Vitamin C and K3 Combination Causes Enhanced Anticancer Activity against RT-4 Bladder Cancer Cells

Karen McGuire1, James Jamison1*, Jacques Gilloteaux2 and Jack L. Summers1

1The Apatone Development Center, St. Thomas Hospital, Summa Health System, USA
2Department of Anatomical Sciences, St Georges’ University School of Medicine, K B Taylor Scholar’s Programme, Newcastle upon Tyne, UK

Abstract

Introduction: Vitamin C (VC), Vitamin K3 (VK3) and the combination (VC:VK3) were evaluated against human bladder cancer cell lines RT-4 and T24 to evaluate their synergistic anticancer activity.

Methods/Results: An MTT assay compared a 1hr pulsed versus a 5hr continuous exposure. VC:VK3 was synergistic, increasing the antitumor activity 12- to 24 fold for RT-4 cells. VC:VK3 pulsed versus continuous exposure produced comparable CD50 values, indicating a triggered response involving a catalase reversible redox mechanism generating hydrogen peroxide. Hydrogen peroxide production caused lipid peroxidation and depletion of cellular thiols. ATP levels were measured over 5hrs to determine metabolic effects where VC:VK3 caused a unique spike in ATP levels. Though the cause of the ATP spike is unknown a possible mechanism is a shunt formed around a defective region of complex III of the ETC from coenzyme Q to cytochrome c, producing a shift from glycolytic to oxidative metabolism and a diminution of lactic acidosis. Analysis of mitochondrial and extra mitochondrial calcium levels revealed a unique calcium pattern for RT4 cells treated with CD50 doses of VC, VK3 or VC:VK3.

Conclusion: VC:VK3 was able to cause autoschizic cell death through oxidative stress, thiol depletion, lipid peroxidation, modification of ATP levels and calcium regulation. Because of these results, VC:VK3 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.

Keywords: Cancer cell lines; Chemokine; H2O2; Vitamin C (VC); Vitamin K3 (VK3)

Introduction

The latest statistics from the National Cancer Institute estimates that 72,570 new cases of bladder cancer will be diagnosed in the United States in 2013 and will result in 15,210 deaths. Bladder cancer is six times more prevalent in developed countries than in under developed countries and is the fifth most common human malignancy. Bladder cancer is also one of the most expensive cancers to treat since the course of therapy requires extensive patient surveillance to monitor for recurrence as well as repeated procedures to remove new tumors or cryptic tumor foci overlooked during the initial transurethral resection [1-4]. These urothelial carcinomas are primarily of epithelial origin (>90%) with multiple genetic pathways leading to disease progression [5]. Patients with high-risk non-muscle invasive bladder cancer receive adjuvant Bacillus Calmette-Guérin (BCG) therapy alone or in combination with interferon α-2b [6], radiation and/or chemotherapy, typically methotrexate, vinblastine, doxorubicin and cisplatin (MVAC), or other targeted strategies [7]. Even with the latest pharmacologic strategies, the relative survival rate for bladder cancer is 5 years, while the median survival for patients with inoperable metastatic bladder cancer is 7 to 20 months [5,8,9].

A new paradigm in cancer therapy is slowly gaining popularity and continues to evolve from the work of Roger Daoust and Henryk Taper [10-13]. Daoust studied the DNase I and DNase II expression patterns of a variety of tumor types and discovered that DNases were often suppressed in tumor cells, despite being active in the surrounding tissues and vasculature. Daoust also found that reactivation of both DNases was associated with successful cancer treatment or spontaneous cancer remission. This work was extended by Henryk Taper and co-workers [14,15] for the treatment of liver and other cancers by using a combination of vitamin C and vitamin K3 (VC:VK3) in a 100:1 ratio. Taper showed that vitamin C reactivated DNase II, while vitamin K3 reactivated DNase I with the combination synergistically causing tumor cell death. Further experimentation showed that VC:VK3 was an effective chemo- and radio-sensitizer [11-13]. Subsequently, these studies were extended to include bladder and [16-22] other cancers [23-27]. Unlike the majority of chemotherapeutic agents which target rapidly dividing cells, VC:VK3 appears to target tumor cells by inflammation [23]. Inflammation is regarded as a “secret killer” and is present in the microenvironment of most neoplastic tissues [28]. A wide variety of stimuli including: microbial infections, viral infections and autoimmune disease can trigger chronic inflammation and the subsequent development of cancer [29]. Chemokine and cytokine production orchestrated by inflammation-sensitive transcription factors are the key players in this cancer-related inflammation (CRI) [30-32] and its role in tumor initiation, promotion, invasion, and metastasis [33]. Therefore, inflammation can be considered an enabling characteristic for the acquisition of the core properties of cancer.

