Evaluation of Potential Redox Modulatory and Chemotherapeutic Effects of a Proprietary Bioactive Silicate Alka-Vita™/Alka-V6™/Alkahydroxy™ (AVAH)

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Summary: A proprietary modified sodium silicate manufactured by Cisne enterprises Inc. (Odessa, TX) was evaluated for its ability to modulate various parameters relevant to establishment and progression of cancer. Antimutagenic effects were determined using Ames test. Prevention of colon cancer cell (HT-29) attachment and growth was done using standard methods. Apoptotic induction was measured by DNA fragmentation (DNAF) assay. Malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) activity were measured using standard assays. Chemical structure determined by nuclear magnetic resonance (NMR) and infra-red (IR) spectroscopy suggested that the product was a mixture of trimeric sodium silicate and sodium silicate pentahydrate. A dose-dependent reduction in attachment (IC₅₀ = 0.15 mM) and growth (IC₅₀ = 0.18 mM) of HT-29 was observed. At low levels (0.029-2.9 mM) the product was able to prevent various sodium azide induced mutations in Ames test. A dose dependent increase in DNAF suggested induction of apoptosis. A drop in MDA levels and increased in GHS, SOD and CAT activities suggested induction of antioxidant response. We conclude that the product may have cancer chemotherapeutic properties in vitro due to its unique structural and electrochemical properties. In vivo tests are imperative to determine true effectiveness.

Industrial relevance: Silicates are extensively used in cosmetic, food and environmental industries. They are also useful in agriculture as anti-fungal agents and have growth promoting functions. Significance of silicates in human health is not very well understood. It has been classified as a trace mineral and may play a role in bone and joint development. Empirical evidence suggests that certain forms of silicates may have therapeutic potential against chronic and infectious diseases. Understanding their health promoting properties and elucidating their mechanism of action may result in development of new strategies for managing these diseases.

Keywords: Sodium silicate; cancer, chemotherapy, antimutagenic

Introduction: Silicon is one of the most abundant mineral in nature and along with their oxides are ubiquitous (Iler 1979). At high temperature and pressure, elemental silicon (Si) and silicon dioxide (SiO₂) can react with oxides and hydroxides of alkaline metals to form different species of silicates (Lee 2006; Doering and Nishi 2007). These silicates which possess unique electrochemical and physical properties, differ in molecular weights and their relative concentration with respect to alkali. Silicates have found extensive applications in glass, cosmetic, and petroleum
industries for long time. Sodium metasilicate is also an approved food additive and has been granted GRAS status by the FDA (FDA 1979). More recently, they have been used in agriculture as anti-fungal agents with insecticidal and growth-promoting functions. The importance of silicon on human health is unclear. Nutritionally it has been categorized as an essential trace mineral, important in bone, structural and connective tissue development (Nielsen 1984, Sahin et al. 2006). Animals consuming silicon free diets have poor skeletal development and joint strength (Carlisle 1970 ; 1972). However, the exact biochemical functionality is not known and daily values for this mineral have not been established.

Alka-Vita™/Alka-V6™/Alkahydroxy™ (AVAH) is a proprietary form of sodium silicate manufactured by CISNE enterprises Inc. (Odessa, TX) and sold as a mineral supplement. Empirical evidence collected by the manufacturing company from testimonials suggests that this product may have therapeutic properties against many diseases. It is reported to be especially effective in prevention, management and treatment of several forms of cancer. Systematic scientific studies evaluating true therapeutic potential of AVAH to back-up these claims do not exist. Therefore, the objective of this research was to investigate the chemotherapeutic properties of this proprietary sodium silicate. We specifically investigated the antioxidant, antimicrobial, antimutagenic and antiproliferative properties in various in vitro model systems.

Materials and methods

Study Compound: Alka-Vita™/Alka-V6™/Alkahydroxy™ (AVAH) is manufactured using a proprietary patent pending process and was supplied by Cisne Enterprises Inc. (Odessa, TX)

Estimation of silicate concentration: The concentration of silicates in the product were quantified by the ammonium molybdate assay at 450 nm (Alexander 1953). Briefly, 500 ml of sample was mixed with 1000 µl of 10% ammonium molybdate solution and allowed to incubate for 5 min at room temperature. Fifty µl of concentrated sulfuric acid was added and immediately vortexed for 10 sec. The absorbance of the yellow colored silicon ammonium molybdate complex was measured at 450 nm. To prevent polymerization of gelling of silicates in the presence of acids, the sample was serially diluted to different concentrations and used as test sample. The concentration of silicates was determined from the slope of a standard curve that was constructed in a similar manner from silicate standards obtained from PQ corporation (Plainview, PA).

