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TEXAS STATE UNIVERSITY-SAN MARCOS
COLLEGE OF APPLIED ARTS
DEPARTMENT OF AGRICULTURE

TITLE

TEST THE CANCER CHEMOTHERAPEUTIC AND ANTI-RETROVIRAL EFFECTS OF ALKA-V6 IN CELL CULTURES AND OTHER IN VITRO SYSTEMS.

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CISNE ENTERPRISES, INC

EVALUATION OF POTENTIAL ANTICANCER AND APOPTOSIS EFFECTS OF ALKA-V6

ALKA-V6 is a modified value-added silicon-based compound developed by the Cisne Enterprises Inc. Recent research with this compound has shown that it has several health enhancing effects in humans and animals. Research conducted by several scientists at universities and private companies have shown that this compound has very high antimicrobial effect and can reduce the risk of infections and poisoning associated with several pathogens in both humans and animals. This proprietary compound has also been shown to be extremely effective in bone remodeling and joint health.

Preliminary work with cancer models in addition to numerous testimonials/case studies has given some empirical evidence that this compound also has anticancer effect. It has been noted in these studies that this compound can reduce tumor size, reduce remissions and aid in chemotherapy by increasing effectiveness and reducing side effects. In spite of these observed empirical effects, systematic studies that detail the exact dose dependency and mechanism of action of this promising compound have not been conducted. Elucidating the mechanism of action and elucidating the dose dependency will further facilitate the accurate administration and application of ALKA-V6 in cancer therapy. This approach can result in development of efficient and more effective alternative/complementary strategies for the management and prevention of different forms of cancer.

MATERIALS AND METHODS

Determination of antimutagenic activity

Salmonella typhimurium (TA 100, 98, 1535, 1537 and 1538) cultures were grown overnight in Nutrient Broth. Voges-Bonner Medium with 1.5% agar was used as the bottom agar. The top agar overlay will consist 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 will be poured. The plates were then incubated at 37^{o}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 (Ames et al 2003) to give 1, 2 and 5 µg per plate. Antimutagen: ALKA-V6 in water, diluted and then added to top agar medium (Ames et al 2003).

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 Lglutamine, 60 U/mL penicillin and 60 µg/mL streptomycin, in a humidified atmosphere of 95% air and 5% CO2 at 37°C.

Cytotoxicity Assay

The ALMAR BLUE proliferation kit (Almar, Sacramento, CA, USA) was used to measure anti-proliferative effects in all the cells. For the cytotoxicity assay, the cells (30.000 cells/well) were be seeded onto a 96-multiwell plate together with DMEM (10% FCS) and incubated overnight at 37°C at 5% CO2. The day after, the cells were washed with PBS and treated with ALKA-V6 (2-4 μM) diluted in DMEM (0.1% FCS) by the adding 100 μl of the extracts to the wells for each condition. Every experiment was performed in octuple and 500 μM deoxycholic acid was used as a positive control. After 4 days of incubation (37°C at 5% CO_2) Almar blue dye (10%) was added and the cells were placed in the same incubator. After 5-6 h, the plates were read at 570 nm in a microplate spectrophotometer (Biot-Tek 808 IUC) (Bio-Tek Instrument, VT). Cell survival will be expressed as the percentage absorbance of the mean absorbance of the negative control (DMEM 0.1% FCS).

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 Lglutamine, 60 U/mL penicillin and 60 μg/mL streptomycin, in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cells were split and seeded at $5x10^6$ with different concentrations of ALKA-V6 in a 8 well plate and incubated in a humidified atmosphere of 95% air and 5% CO_2 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 and counted under a hemocytometer.

Assays for effects on apoptosis

Evasion of normal apoptotic process is involved in tumorigenesis and therefore the effect of ALKA-V6 on induction of apoptosis by ALKA-V6 was investigated.