*Corresponding author: James M. Jamison, Ph.D, Summa Health System Foundation, St. Thomas Hospital 444 N. Main Street, Akron, Ohio 44310, USA, Tel: (330) 379-8178; Fax: (330) 379-8177; E-mail: jamisonj@summa-health.org

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corning 96-well titer plates were seeded with tumor cells (5 x 10^4 per well) and allowed to grow and spread overnight. Cells were consequentially incubated for 1-h or 5-days with VC or VK alone or in combination. Formazan crystals were dissolved in DMSO and plates were read at 590nm and 620nm on a Biotek Synergy HT plate reader. The CD_{50} was determined based on the line of best fit. The fractional inhibitory concentration index (FIC) was employed to evaluate synergism.

**Analysis of protein thiols**

Thiol levels were determined according to Nagelkerke et al. [40]. In brief, RT4 cells were treated with CD_{30} concentrations of the vitamins alone or in combination. Cells were washed with PBS, culture media and trypsinized every hour up to 6 hrs. Cells were subsequently pelleted for 5 min at 1000 rpm, washed twice with 6.5% TCA (trichloroacetic acid) and resuspended in 1 mL of 0.5 M Tris-HCl (pH 7.6). To detect thiols 50 µL of 10 mM ethanolic Ellman’s Reagent was added to each sample and incubated for 20 min at room temperature. The solution was then centrifuged for 5 min at 1000 rpm and the absorbance of the resulting supernatant was measured at 412 nm. Thiol content was determined based on a reduced glutathione (GSH) standard curve and was expressed as µM thiols per mg of protein.

**Analysis of ATP**

RT4 cells were seeded at a density of 1.0 x 10^4 and allowed to grow and spread overnight at 37°C and 5% CO₂. Culture medium was removed; the cells were treated with vitamins alone or in combination and ATP content were determined every hour for 5 hrs. Cells were then washed with 1xPBS covered with vitamin-free media and solubilized in somatic cell ATP releasing reagent (Sigma Chemical Co, St Louis, MO). Cellular ATP content was determined using an ATP bioluminescent assay kit (Sigma, St. Louis, MO) [41] and bioluminescence was then measured using a Beckman LS 9000 scintillation counter set for single photon counting. ATP content was then calculated based on an ATP standard curve and was expressed as nM ATP per mg of protein.

**Lipid peroxidation**

Lipid peroxidation was evaluated using the thiobarbituric acid (TBA) method [42]. 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.

**Calcium**

Calcium was assayed according to the method of Scott et al. [43]. Briefly, 4x10^4 cells were suspended in 1mL of calcium and magnesium free HBSS containing CD_{30} vitamin concentrations and incubated at 37°C for 15, 30, 45 and 60 min. Following incubation the cell suspension was treated with 100 µL of 390 µM arsenazo III (2,2’-[1,8-dihydroxy-3,6-disulpho-2,7-naphthalene-bis(azo)]-dibenzenearsonic acid). Followed by the addition of 100 µL of 130 µM FCCP (carbonyl cyanide-p-(trifluoromethoxy) phenylhydrazone) to the cell suspension and the mitochondrial calcium release was recorded until no further change in absorbance was observed at 675-685 nm using a HP8451A diode array spectrophotometer. Then 100 µL of 195 mM A23187 (a calcium ionophore) was added to the suspension to depolarize the mitochondrial membrane. 

**Materials and Methods**

**Cell lines**

Grade I (RT-4) and grade III/IV (T24) human bladder cancer cell lines were purchased from the American Type Culture Collection (Rockville, MD, U.S.A.) and were grown in Eagle’s minimum essential medium (MEM) and McCoy’s 5A respectively (Gibco, Grand Island, NY, U.S.A.). All media was supplemented with 10% fetal bovine serum (Gibco) and 50 µg/mL Gentamycin sulfate (Sigma Aldrich, St. Louis, MO).