2,2′-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) Assay: The ABTS assay was conducted by modifying a previously described method (Vattem 2007). Briefly, to 1 mL of 7 mM ABTS (in water, activated overnight with 140 mM potassium persulfate) was added 50 µL of sample, and the mixture was incubated for 2.5 min (RT). The absorbance of the sample was measured at 754 nm and free radical quenching activity was expressed by calculating the percent change in absorbance compared to the control, which contained only ethanol in place of the sample. The percentage inhibition in ABTS radical due to the extract was calculated by:

\[
\text{% inhibition} = \left( \frac{A_{754}^{\text{Control}} - A_{754}^{\text{Extract}}}{A_{754}^{\text{Control}}} \right) \times 100
\]

Structural determination: Nuclear magnetic resonance (NMR) and Infrared Spectroscopy (IR) were used for structural determination. In the case of NMR a Magic Angle Spinning (MAS) technique of high resolution was used in the ¹H nuclei; meanwhile, for IR study a Fourier Transform (FT) methodology was performed to analyses the spectra (Tanaka and Takahasi, 1999 and 2005).

Determination of antimutagenic activity: Salmonella typhimurium (TA 100, 98, 1535, 1537 and 1538) cultures were grown overnight in Nutrient Broth (Ames et al 2003). Voges-Bonner Medium with 1.5% agar was used as the bottom agar. The top agar overlay consisted of 0.6% agar with trace amounts of biotin and histidine. In a petri plates, containing 20 ml of agar a 3 ml of top soft agar overlay mixed with 0.1 ml each of bacteria, mutagen, antimutagen was poured. The plates were then incubated at 37°C for 48 h. The number of colony forming units (c.f.u.) after incubation were counted. Controls for spontaneous reversions, mutagen and antimutagen treatments were run along with the evaluated treatments in various combinations mentioned previously. Mutagen: Different concentrations of NaN3 (in distilled water) were added to the top agar to give 1, 2 and 5 µg per plate. Antimutagen: AVAH was diluted in water to 2.9 mM, 0.29 mM and 0.029 mM, and then added to top agar medium (Vattem et al, 2006). The antimutagenic activity of the test compound was expressed as percent change decrease in mutagen induced reversions with respect to the control.

Cells and Culture: Colon cancer cell line (HT-29) was purchased from ATCC, Rockville, MD, USA and used between passages 3-25 for all experiments. Cells were grown as monolayers in Dulbecco’s modified eagle medium (DMEM), 4500 mg/L glucose (Gibco, Life Technologies Ltd, UK) with 10% fetal calf serum (FCS), 2 mmol/L
L-glutamine, 60 U/mL penicillin and 60 µg/mL streptomycin, in a humidified atmosphere of 95% air and 5% CO2 at 37°C.

**Cell Viability Assay:** The trypan blue exclusion assay was used to measure anti-proliferative effects of the test compound (Cavagis et al. 2006). Cells were split and seeded at 5x10^6 in DMEM with different concentrations of AVAH (2.9 mM, 0.29 mM and 0.029 mM) in a 8 well plate and incubated in a humidified atmosphere of 95% air and 5% CO2 at 37°C for 24 h. After 24 h floating and attached cells were harvested and combined for 10 min centrifugation at 1000 g. Cell pellets were resuspended in PBS, and were mixed at a ratio of 1:1 with 0.04% trypan blue (0.004 g/ 10 mM PBS) and incubated for 5 min. The number of cells was counted by using a hemocytometer. Unstained cells were read as viable cells; and stained blue cells as dead cells. The percentage of dead cells was calculated as the ratio between the number of stained cells and the total cell counts.

**Adhesion assay:** Colon cancer cell line (HT-29) was purchased from ATCC, Rockville, MD, USA and used between passages 3-25 for all experiments. Cells were grown as monolayers in Dulbecco’s modified eagle medium (DMEM), 4500 mg/L glucose (Gibco, Life Technologies Ltd, UK) with 10% fetal calf serum (FCS), 2 mmol/L L-glutamine, 60 U/mL penicillin and 60 µg/mL streptomycin, in a humidified atmosphere of 95% air and 5% CO2 at 37°C (Fabre et al., 1993). Cells were split and seeded at 5x10^6 with different concentrations of AVAH in a 8 well plate and incubated in a humidified atmosphere of 95% air and 5% CO2 at 37°C for 24 h. After 24 h the plates were washed to remove non-adherent cells and the adherent cells were trypsinized and counted under a hemocytometer.