DNA Fragmentation Analysis

This assay uses centrifugal sedimentation to separate fragmented double-stranded DNA from intact DNA. Upon lysis of cells, cytosolic DNA is released and a centrifugation step will generate two fractions corresponding to intact and fragmented DNA (present in cytosol). Acid hydrolysis allows for deoxyribose sugars to bind with DPA, and the percentage of fragmented DNA can be quantified spectrophotometrically. Amount of fragmented DNA is directly proportional to apoptotic activity. The cell pellets ($5x10^6$) were lysed in 0.5 ml of lysis buffer containing 5 mM Tris -HCl, 20 mM ethilenediaminetetraacetic 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 and, to the supernatants (S), 20 μL of 6 M perchloric acid was added. Then, 500 μL of 10% trichloroacetic acid (TCA) were added to the pellets (P). The samples were then centrifuged for 10 min at 5,000 rpm and the pellets were resuspended in 250 μL of 5% TCA followed by incubation at 100oC for 15 min. Subsequently, to 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 ^oC 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 by Tamagnone et al., 1998 (35). In a test tube 200 µl of the briefly treated and untreated cell homogenate was mixed with 800 µl of water, 500 µl of 20 % (w/v) trichloroacetic acid and 1 ml of 10 mM thiobarbutyric acid. The test tubes were incubated for 30 min at 100 ^{o}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 $\mu mol^{-1}cm^{-1}$ 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. Briefly, 20µL of treated and untreated cell suspension 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. 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 .

Reduced glutathione determination

The concentration of reduced glutathione in cells was determined after treatment of the cells for 24 h. The cells (3×105/ml) were washed with physiological solution and lysed with water; 3 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) were added to the lysate (2 ml). After 5min, this mixture was centrifuged and 0.4 ml of the supernatant was added to 1.6 ml of reaction medium (0.2M Na2HPO4 buffer, pH 8.0; 0.5 mM DTNB dissolved in 1% sodium citrate).

Subsequently, the absorbance of the product (NTB) was measured at 412 nm and reduced glutathione concentration calculated using the extinction coefficient *E* = 13.6 mol-1 cm-1 (19).

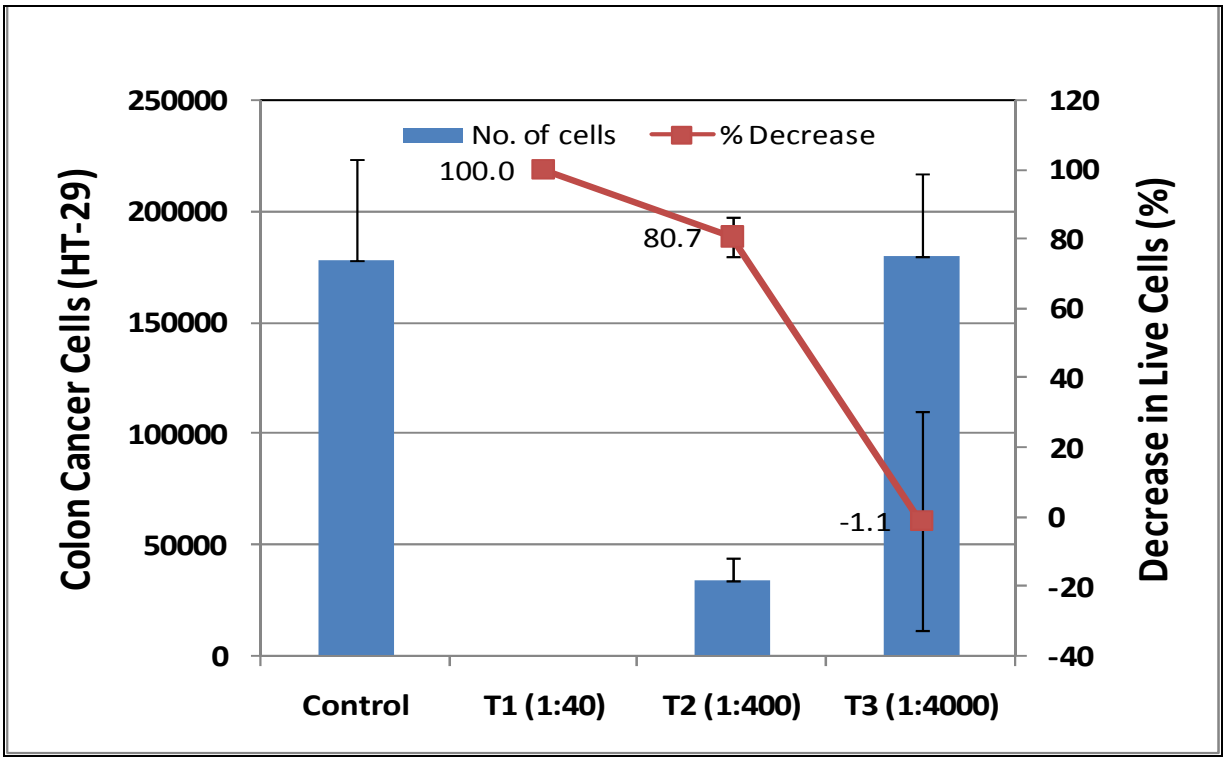

Figure 1. Effect of ALKA-V6 on survival of colon cancer cell line HT-29.