**Test solutions**

Sodium L-Ascorbate (VC) and menadione sodium bisulphite (VK) were purchased from Sigma Chemical Company (St Louis, MO, U.S.A.) and were dissolved in 1X phosphate-buffered saline (PBS). For the cytotoxicity assay vitamins were diluted to a final concentration of 10,000 µM VC and 500 µM VK, alone and the combination was diluted to a final concentration of 8,000 µM VC and 80 µM VK, respectively. Two fold serial dilutions were then performed and solutions were added to the plate. The CD_{30} concentrations determined by the cytotoxicity assay (VC: 8,750 µM), VK: 90 µM and VC:VK: (520 µM:5.2 µM) were used for all additional experiments.

**Protein concentration assay**

Total protein content for each sample was determined using the method of Bradford [39] and sham treated cells served as control for all experiments.

**Cytotoxicity assay**

Tumor-cell cytotoxicity was performed using the microtiterazolium assay [MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-diphenyltetrazolium bromide] as described previously [16]. Briefly,
ionophore) was added and extra mitochondrial calcium release was recorded until no further change in absorbance was recorded at 675-685 nm. Calcium concentration was determined using a calcium standard curve. The linear range of the standard curve was used to express the calcium concentration as nM calcium/mg protein [44].

**Statistics**

A 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. All other effects were tested at the 0.0022 level of significance.

**Results**

VC, VK, and the combination of VC:VK, in a ratio of 100:1 have been evaluated for their cytotoxicity against both the low grade (RT-4) and high grade (T-24) bladder cancer 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 vitamin treatment of the RT-4 cells resulted in CD$_{50}$ values of 2.430 µM for VC, 12.8 µM for VK, and 110 µM:110 µM for the VC:VK combination. These results represented a 22-fold decrease of the CD$_{50}$ of VC and a 12-fold decrease in the CD$_{50}$ of the VK. The fractional inhibitory concentration index (FIC) was used to assess the synergism of the combination. For the RT-4 cells, the VC:VK resulted in an FIC value of 0.136. Continuous 5-day vitamin treatment of the T24 cells produced a CD$_{50}$ value of 1,490 µM for VC, 13.1 µM for VK, and 212 µM:212 µM for VC:VK. These results correspond to a 41-fold decrease of the CD$_{50}$ of VC and a 6-fold decrease in the CD$_{50}$ of the VK, with an FIC value of 0.158 (Table 1).

Studies by Taper and co-workers [11] demonstrate that exposure to the combination for as little as 1 h, results in significant anti-tumor activity. A similar experiment was performed to determine if this effect was repeatable against RT-4 and T24 cells following a 1-h vitamin exposure. A 1-h vitamin treatment of the RT-4 cells resulted in CD$_{50}$ values of 4,740 µM for VC, 60.7 µM for VK, and 267 µM:267 µM for VC:VK. These values correspond to an 18-fold decrease in the CD$_{50}$ of VC and a 12-fold decrease in the CD$_{50}$ of the VK. The fractional inhibitory concentration index (FIC) value of VC, a 22-fold decrease in the CD$_{50}$ value of VK, and produced an FIC value of 0.100. In the case of the T24 cells, the CD$_{50}$ values were 4,970 ± 27.4 µM for VC, 73.2 ± 5.9 µM for VK, and 120 ± 7.0 µM:120 µM for VC:VK. These values correspond to a 41-fold decrease in the CD$_{50}$ of VC and a 59-fold decrease in the CD$_{50}$ of the VK, with a corresponding FIC for the vitamin combination of 0.093 (Table 1).

VC, VK, or the VC:VK combination has been shown to generate hydrogen peroxide (H$_2$O$_2$) and other reactive oxygen species (ROS) in tumor cells and these species may initiate peroxidation of lipid membranes [15,17]. Vitamin induced lipid peroxidation (Figure 1) was examined using the thiobarbituric acid method. The lipid peroxidation of sham-treated RT-4 cells displayed an average value of 3.17nM (MDA)/mg of protein. However, this is only a measure of the lipid peroxidation that occurs during the heating of samples to 95 °C during the assay and can, therefore, be considered as a baseline for MDA production. VC treatment resulted in MDA peak at 4.27 nM/mg with an average value of 3.67 nM/mg. Lipid peroxidation of VK$_3$-treated cells was 4.34 nM/mg at 1 h increased to near 5.84 nM/mg by hours 3 and 4 and decreased to 4.27 by hour 5. The average VK$_3$-induced lipid peroxidation was 4.98 nM/mg. This spike in lipid peroxidation was attributed to a concomitant increase in ROS production due to redox cycling and a decrease in catalase activity [45]. VC:VK$_3$ lipid peroxidation peaked at 6.7 nM/mg with an average value of 5.58 nM/mg of protein. Overall, the VC:VK$_3$ treatment resulted in a statistically significant alteration in lipid peroxidation (p<0.0022) for all time points compared to control.