**Growth and culture of Lumbricus terrestris:** Standard growth and culture conditions as described previously (Hutton, 2009) were used for culture, maintenance and treatment of *L. terrestris*. Briefly, sexually mature *L. terrestris* with a fully developed clitellum were primed on petri dishes containing 15 ml of Lumbricus growth medium (LGM; 1.25% agar, 0.31% Gerber oatmeal, single grain) for 48 hrs at 10 °C. They were then transferred to treatment plates containing LGM with different concentrations of test compound (1.14 mM, 0.114 mM, 0.0045 mM and 0.0023 mM). Seminal vesicles were harvested and homogenized in calcium free –Lumbricus balanced salt solution (LBSS; LBSS (1.5 mM NaCl, 4.8 mM KCl, 1.1 mM MgSO4, 0.4 mM KH2PO4, 0.3 mM Na2HPO4, 4.2 mM NaHCO3, and adjusted to pH 7.3 with HCl) and used for further analysis. The homogenate is centrifuged at 1100 rpm for 2 minutes at 4°C. The supernatant was then transferred to a clean microcentrifuge tube and stored -80°C for future analyses. The remaining pellet is used for DNA fragmentation analysis.

**Assays for effects on apoptosis:** Evasion of normal apoptotic process is involved in tumorigenesis and therefore the effect of AVAH on induction of apoptosis by ex-vivo DNA Fragmentation Analysis in the rapidly proliferative seminal vesicle cells of *Lumbricus terrestris* treated with different concentration of the compound. The cell pellets obtained from homogenization and centrifugation of seminal vesicles of *Lumbricus terrestris* treated with different concentrations of test compound were resuspended in 20 mM ethylenediaminetetraacetic acid (EDTA) and 0.5% Triton X 100. After centrifugation at 1,500 x g for 10 min, the pellets were resuspended in 250 µL of lysis buffer containing 5 mM Tris -HCl, 20 mM ethylenediaminetetraacetic acid (EDTA) and 0.5% Triton X 100. After centrifugation at 1,500 x g for 10 min, the pellets were resuspended in 250 µL of lysis buffer containing 5 mM Tris -HCl, 20 mM ethylenediaminetetraacetic acid (EDTA) and 0.5% Triton X 100. After centrifugation at 1,500 x g for 10 min the pellets were resuspended in 250 µL of 5% TCA followed by incubation at 100°C for 15 min. Subsequently, each sample, 500 µL of solution (15 mg/ml DPA in glacial acetic acid), 15 µL/ml of sulfuric acid and 15 µg/ml acetaldehyde were added and incubated at 37 °C for 18 h (20). The proportion of fragmented DNA was calculated from the absorbance at 594 nm using the following formula: Fragmented DNA (%) = 100 x (amount of the fragmented DNA in the supernatant) / (amount of the fragmented DNA in the supernatant + amount DNA in the pellets).

**Malondialdehyde (MDA) assay:** Malondialdehyde was measured by modifying the method discussed previously (Vattem 2005). Briefly, in a test tube 200 µl of the cell lysate was mixed with 800 µl of water, 500 µl of 20 % (w/v) trichloroacetic acid and 1 ml of 10 mM thiobarbituric acid. The test tubes were incubated for 30 min at 100°C and then centrifuged at 13,000 rpm for 10 min. The absorbance of the supernatant was measured at 532 nm and the concentration of MDA was calculated from its molar extinction coefficient (ε) 156 µmol−1cm−1 and expressed as µmol/g FW.

**SOD-Riboflavin-NBT Assay:** The superoxide dismutase (SOD) activity was measured by its ability to prevent superoxide mediated oxidation of NBT to Diformazan as a result of the photooxidation of riboflavin (Martinez et al., 2001). Briefly, 20µL cell lysate was transferred into a well of a 96 well plate. 150µL of riboflavin reaction mixture (2 mM riboflavin, 50 mM KH2PO4 buffer (pH 8.0), 0.1 mM EDTA, 200 µM DTPA and 57 µM NBT) was transferred to the well. Then, 170µL of riboflavin reaction mixture was added to a well to serve as the blank. Plates were incubated in dark in a chamber which exposed the 96 well plate to fluorescent lamps for 20 minutes and absorbance was read at 560nm in a microplate spectrophotometer (Biot-Tek 808 IUC) (Bio-Tek Instrument, VT). Calculation of the concentration of Diformazan was determined using its molar extinction coefficient, 26478 mol-1 cm-1. The concentration of Diformazan was expressed as µmol/mg of protein.
**Catalase assay:** Catalase activity was measured by modifying an assay described elsewhere (Sinha 1972). Briefly, 100 µL of supernatant was transferred to a test tube. One mL of peroxide reaction mixture (30% hydrogen peroxide, 0.1M sodium phosphate buffer) was transferred into the test tube and the mixture incubated at room temperature for 10 minutes. Three mL of potassium chromate-acetic acid reagent (2.5% aqueous potassium chromate in glacial acetic acid) to the test tubes and incubated a water bath at 100°C for 15 minutes. Samples were cooled to room temperature and absorbance measured spectrophotometrically at 570nm. Catalase activity was then calculated as follows: % decrease = \((A_{570}^{Blank} - A_{570}^{Treatment}) / (A_{570}^{Blank})\) x 100. The concentration of catalase was expressed as mU/mg of protein.

