>
<td>5.70 ± 0.21</td>
</tr>
<tr>
<td>3</td>
<td>3.01 ± 0.64</td>
<td>3.98 ± 0.20</td>
<td>5.84 ± 0.21</td>
<td>5.27 ± 0.21</td>
</tr>
<tr>
<td>4</td>
<td>3.42 ± 0.46</td>
<td>4.27 ± 0.60</td>
<td>5.77 ± 0.50</td>
<td>5.55 ± 0.61</td>
</tr>
<tr>
<td>5</td>
<td>3.39 ± 0.72</td>
<td>3.48 ± 0.30</td>
<td>4.27 ± 0.21</td>
<td>4.98 ± 0.41</td>
</tr>
</tbody>
</table>

†nM of MDA/mg of protein. RT-4 cells were treated with the vitamins at their CD90 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.

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 ± 8.64 nM during the first hour. Subsequently, the ATP levels decreased to 86.0 ± 4.73 nM during the second hour and remained relatively constant during the third and fourth hours and then fell to 56.1 ± 4.09 nM during the final hour. VK3 treatment lowered ATP levels to 39.9 ± 0.99 nM during the first hour. ATP levels rose slightly to 48.8 ± 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:VK3 combination produced a slight decrease in ATP concentration to 46.7 ± 2.13 nM during the first hour. ATP levels increased during the second and third hours to 134 ± 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:VK3 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 VK3 to hepatocytes is known to induce a variety of effects including: depletion of GSH and oxidation of protein sulphydryl groups in cytoskeletal 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 µM/mg of protein. VC treatment resulted in a decrease in cellular thiol levels to 0.92 ± 0.31 µM/mg during the first hour. Subsequently, the thiol level remained constant during the second hour, dropped precipitously to 0.47 ± 0.03 µM/mg during the third hour, rebounded to 0.73 ± 0.12 µM/mg of during the fourth hour and then returned to 0.45 ± 0.03 µM/mg during the final hour. VK3 treatment lowered thiol levels to 0.62 ± 0.5 µM/mg during the first hour. Thiol levels remained constant during the next three hours and then dropped slightly to 0.54 ± 0.1 µM/mg during the final hour. The VC:VK3 combination produced a decrease in thiol concentration to 0.63 ± 0.05 µM/mg during the first hour. Thiol levels gradually decreased to 0.45 ± 0.03 µM/mg during the second hour and then remained constant. VC:VK3 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 antitumour activity of VC appears to be related to the prooxidant properties of ascorbate and dehydroascorbate, the oxidative product of ascorbate, which generates intracellular H2O2 and other reactive oxygen species (ROS) which may deplete cellular thiol levels and initiate membrane lipid peroxidation [45,66].

Likewise, VK3 is a synthetic derivative of phylloquinone (VK1) that exhibits in vitro cytotoxic activity against a variety of tumor cell lines [69] as well as in 

![Figure 2. Cultures of exponentially growing RT4 cells were treated for 1 hour with the vitamins at their CD₉₀ 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 ± 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).](image-url)
vivo antitumor activity [70]. VK$_3$ is also a chemosensitizer for most of traditional chemotherapeutic agents [70]. Administration of VK$_3$ to tumor cells induces depletion of glutathione, reduction of nicotinamide adenine dinucleotide phosphate and adenosine triphosphate pools, oxidation of sulphydryl groups in cytoskeletal proteins, and induction of single-stranded DNA breaks [71-73].

When VC and VK$_3$ 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$_3$ 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$_3$ 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$_3$ combination in the catalase-deficient cancer cells [35]. When VC and VK$_3$ were combined in a 100:1 ratio, the VC:VK$_3$ 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$_2$O$_2$ 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$_3$ combination. Modification of one of the constituents (i.e. brominated VK$_3$) or replacement of the VK$_3$ 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$_3$ and the VC:VK$_3$ 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$_3$ combination with a VC:VK$_3$ 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$_{50}$ values of the VC:VK$_3$ 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$_3$ 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$_0$ - G$_1$/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 UMC-14 tumorigenic urothelial carcinoma cells were implanted into the subcutis of nude mice and the mice were treated with gemcitabine GEM), VC:VK$_3$ or a GEM-VC:VK$_3$ 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$_3$, these effects can be attributed to the combined effects of H$_2$O$_2$ and the vitamins [26].

When RT4 cells were co-incubated with catalase and VC, VK$_3$ or the VC:VK$_3$ 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$_3$ than was required to destroy the antitumor activity of the vitamin combination, suggested that while H$_2$O$_2$ 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$_2$O$_2$ 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:VK₃ 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 H₂O₂ 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 VK₃, and 1.5- to 2-fold for VC:VK₃. 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 autoschizis.

Since VC:VK₃ 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 VK₃ 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:VK₃ 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:VK₃-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:VK₃ to form a shunt around a defective complex III of the electron transport chain by having menadione accept electrons from coenzyme Q (ubiquinone), shuttle them to ascorbate and then 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 shuttle 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 VK₃ led to a drop in intracellular levels to 45% of that of control levels. Following a one hour VC:VK₃ 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:VK₃ was observed in 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:VK₃ 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:VK₃ 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 H2O2 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:VK3. Because of these results, efforts are underway to conduct a phase II clinical trial for this indication.

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Synergistic Antitumor Activity of Vitamins C and K₃ on Human Prostate Cancer Cell Lines

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