Administration of VK$_3$, or menadione has been shown to cause depletion of GSH and oxidation of protein sulphhydril groups in cytoskeletal proteins [46,47]. Therefore, the effect of vitamin treatment on cellular thiols has been examined (Figure 2). The sham-treated RT-4 cells presented with an average thiol content of 1.39 ± 0.02 µM thiol/mg of protein. All other cells were exposed to the vitamins for 1 h and then incubated in vitamin-free culture media for 5 h. VC treatment in the first hour depleted thiol levels to 0.92 ± 0.03 µM thiol/mg of protein which was not statistically significant compared to the control value at 1 h. Thiol levels remained constant during the second hour and dropped steeply to 0.47 ± 0.03 µM thiol/mg of protein during the third hour. These levels rebounded to 0.73 ± 0.12 µM thiol/mg of protein during the fourth hour and then returned to second and third hour levels of 0.45 ± 0.03 µM thiol/mg of protein during the final hour. The values for the remaining hours are statistically significant (p<0.0022) compared to their corresponding control values.

VK$_3$ treatment decreased thiol levels to 0.62 ± 0.05 µM thiol/mg of protein during the first hour, where they remained constant for the next three hours. By five hours thiol levels lowered slightly to 0.54 ± 0.1 µM thiol/mg of protein. The VC:VK$_3$ combination produced a stepped decrease in thiol levels during the first and second hour from 0.63 ± 0.05 µM thiol/mg to 0.45 ± 0.03 µM thiol/mg of protein. Overall, VC:VK$_3$ treated cells induced a significant (p<0.0022) depletion of cellular thiols.

Investigation using transmission electron microscopy has shown that mitochondrial ultrastructure is altered by vitamin treatment resulting in autophagic cell death [15,48]. To determine the role of mitochondria in VC:VK$_3$ induced cell death the intracellular levels of ATP were measured over the course of 5hrs to look for an ATP-less cell death as a result of mitochondrial damage (Figure 3). The ATP content of sham treated RT-4 cells varies from 58.10 to 62.20 nM ATP/mg of protein with an average value of 59.64 ± 2.4 nM ATP/mg of protein. VC exposure results in an increase in ATP levels to 147 ± 8.64 nM during the first hour. Subsequently, the ATP levels decreased to 86.0

| Cell Line | Incubation Time | Vitamins Alone | Vitamin Combination |
|-----------|----------------|----------------|---------------------|
|           | CD$_{50}$ (µM) | CD$_{50}$ (µM) | CD$_{50}$ (µM)      |
| RT4       | 1 h            | 4,740 ± 27.2   | 60.7 ± 4.01         | 267 ± 4.04          | 2.68 ± 0.05 | 0.100 |
|           | 5 days         | 2,430 ± 28.3   | 12.8 ± 0.03         | 110 ± 9.73          | 1.10 ± 0.10 | 0.136 |
| T24       | 1 h            | 4,970 ± 27.4   | 73.2 ± 5.91         | 120 ± 7.0           | 1.21 ± 0.07 | 0.093 |
|           | 5 days         | 1,490 ± 141    | 13.1 ± 0.01         | 212 ± 7.6           | 2.13 ± 0.06 | 0.158 |

FIC = CD$_{50}$ (cont) / CD$_{50}$ (alone) + CD$_{50}$ (cont) / CD$_{50}$ (alone), where CD$_{50}$ (cont) and CD$_{50}$ (alone) are 50% cytotoxic doses of each vitamin alone; CD$_{50}$ (cont) and CD$_{50}$ (alone) are the 50% cytotoxic doses of the vitamins administered together. FIC<1.0 is synergistic, FIC>1 is antagonistic and FIC=1 is indifferent.

**Table 1:** Antitumor Activity of Vitamins Against RT-4 Bladder Carcinoma Cells.
of the cells with the VC:VK₃ combination resulted in a significant (p < 0.0022) alteration in ATP levels for all hours except for 5h when the difference from control is not significant.