**Protein concentration:** Protein concentration was determined by the Bradford’s assay (Bradford 1985). Briefly, 20 ml of supernatant is transferred to a 96 well microplate to which 200 ml of diluted dye reagent (Bio-Rad Protein Assay Kit, 1:4 DH2O) is added. The mixture is incubated at room temperature for 5 minutes and the absorbance is measured in a microplate reader at 595nm. The protein concentration was calculated based on a standard curve prepared using different dilutions of BSA.

**Reduced glutathione determination:** The concentration of reduced glutathione in cells was determined using Ellman’s reagent (Mossman, 1983). Briefly, to 100 ml of supernatant, 0.75 ml of precipitant solution (1.67 g glacial metaphosphoric acid, 0.2 g ethylenediaminetetraacetic acid (EDTA) and 30 g NaCl in 100 ml MilliQ water) is added in a microcentrifuge tube. The mix is vortexed and incubated at room temperature for 5 minutes and centrifuged at 3000xg for 15 minutes at 4°C. 500 µL of the supernatant is transferred to a test tube. 2.0 mL of 0.2M Na2HPO4 buffer (pH 8) is added to a small test tube. 250 µL of 0.5mM DTNB solution (Ellmans Reagent) is transferred to the test tube and vortexed. 250 µL of the mixture was immediately transferred to a microplate. The blank consisted of 500µL of 0.2M Na2HPO4 buffer (pH 8), 500µL DH2O, 250µL of precipitant solution, 125µL of DTNB. The absorbance was reading spectrophotometrically at 412nm. The reduced glutathione concentration was calculated using the extinction coefficient \(E= 13.7 \text{ mol}^{-1} \text{ cm}^{-1}\).

**Results and Discussion**

**Sodium Silicate Concentration:** The concentration of sodium silicate in AVAH was determined using the ammonium molybdate method by constructing a standard curve from four different sodium silicates with different molar SiO₂/Na₂O purchased from PQ Corporation (Valley Forge, PA). Star™ (37.1% sodium silicate); N® (37.1% sodium silicate), BW-50® (42.5 % sodium silicate) and D™ (44.1% sodium silicate) were serially diluted and standard curve was constructed at different dilutions (Figure 1). The average concentration of sodium silicate in AVAH was determined using these standard curve and was found to be 21.3 % in the supplied liquid.

![Figure 1: Sodium silicate standard curve using the ammonium molybdate method.](image-url)
Structural determination of AVAH: To determine the composition of the AVAH compound, nuclear magnetic resonance (NMR) and Infrared Spectroscopy (IR) were used. In the case of NMR a high resolution Magic Angle Spinning (MAS) technique was used in the $^1$H$_1$ nuclei; meanwhile, for IR study a Fourier Transform (FT) methodology was performed to analyze the spectra. The target solution was analyzed by IR spectrometer operated in transition mode; Figure 2 shows three well-resolved vibration signals, the first one at 3311 cm$^{-1}$ normally is attributed to the water molecule in the sample according to the discrimination rules. The next signals at 1645 and 1006 cm$^{-1}$ can be assigned to the vibration of the Na and Si ions, respectively. It is important to remember that these signals can be either due to the free OH group or due to the hydroxy compounds based on Na and Si. The result shown in figure 1 suggests that the only functional groups presented in this sample are water and OH groups, however deeper analysis using $^1$H$_1$ MAS-NMR was carried out to complement these results. Through $^1$H$_1$MAS-NMR spectroscopy was possible to confirm that in the sample there is no functional groups other than OH groups. Figure 3 shows the main resonance of the proton at 0 ppm is attributed to the water. On the other hand, the tiny peaks observed up 7 ppm can be related to the resonance of the hydroxy group attached to inorganic components as Na and/or Si. We will perform $^{23}$Na and $^{29}$Si NMR experiment to study the nature and number of the substitutes present in the Na and Si shell.

Figure 2. FTIR spectra of AVAH

Figure 3: $^1$H$_1$ MAS-NMR spectra of AVAH
Bioactive properties of a Sodium Silicate (Alka-V6/Alkahydroxy)-AVAH

MS and NMR analysis generated a putative formula of the compound to be Na_{8.2}Si_{4.4}H_{9.7}O_{17.6} and molecular weight of 563.4 mol\(^{-1}\). An exact structural formula could not be obtained from these structural analysis as the formula suggests that AVAH is not a single compound but a mixture of two different compounds that are in equilibrium with each other. The molecular formula provides the empirical equation for the monomer. We deduced that AVAH appears to be a stoichiometric mixture of (a) Trimeric Sodium Silicate (Na\(_2\)SiO\(_3\)) (b) Sodium Silicate Pentahydrate (Na\(_2\)SiO\(_3\)).5H\(_2\)O. We postulated that Sodium Silicate Pentahydrate (Na\(_2\)SiO\(_3\)).5H\(_2\)O to exist in equilibrium with two structural forms, with one form containing one ionized water molecule and the other form containing 3 ionized water molecules (Figure 4). We hypothesize that observed biological activities AVAH could be due to the presence of multiple ionizable forms, which can accept and donate electrons modulating cellular redox homeostasis.