ALKA-V6 was diluted in distilled deionized (DDI) water 1:40; 1:400 and 1:4000 times. This diluted product 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 surviving cancer cells were counted and recorded. Compared to the control, it was observed that cells treated with ALKA-V6 at 1:40 diluted completely killed all the cancer cells (100% lethality). At 1:400 dilution of ALKA-V6 only 20% of the cancer cells survived (80% lethality) and at 1:4000 dilution it was not effective in killing colon cancer cells, which suggests that the EC50 (concentration at which 50% of the cells were killed) is approximately 1:250 dilution (Figure 1).

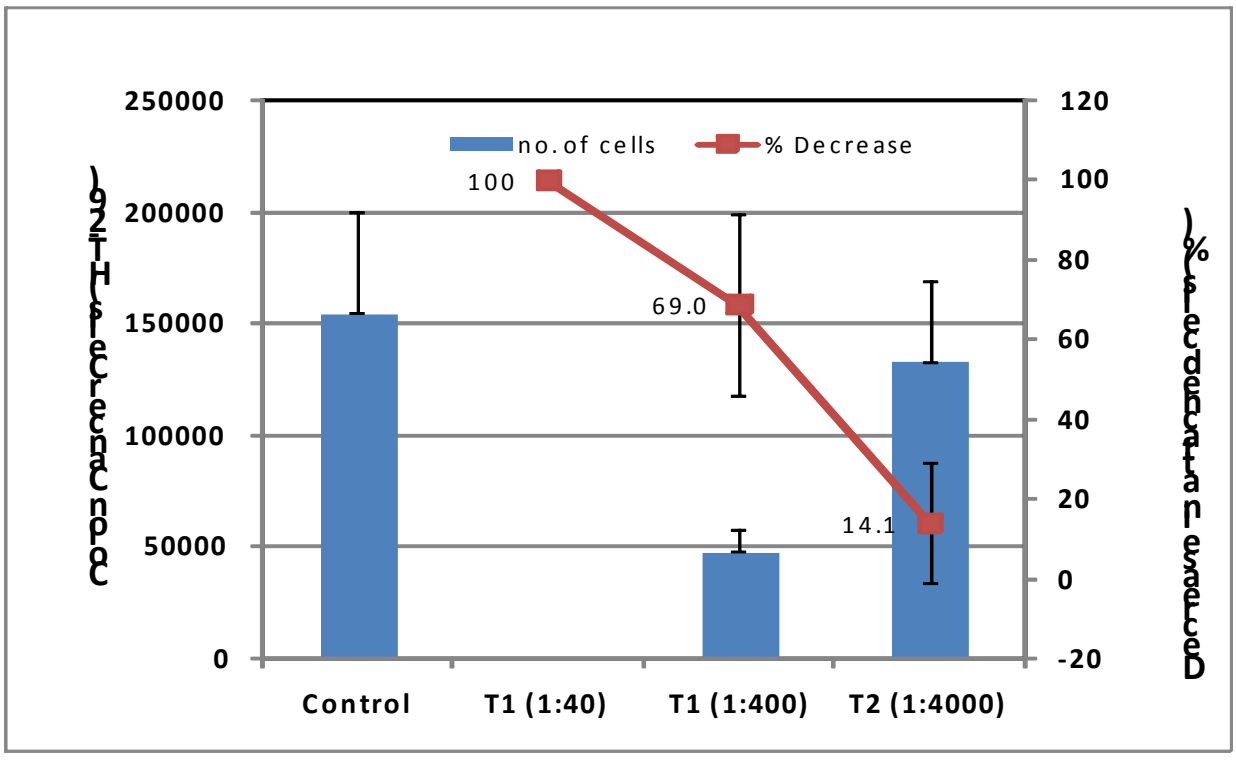

Figure 2. Effect of ALKA-V6 on attachment of colon cancer cell line HT-29 to surfaces.