These results demonstrate that pulse treatment of RT-4 cells with VC alone or with the VC:VK₃ combination resulted in significant amounts of lipid peroxidation compared to control (P<0.0022).

Figure 1: RT-4 cells were treated with the vitamins at their CD₉₀ doses VC (8,750µM), VK₃ (90µM) and VC:VK₃ (520µM:5.2µM) and 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 was expressed as nM MDA per mg of protein and calculated based on an MDA standard curve. Values are the mean ± standard error of the mean of three experiments with three readings per experiment and compared to the control. VC:VK₃ treatments resulted in significant amounts of lipid peroxidation compared to control (P<0.0022).

Figure 2: RT4 cells were treated for 1 hour with the vitamins at their CD₉₀ doses, VC (8,750µM), VK₃ (90µM) and VC:VK₃ (520µM:5.2µM) 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 µM Thiol/mg of protein, calculated on the basis of a GSH standard curve. Values are the mean ± standard error of the mean of three experiments with three readings per experiment. VC:VK₃ causes significant depletion of cellular thiols compared to control (P<0.0022).

Figure 3: RT-4 cells were treated for 1 hour with the vitamins at their CD₉₀ doses (VC (8,750µM), VK₃ (90µM) and VC:VK₃ (520µM:5.2µM)) and 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 and calculated based 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<0.0022 between VC:VK₃ and control from 2-4 hrs).

Mitochondrial Calcium in RT4 cells

Figure 4: RT-4 cells were exposed to vitamins 15, 30, 45 and 60 min their CD₉₀ doses, (VC (8,750µM), VK₃ (90µM) and VC:VK₃ (520µM:5.2µM)) harvested and analyzed for mitochondrial calcium content. Calcium content was assayed using arsenazo III. Data has been expressed as nM Calcium per mg of protein calculated on the basis of a calcium 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<0.0022 between treatment and control for all groups by 45-60 minutes).

ATP levels rose slightly to 48.8 ± 4.52 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. Subsequently, 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 RT-4 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 VC:VK₃ combination resulted in a significant (p =0.0022) alteration in ATP levels for all hours except for 5h when the difference from control is not significant.

To determine the role of calcium in VC:VK₃ induced cell death the mitochondrial and extra mitochondrial calcium levels were measured during the first 1hr to look for differences in apoptotic and autopschizic calcium patterns (Figure 4 and 5). The mitochondrial calcium (Figure 4) content of sham treated RT-4 cells varies from 7.11 to 7.95 nM calcium/mg of protein with an average value of 7.47 ± 0.63 nM calcium/mg of protein. VC exposure results in a ~70% decrease in calcium levels to 2.32 ± 0.3 nM during the first thirty minutes compared to control. Subsequently, by 45min to 1 hour the calcium levels rebound to ~50% of control values with an average of 3.67 ± 0.35 nM. VK₃ treatment also lowered calcium levels by ~75% compared to control values to 1.66 ± 0.3 nM during the first thirty minutes. Subsequently, by 45min to 1 hour the calcium levels rebound to ~40% of control values with an average of 4.32 ± 0.3 nM by 1hr.

Unlike VC and VK₃ alone, the VC:VK₃ combination showed no statistically significant change in mitochondrial calcium concentration during the first thirty minutes with an average of 7.0 ± 0.25 nM. This lack of change in mitochondrial calcium concentration during the first 30 minutes probably reflects a slower rate of mitochondrial accumulation...
These results demonstrate that treatment of RT-4 cells with VC or VK3 calcium level slowly rises upward to reach ~75% of the control level. By 45 minutes to 1hr the extra mitochondrial calcium concentration by thirty minutes with an average of 5.5 ± 0.2 nM. By 45 minutes to 1 hour the calcium levels plummet ~20% of control values with an average of 3.75 ± 0.45 nM. Subsequently, by 45min the calcium levels rebound to ~40-50% of the control value. The treatment of the cells with the VC:VK3 combination displayed a calcium pattern that was distinctly different from the mitochondrial calcium pattern created by either vitamin administered alone with, combination mitochondrial calcium levels never dropping below 40% of control levels.