**Figure 4.** Possible chemical sodium silicate speciation in AVAH sample

![FTIR and NMR formula]

\[ \text{FTIR and NMR formula} \]

\[ \text{Na}_{8.2}\text{Si}_{4.4}\text{H}_{9.7}\text{O}_{17.6} \]

1. Trimeric Sodium Silicate (Na\(_2\)SiO\(_3\))\(_3\)
2. Sodium Silicate Pentahydrate (Na\(_2\)SiO\(_3\)).5H\(_2\)O

**Figure 5:** Free radical quenching capacity of different silicates as measured by the ABTS assay.

![Figure 5: Free radical quenching capacity of different silicates as measured by the ABTS assay.](image)
**ABTS Free radical quenching activity:** To determine if the silicate speciation unique to AVAH as determined by structural analysis had any effect on its biological functionality, we compared different silicates for their ability to quench ABTS•⁺ radical. We found that the ABTS radical quenching potential of silicates did not depend on the amount of silicates as evidenced from the IC50 values (Figure 5). Among all the silicates, BW-50° (42.5% sodium silicate) had the lowest IC50 value of 0.69 mM. This was followed by AVAH (21.3% sodium silicate) with an IC50 of 0.95 mM. DM even though had the highest sodium silicate concentration, (44.1% sodium silicate) had an IC50 of only 1.02. Star™ with the lowest concentration of silicates among all the PQ samples, (37.1% sodium silicate); had an IC50 of 1.23 mM. N® (37.5% sodium silicate) with an IC50 of 2.5 mM was least effective in quenching ABTS radical (Figure 5). Eventhough, silicates are known to complex with metals, their free radical quenching potential has not been demonstrated previously (Farmer and Lumson 1994). Our results suggest that silicates are very effective in quenching free radicals and their free radical quenching potential depends both on the speciation and concentration of silicates. AVAH which had the lowest amount of silicates in our samples tested had the second lowest IC50 among all the samples tested. These results provide a further insight into the probable electrochemical characteristics of the AVAH silicates and its probable mechanism of action.

**Antimutagenic activity of AVAH silicates:** ALKA-V6 was tested at various concentrations for its ability to inhibit various types of mutations induced in the salmonella tester strains in response to sodium azide. In all the strains except in TA102, the product was able to prevent mutagen induced reversions into wild type by 80-100% at 2.9 mM effective concentration. At 0.29 mM concentration, AVAH reduced 97-100% missesense (TA1535) deletion-frameshift mutations (TA1537) and 17% of missense mutations in TA100. At the lowest concentration of 0.029 mM, AVAH was still effective and prevented 20% missense mutations in TA100; 60% missense mutations in TA1535 and 86% deletion frame-shift mutations in TA1537. It is possible that silicates in AVAH may be inhibiting the binding of the mutagen (sodium azide) to DNA by blocking the mutagen binding sites. NaN₃ is known to cause a mismatch in DNA replication by substituting for natural thiol groups from cysteine and methionine and then binding to the DNA. It is also likely that AVAH silicates could possibly inhibit the mutation induced by NaN₃ by preventing the binding of β-azidoalanine to the DNA bases by blocking its DNA binding site and by maintaining the activity of the enzyme O-acetyl serine thiol lyase which has DNA protective functions. A consequence of DNA mutation in response to NaN₃ is the induction of recA dependent SOS response which identifies the mutated base and removes it creating an “empty” base (Vattem 2006). As a more effective redox modulator, the mechanism for inhibition of mutagen induced SOS response by AVAH could be by the suppression of inactivation of the LexA repressor by the RecA protease, suppression of the transcription of the recA gene, and suppression of RecA protein synthesis as well as induction of adaptive/inducible repair systems consisting of several proteins that recognize very specific modified bases (Vattem 2006).