Attachment of cancer cells is a fundamental process involved in the establishment of cancer, its spreading and eventual metastasis. All these processes are imperative for cancinogenesis and eventual morbidity and mortality that results from it. ALKA-V6 was diluted in distilled deionized (DDI) water 1:40; 1:400 and 1:4000 times. This diluted product 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 ALKA-V6 at 1:40 dilution it completely prevented attachment of all the cancer cells (100% effective) (Figure 2). At 1:400 dilution of ALKA-V6 only 31% of the cancer cells attached (69% effective) and at 1:4000 dilution only 86 % of the cancer cells attached (14 % effective), which suggests that the EC50 (concentration at which 50% of the cells were killed) is approximately 1:290 dilution.

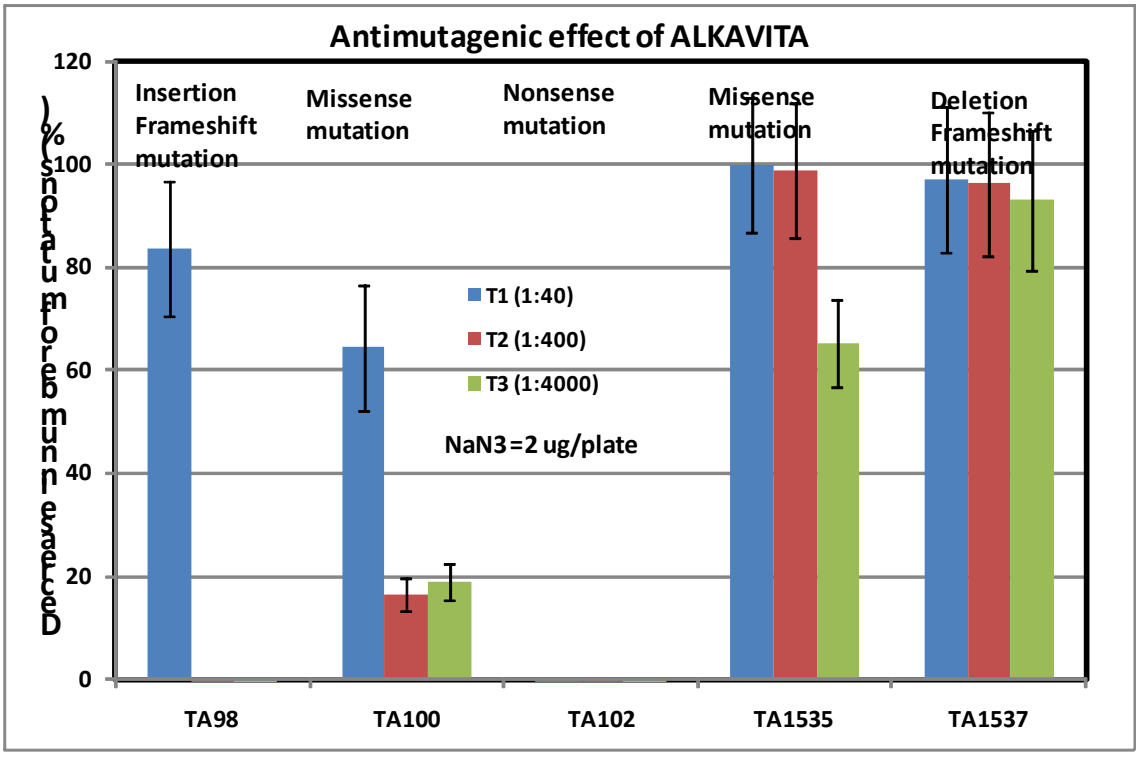

Figure 3. Effect of ALKA-V6 against various type of mutations induced by sodium azide in Ames test.

ALKA-V6 was tested at various dilutions 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 reduce mutations by 80-100% at 1:40 times dilution. At 1:400 dilution, it was able to prevent 97-100% missesense (TA1535) deletion- frameshift mutations (TA1537) and 17% of missense mutations in TA100. At the highest dilution (1:4000), the product was able to prevent 20% missense mutations in TA100; 60% missense mutations in TA1535 and 86% deletion frame-shift mutations in TA1537. ALKA-V6 may be inhibiting the binding of the mutagen to DNA by blocking the mutagen binding sites . NaN_3 is known to cause a mismatch in DNA replication by substituting for natural thiol groups from cysteine and methonine and then binding to the DNA . It is also likely that ALKA-V6 could possibly inhibit the mutation induced by NaN_3 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 NaN_3 is the induction of *recA* dependent SOS response which identifies the mutated base and removes it creating an "empty" base . As a more effective redox modulator, the mechanism for inhibition of mutagen induced SOS response by ALKA-V6 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.