Changes in extra mitochondrial calcium were also examined to look for calcium release from other cellular compartments including the endoplasmic reticulum and cell membrane. The extra mitochondrial calcium content of sham treated RT-4 cells varies from 8.23 to 8.99 nM calcium/mg of protein with an average value of 8.63 ± 0.63 nM calcium/mg of protein. VC exposure results in a ~80% decrease in calcium levels to 1.43 ± 0.2 nM during the first thirty minutes compared to control. Subsequently, by 45min to 1 hour the calcium levels rebound to ~30-35% of control values with an average of 3.75 ± 0.45 nM. VK treatment also lowered calcium levels by ~55-60% compared to control values to an average of 3.75± 0.75 nM during the first thirty minutes. Subsequently, by 45min the calcium levels plummet ~20% of control values with an average of 1.33±0.6 nM. Finally, by 1hr extra mitochondrial calcium levels slightly rebound to ~30% of control but remain low.

The VC:VK3 combination showed a maximum ~36% decrease in extra mitochondrial calcium concentration by thirty minutes with an average of 5.5 ± 0.2 nM. By 45 minutes to 1hr the extra mitochondrial calcium level slowly rises upward to reach ~75% of the control level. These results demonstrate that treatment of RT-4 cells with VC or VK3 alone caused a decrease in extra mitochondrial calcium levels while the combination displayed only a slight decrease in extra mitochondrial calcium over the course of 1hr.

Discussion

VC is cytotoxic to a variety of tumor types [49-51] when administered as a monotherapy or as a sensitizer of tumor cells to radiation and chemotherapy [49,52,53]. At megadoses, VC generates hydrogen peroxide, ROS, depletes cellular thiols and initiates lipid peroxidation (LPO). One problem commonly associated with VC therapy is achieving and maintaining clinically active doses in the bloodstream. For example, following oral VC administration, VC concentrations in the blood peak at ~ 220 µM which is below the concentration required for clinical efficacy. Conversely, following intravenous (iv) VC administration, VC concentrations in the blood peak at ~ 885 µM which is sufficient for clinical efficacy. However, the half-life of this iv dose of VC is short with circulating VC doses returning to control levels within 4 to 6 h [54]. This problem of achieving and maintaining clinically active doses in the bloodstream has hindered VC monotherapy from becoming a widely acceptable cancer therapy.

VK3 also exhibits in vitro cytotoxic activity against a variety of tumor cell lines [55] as well as in vivo antitumor activity [56]. VK3 can act to detoxify ROS (reduced environment) or act as a ROS generator (prooxidant environment) through single electron (1e-) and two electron (2e-) cycling. At doses greater than 50 µM, VK3 causes tumor cell death [57] by depleting cellular pools of ADP, ATP and glutathione (GSH); inducing single stranded DNA breaks and oxidizing protein sulphhydryl groups [50]. VK3 is also a chemosensitizer for most traditional chemotherapeutic agents [58]. The MTD for menadione was determined in phase I and II studies to be 2.5 g/m², but once the dose was increased to between 4 and 8 g/m² hemolysis occurred despite the presence of red blood cell glucose-6-phosphate dehydrogenase with no notable coagulopathy [59-61].

Combining VC and VK3 in a ratio of 100:1 lowered the CD₅₀ values of VC and VK3 6 to 41 fold. This drop in CD₅₀ values places the effective antitumor activity [56] of VC and VK3 6 to 41 fold. This drop in CD₅₀ values places the effective antitumor activity of the VC:VK3 combination above the VC or VK3 alone in vitro and in vivo. The combination is more effective than each of the constituents alone and targets tumor cells thus, avoiding indiscriminate redox damage. Finally, the VC:VK3 combination is an effective chemotherapeutic and radiation sensitizer in hepatoma bearing mice [11-13]. Taper and his associates have shown that the VC:VK3 combination exhibited antitumor activity with exposure times as short as 1 h [11]. The results of previous studies with bladder cancer and other tumor cell lines demonstrated that VC:VK₃ induced cell death via a caspase independent process that was not apoptosis [27,62,63]. In addition, VC:VK₃ treatment did not lead to the conversion of soluble LC3-I to autophagic vesicle associated LC3-II and thus tumor cell death was not due to autophagy [64-66]. Instead, cell death was due to autosis with cathepsins, not caspases, as the cell executioners [65,66]. In the case of RT4 cells, the antitumor activity of VC:VK₃ was due to cell death by autosis [20]. Ultrastructural studies of vitamin-treated RT4 cells undergoing autosis revealed exaggerated membrane damage and an enucleation process in which the perikarya separated from the main cytoplasmic body by self-excision. These self-excisions continued until all that remained was an intact nucleus surrounded by a narrow rim of cytoplasm that contained damaged organelles. The nucleus exhibited nucleolar segregation and chromatin decondensation followed by nuclear karyorrhexis and karyolysis [20].