**Figure 6.** Effect of ALKA-V6 against various type of mutations induced by sodium azide in Ames test.
Effect of AVAH on survival and attachment of HT-29 cells: AVAH was serially diluted in distilled deionized (DDI) water to 2.9 mM, 0.29 mM and 0.029 mM. At these concentrations, AVAH was added to cell culture media seeded with colon cancer cells (HT-29) in a 16 well plate and allowed to grow overnight at the conditions described above. The following day, the number of viable cells were counted and recorded. Compared to the control, it was observed that cells treated with AVAH at 2.9 mM concentration caused complete (100%) lethality to HT-29 cells. At 0.29 mM concentration, in the presence of AVAH only 20% of the cancer cells survived (80% lethality) and at 0.029 mM concentration the product did not exhibit any cytotoxic effect and was not effective in killing colon cancer cells (Figure 7). From the data obtained, the IC\textsubscript{50} of AVAH was calculated 0.18 mM (Figure 7). Attachment of cancer cells is a fundamental process involved in the establishment of cancer, its growth and eventual metastasis (ref). To study the effect of AVAH silicates on attachment of HT-29 cancer cells, it was serially diluted in distilled deionized (DDI) water to 2.9 mM, 0.29 mM and 0.029 mM. AVAH at different concentrations was added to cell culture media seeded with colon cancer cells (HT-29) in a 16 well plate and allowed to grow overnight at the conditions described above. The following day, plates were washed to remove detached cells. The cells that were still attached were trypsinised and enumerated under the microscope using a hemocytometer. Compared to the control it was observed that cells treated with AVAH at 2.9 mM concentration completely prevented attachment of all the cancer cells (100% effective) (Figure 7). At 0.29 mM only 31% of the cancer cells attached (69% effective) and at 0.029 mM only 86 % of the cancer cells attached (14 % effective). From the data obtained, the IC\textsubscript{50} of AVAH was calculated 0.15 mM (Figure 7).

Figure 7. Effect of AVAH sodium silicate on attachment and survival of colon cancer cell line HT-29.

Apoptotic effects of AVAH: Induction apoptosis or programmed cell death is an effective mechanism to control cancer growth and is the target of many anti-cancer drugs. A characteristic of cells undergoing is the fragmentation of nuclear DNA as a result of the initiation of apoptotic signaling pathways culminating into the activation of caspases and chromatin condensation (ref). To study the effect of AVAH on apoptosis induced DNA fragmentation (DNAF), AVAH was serially diluted to DDI water to 1.14 mM, 0.114 mM, 0.0045 mM and 0.0023 mM concentrations and sustained apoptotic effect was measured. It was observed that AVAH had exhibited a classic Gaussian response curve. In general, at all dilutions, the compound was very effective in inducing DNAF with the product being most effective at 0.114 mM (Figure 8). As discussed before, the possible mechanism by which this product may be cytotoxic to HT-29 cells may be due to induction of apoptotic pathways resulting in a programmed cell death and lower survival and proliferation rates. At 1.14 mM dilution, the amount of fragmented DNA detected decreased (Figure 8). We believe that this phenomenon could be due to the ability of AVAH to induce cell death in a process that is independent of apoptosis, which is a commonly observed effect since beyond a critical concentration ALKA-V6 might be promoting death of cancer cells independent of induction of apoptosis.
Effect of AVAH on Redox homeostasis: Oxidation of biological molecules by reactive oxygen species results in initiation of tumorigenic and carcinogenic processes (Vattem 2007). Reduction of molecular oxygen is inevitably associated with the formation of reactive oxygen species (ROS) via enzymatic or metal-catalyzed reactions such as in beta oxidation, electron transport chain, or the fenton reaction (Gershman et al, 1954; Harman, 1956; Rae et al, 1999; Valko et al, 2005). The term reactive oxygen species includes free radicals, non-radical molecules, reactive nitrogen species, and chlorine species (Karihtala and Soini, 2007; Valko et al, 2007). Free radicals are characterized by having one or more unpaired electrons and possess unique configurations and chemical structures that affect its ability to diffuse through cells and react with macromolecules (Halliwell and Gutteridge, 1989; Tsukahara, 2007). A few examples of ROS and free radicals include the superoxide anion, hydrogen peroxide, the hydroxyl radical, peroxynitrite radical, hypochlorous acid, and nitrogen dioxide (Cadenas and Sies, 1998; Lieberman and Marks, 2009; Karihtala and Soini, 2007). ROS are mainly formed during mitochondrial respiration, mitochondrial beta-oxidation, NAD(P)H oxidase activity in neutrophils, myeloperoxidase activity, peroxisomal beta-oxidation, lysosomal oxidative degradation, cytochrome P450 activity in the liver endoplasmic reticulum, and lipoxygenase activity. Being unstable and reactive, free radicals participate in spontaneous electron abstraction from surrounding macromolecules causing oxidative modification to lipids, proteins, and nucleic acids which eventually alter normal cell physiology (Halliwell and Gutteridge, 1989; Siems et al, 1995). Proteins, DNA, and lipids are oxidized upon formation of excess ROS. Protein oxidation leads to decreased protein activity, improper protein activity, transcription factor activation, and abnormal and untimely cell proliferation. Proteins that are especially susceptible to oxidation include those that contain the amino acids cysteine, methionine, arginine, histidine, tryptophan, and tyrosine. Upon oxidation of proteins containing cysteine, the cysteine sulfhydryl groups crosslink to form disulfide bridges, which cause unusual protein folds. This crosslinking causes the alterations in protein function. The relationship between protein oxidation and protein glycosylation can lead to inactivation of antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD), leading to increased levels of ROS. DNA oxidation occurs upon hydrogen abstraction, which is activated by a transition metal such as copper, is also initiated by ROS. Hydrogen abstraction causes strands of DNA to break, crosslink, or alterations in base modification. Base modification alterations occur upon hydrogen abstraction; this causes the DNA repair system, which proofreads copies of DNA, to misread the copied DNA which leads to DNA mutation and possibly cancer. In lipid oxidation, radicals are involved in the reaction and are produced as byproducts. Malondialdehyde (MDA) is a secondary oxidation product of lipids and serves as a good marker for oxidation and cell injury and possible risk for diseases resulting from oxidative damages. Living organisms have evolved many antioxidant and protective mechanisms against oxygen and its highly reactive metabolites (Cadenas, 1997). Mechanisms for ROS removal include primary, secondary, and tertiary antioxidant defenses. These include the endogenous enzymes superoxide dismutase, catalase, glutathione peroxidase (GP), and glutathione transferase (GT) that can metabolize free radicals, these are referred to as primary antioxidant systems. There are four isoforms of the enzyme which have been identified and they include Ni-SOD (cytoplasmic), Mn-SOD (mitochondrial), Cu/Zn-SOD (cytoplasmic), and EC-SOD (extracellular) (Mates and Sanchez-Jimenez, 1999). The product of the dismutation of two superoxide anions carried out by SOD is the ROS, hydrogen peroxide, therefore biologically this enzyme is often coupled with antioxidants that remove hydrogen peroxide such as catalase and glutathione peroxidase. Recently, Ni SOD has also been discovered. GP is also responsible for converting hydrogen peroxide to water; however, selenium is necessary for this function to occur. GP is found in high concentration in the cytosol and mitochondria because high amounts of hydrogen peroxide are produced in those locations. Additionally, secondary antioxidant systems such as as glutathione, selenium, coenzyme Q, and vitamins C and E have high electron donating potentials and they also participate in the removal of ROS (Mates and Sanchez-Jimenez, 1999; Mates, 2000; Halliwell, 1994).