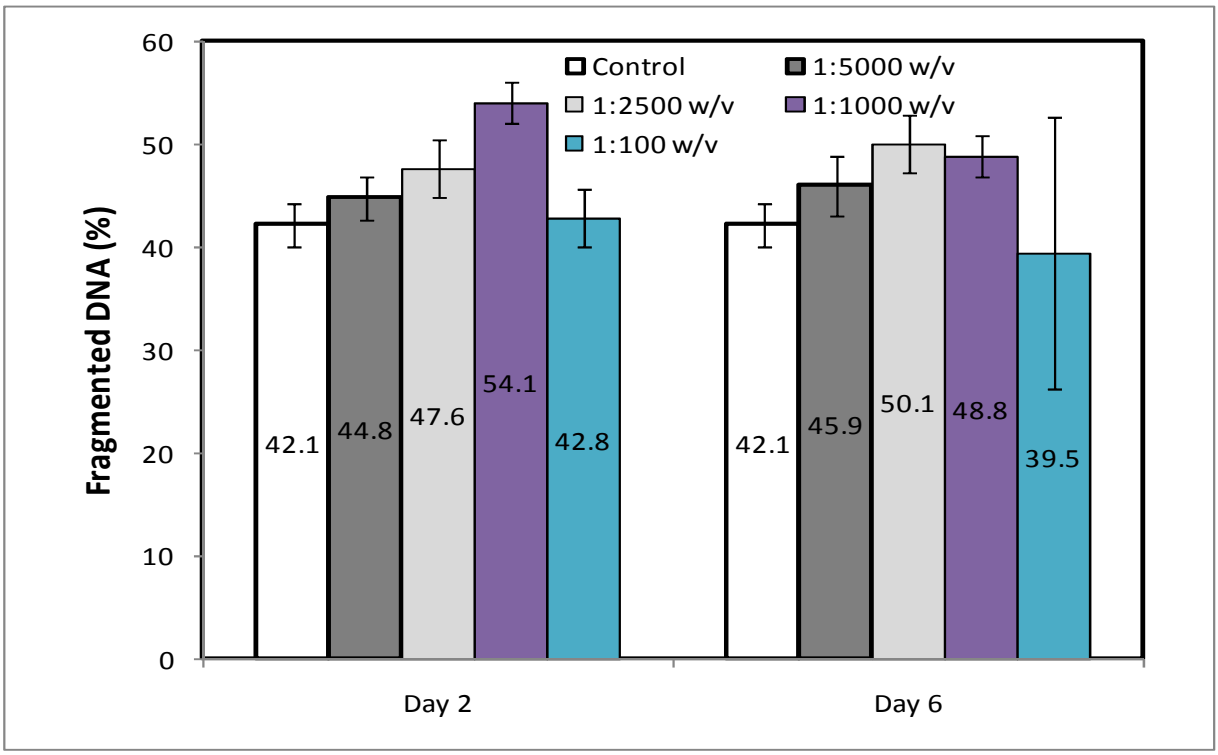

Figure 4. Apoptotic Effect of ALKA-V6 at various concentrations as measured by fragmented DNA.

Induction apoptosis or programmed cell death is an effective mechanism by which cancer development and progression can be controlled. One of the hallmarks of apoptosis is the formation of fragmented DNA due to the induction of apoptotic processes such as chromatin condensation. ALKA-V6 was diluted in DDI water 1:100; 1:1000, 1:2500and 1:5000 times and sustained apoptotic effect of ALKA-V6 was measured for 6 days. It was observed that both at day 2 and day 6 the compound exhibited a classic response curve. In general at all dilutions the compound was more effective on day 2 compared to day 6, however, even at day 6 the product retained significant activity. On both day 2 and day 6, the apoptotic activity increased with increase in concentration of ALKA-V6 (Figure 4), with the product being most effective at 1:1000 dilution. At 1:100 dilution, the amount of fragmented DNA decreased, 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.