In previous studies, including those with RT4 cells, it was determined that H₂O₂ and other ROS were essential effectors of VC, VK₃ and VC:VK₃ activity [46,67] and the anti-cancer activity of the
vitamins could be destroyed by addition of exogenous catalase at doses as low as 100 µg/mL [68]. While VC generated H₂O₂ peroxide primarily outside of the cell and VK₃ generated primarily intracellular H₂O₂, the VC:VK₃ combination appeared to produce both extracellular and intracellular H₂O₂ with total H₂O₂ production being additive [45]. In addition, VC and VK₃ formed a redox pair resulting in both one and two electron cycling and the depletion of cellular thiols as well as the generation of hydrogen peroxide, superoxide and other ROS [23]. The fact that a greater amount of catalase was required to destroy the antitumor activity of VK₃ than was required to destroy the antitumor activity of the vitamin combination, suggested that while H₂O₂ 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 H₂O₂ production.

In an initial attempt to elucidate the H₂O₂-mediated forces underlying these mechanism(s), tumor cells were treated with VC alone, VK₃ alone or with the VC:VK₃ combination for 1 h to allow triggering of autoschizis. Subsequently, the vitamins were removed, culture medium was added, and changes in thiol levels, lipid peroxidation and ATP content were monitored for 5 hrs (a time by which most cells would be undergoing autoschizis). The effects of continuous vitamin exposure over a 5 h time period have already been described in a previous manuscript [68]. Since vitamin administration induces H₂O₂ production, the amount of LPO has been evaluated. While the increase in lipid peroxidation values for cells were significantly higher than control levels after 1 hr of vitamin exposure, significant levels of lipid peroxidation and damage to the cell membrane occur only after 2–3 hr vitamin exposure and suggest that wholesale, indiscriminate lipid peroxidation was a late event in the cell death process. However, TEM micrographs of RT4 cells, that were treated at the same time and dose as those employed in this paper, demonstrated that the architecture of the mitochondria, lysosomes and endoplasmic reticulum (ER) was rapidly disrupted by vitamin-induced lipid peroxidation and/or disruption of the glutathione redox balance in the ER as well as diminution of reduced thiols in the membranes of these organelles [20,45,66]. The resultant damage to the membranes as well as Ca²⁺ transport channels of the lysosomes, mitochondria and ER membranes leads to increased intracellular Ca²⁺ levels. Ca²⁺ deregulation also leads to the activation of a number of phospholipases, proteases, and DNases [69]. This Ca²⁺ release occurs within the first 5 minutes of vitamin treatment and ultimately leads to cell death [68].

To further differentiate autoschizis from other types of cell death, vitamin-induced changes in ATP levels were determined. While ATP levels in sham treated cells remained constant, ATP levels in VK₃ treated cells showed a steady decline (1.5 fold decrease) during the 5 h. VK₃ has been shown to induce either apoptosis or necrosis depending upon the dose employed [18,19]. This cytoskeletal disorganization is reflected by blister and induced ultrastructural changes in both the smooth and rough endoplasmic reticulum (SER, RER) as well. As a consequence of the resultant damage to the membranes of these organelles, Ca²⁺ transport systems of the mitochondria, SER and RER was rapidly altered mitochondrial architecture

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leads to the reactivation of DNases [78]. While lipid peroxidation and subsequent loss of membrane integrity may be responsible for the release of \( \text{Ca}^{2+} \) into the cytoplasm, the fact that ATP production by the mitochondria increases 1h after VC treatment and 3h after combined vitamin treatment suggests that the \( \text{Ca}^{2+} \) release occurs via modulation of the voltage-dependent anion channel (VDAC) [66]. In addition to these processes mentioned in this study, a number of cellular processes were affected by the presence of AA and especially DHA, including: modulation of signal transduction, cell cycle arrest and inhibition of glycolytic respiration, inhibition of metastasis [10,26,62,71,79,80]. Taken together these results indicate that autoschizis (the type of cell death induced by the vitamin combination) entails the coordinated modulation of cell signaling and metabolism by VC, VK, in their various redox states coupled with the attack of \( \text{H}_2\text{O}_2 \) and ROS on cellular thios, membranes, cytoskeleton, and DNA that continues until cell death by self-morcellation ensues.

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