Glutathione (GSH) has been described for a long time just as a defensive reagent against the action of toxic xenobiotics (drugs, pollutants, carcinogens). It is a tripeptide that is generated within the cytosol via glutamate-cystein ligase and glutathione synthetase (Valko et al, 2007). This molecule has several important functions in the cell which include it acting as a cofactor for antioxidant enzymes, an aide for amino acid transfer through plasma membranes, a scavenger of free radicals, and a regenerator of oxidized vitamins C and E (Masella et al, 2005). As a prototype antioxidant, it has been involved in cell protection from the noxious effect of excess oxidant stress, both directly and as a cofactor of glutathione peroxidases. In addition, it has long been known that GSH is capable of forming disulfide bonds with cysteine residues of proteins, and the relevance of this mechanism (“S-glutathionylation”) in regulation of protein function. Tertiary antioxidant defense functions in repair and include lipases, proteases, transferases, and DNA repair enzymes. CAT is responsible for converting the hydrogen peroxide ROS to water and oxygen; it is a very efficient enzyme because it has a very low Km for hydrogen peroxide. Hydrogen peroxide, if not removed from a system, can lead to the formation of the extremely reactive hydroxyl radical through the Fenton reaction or Haber-Weiss reaction (Cohen and Heikkila, 1974). This enzyme is distributed...
in all tissues in most species, and it helps prevent cell damage and the further propagation of free radicals (Harris, 1992). Glutathione peroxidase is another enzyme capable of catalytically removing hydrogen peroxide and other hydroperoxides using glutathione (Ladenstein et al, 1979). Its primary function is in converting lipid hydroperoxides to non-toxic molecules via glutathione oxidation. There are at least five known isoforms of this enzyme distributed in various tissues of organisms (Mates and Jimenez-Sanchez, 1999). For constant removal of reactive oxygen species (ROS) from the system, it is essential for the cells to replenish cellular antioxidant pools either by reducing oxidized antioxidants or by inducing synthesis of cellular antioxidants and antioxidant enzymes. As explained above in an actively metabolizing tissue, this ROS is quickly removed with the help of several cellular antioxidants and cellular antioxidant enzymes such as GSH, SOD and CAT. Together these antioxidant enzymes and molecules maintain a homeostatic balance by neutralizing the constant flux of radicals generated through cellular metabolic processes and exogenous sources (Droge, 2002). We investigated the role of AVAH in maintaining cellular redox homeostasis by increasing the antioxidant response systems and decreasing the formation of free radicals.