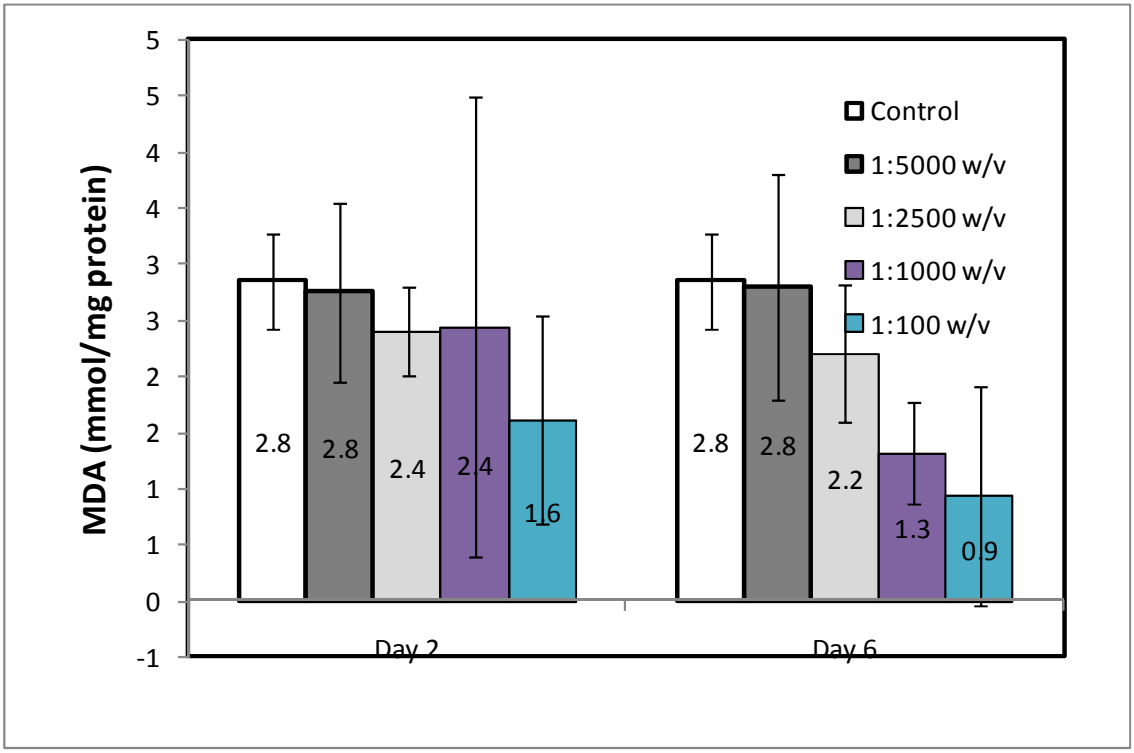

Figure 5. Effect of ALKA-V6 at various concentrations on free radical formation .

We investigated if the effect of ALKA-V6 on the redox homeostasis, by measuring the amount of free radical induced formation of malondialdehyde (MDA). Oxidation of biological molecules by reactive oxygen species results in initiation of tumerogenic and carcinogenic processes. MDA is a secondary oxidation product of lipids and serves as a good marker for oxidation and cell injury. 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. 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. In our studies, we diluted ALKA-V6 in DDI water 1:100; 1:1000, 1:2500and 1:5000 times and sustained effect of ALKA-V6 on reducing free radical induced oxidation for 6 days was evaluated. It was observed that both at day 2 and day 6 the compound exhibited a dose response curve. The compound was equally effective on day 2 and day 6 . On both day 2 and day 6, the MDA values decreased linearly with an increase in concentration of ALKA-V6 (Figure 5), with the product being most effective at 1:100 dilution.

Antioxidant Systems

Secondary to the necessary production of ROS in vivo, the body must provide a mechanism for removal of excess ROS to prevent the damaging effects of oxidation. Mechanisms for ROS removal include primary, secondary, and tertiary antioxidant defenses. Primary antioxidant defense prevents oxidation by ROS; primary defense encompasses specific antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GP), and glutathione transferase (GT). Proteins, DNA, and lipids are oxidized upon formation of excess ROS which leads to decreased protein activity, improper protein activity, transcription factor activation, and abnormal and untimely cell proliferation and apoptosis. Proteins that are especially susceptible to oxidation include those that contain the amino acids cysteine, methionine, arginine, histidine, tryptophan, and tyrosine. DNA oxidation occurs upon hydrogen abstraction, which 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. The DNA copy is misread by the DNA repair system because, when the DNA copy is oxidized, it is

unrecognizable to the repair system. This leads to incorrect pairing of DNA bases, which leads to DNA mutation and possibly cancer.