**Effect of AVAH on Malondialdehyde formation:** We investigated if the effect of AVAH on the redox homeostasis, by measuring the amount of free radical induced formation of malondialdehyde (MDA). AVAH was serially diluted in DDI water to 1.14 mM, 0.114 mM, 0.0045 mM and 0.0023 mM and sustained effect on reducing free radical induced oxidation was evaluated. It was observed that AVAH exhibited a dose dependent effect on reducing the formation of MDA (Figure 8). This highest reduction in MDA formation compared to the control was observed at a concentration of 1.14 mM.

![Figure 8](image)

**Figure 8.** Apoptotic and antioxidant effect of AVAH at various concentrations as measured by fragmented DNA and malondialdehyde (MDA) levels.
Antioxidant Enzymes: We investigated the ability of various concentrations of AVAH on an important antioxidant enzyme, SOD and CAT. AVAH was serially diluted in DDI water to 1.14 mM, 0.114 mM, 0.0045 mM and 0.0023 mM used in these studies. SOD is important in antioxidant defense in nearly all cells exposed to oxygen and high activity of SOD is linked to lower incidences of several forms of cancer (ref). Our results showed a dose dependent effect of AVAH on SOD activity. At 1.14 mM AVAH was most effective and increased the activity of SOD by 1.45 fold compared to the control. At a concentration of 0.0045 mM and 0.114 mM AVAH treatment resulted in a fold
change of 1.15 and 1.14 in SOD activity (Figure 9). At the lowest concentration of 0.0023 mM AVAH did not have any statistically significant effect on increasing SOD activity. We also investigated the effect of AVAH on the activity of CAT. Deficiency of CAT is observed in several types of cancer and increased activity is related to lower vigor in cancer cells (ref). At a concentration of 0.0023 mM and 0.0043 mM AVAH silicates did not have any effect on CAT activity, surprisingly, CAT activity went down at 0.0045 mM. At 0.114 mM AVAH silicates the CAT activity was 2.32 fold higher than the control, which was higher than the CAT activity observed at 1.14 mM.

**Effect of AVAH on cellular reduced glutathione levels:** Effect of 1.14 mM, 0.114 mM, 0.0045 mM and 0.0023 mM of AVAH silicates was measured on reduced glutathione levels. Our results indicated that the product was very effective in increasing the levels of GSH in the cells by 1.24 fold and 1.44 fold at 0.114 mM and 1.14 mM GSH and reduce CAT activity. respectively. At lower concentrations of 0.0045 mM and 0.0023 mM lower levels of GSH were observed when compared to control. A low GSH and a concomitant reduction in CAT activity, may suggest a possible activation of the glutathione peroxidase (GP) based removal of hydrogen peroxide in which would result in increased depletion of GSH.

**Conclusions**

Chemical and structural analysis of AVAH by $^1$H$_2$-MAS-NMR and FTIR spectroscopy indicated that it is a unique sodium silicate based product. We postulated that the product may contain two silicate species, trimeric sodium silicate and sodium silicate pentahydrate. Using spectrophotometric analysis, we estimated the product to be 21.3 % (w/v) sodium silicate. Our results showed that sodium silicates may have free radical quenching activity. Moreover, we also demonstrated that the speciation of silicates in addition to the concentration of silicates determined the free radical reducing potential of silicates. AVAH sodium silicates were very effective in quenching free radicals and had the ability to quench free radicals compared to other silicates tested. AVAH was also very effective in inhibiting processes that were important in initiation and development of cancer. At the concentrations tested, the product was effective in reducing sodium azide induced mutations and reduce the survival and attachment of cancer cells. In our studies we also observed that the AVAH silicates increases apoptotic cell death and decreases oxidative lipid damage. DNAF, an indicator of apoptosis increase significantly upon treatment with AVAH silicates. MDA a secondary oxidation product of cellular lipid oxidation was found to decrease upon treatment with AVAH. In addition to reduced oxidative stress, we also observed a significant increase in the activity of antioxidant enzymes SOD and CAT upon treatment with AVAH silicates. The cellular levels of important antioxidant molecule GSH also increased in the presence of AVAH treatments, suggesting a redox modulating effect of these silicates. Preliminary in vitro results reported in this study suggest that the product may be effective in positively modulating biological processes which may have health promoting effect. Further investigations using in vivo models to understand the mechanism of action of Alka-Vita™/Alka-V6™/Alkahydroxy™ (AVAH) silicates is underway.

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Bioactive properties of a Sodium Silicate (Alka-V6/Alkahydroxy)-AVAH