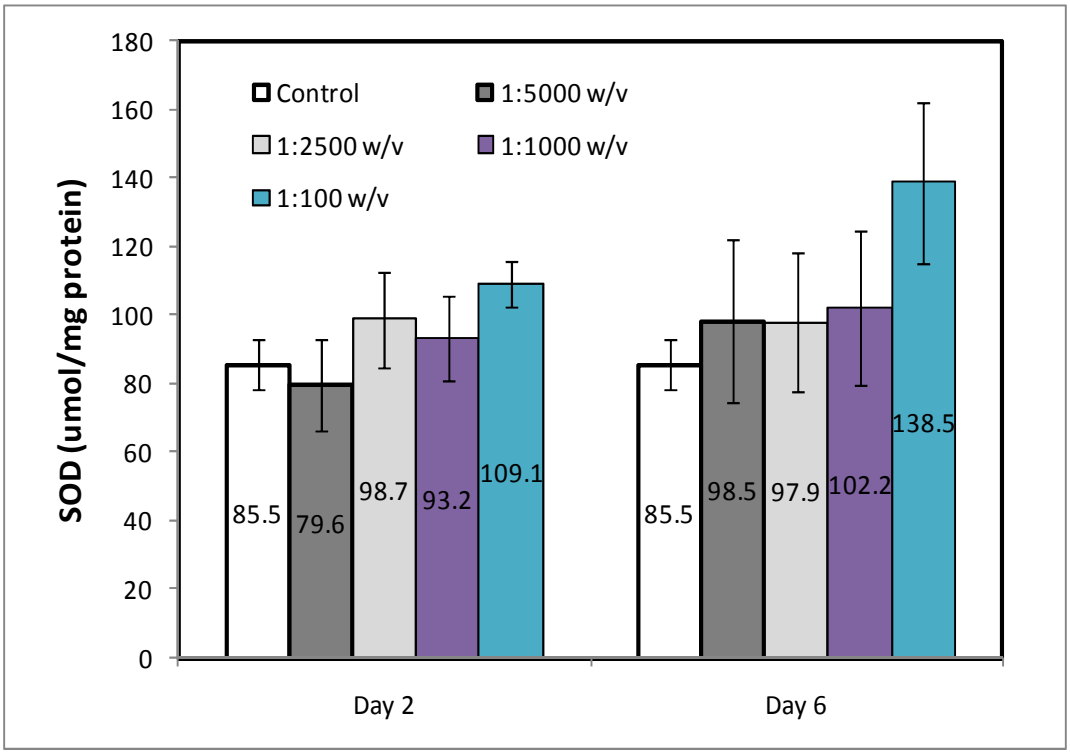

Figure 7. Effect of ALKA-V6 at various concentrations on SOD activity.

SOD is responsible for converting superoxide to hydrogen peroxide, which is then degraded by CAT. SOD exists in combination with a transition metal that corresponds to the transition metal that catalyzes the reaction that forms the ROS; for example, Mn SOD, CuZn SOD, Fe SOD, and Ni SOD. CuZn SOD is restrained in the presence of hydrogen peroxide. It is an important antioxidant defense in nearly all cells exposed to oxygen and high activity of SOD is linked to lower incidences of several forms of cancer. We investigated the ability of various concentrations of ALKA-V6 on increasing the expression and activity of an important antioxidant enzyme, SOD. In our studies, we diluted ALKA-V6 in DDI water 1:100; 1:1000,

1:2500and 1:5000 times and measured sustained effect of ALKA-V6 on SOD activity for 6 days. It was observed that both at day 2 and day 6 the compound exhibited a dose response curve. The effectiveness of the treatment increased with the time and on day 6 the SOD activity was higher than compared to day 3 levels. On both day 2 and day 6, SOD activity in the cells increased linearly with an increase in concentration of ALKA-V6 (Figure 7) with the product being most effective at 1:100 dilution. At this dilution the increase in SOD activity was 109% on day 2 and 138% on day 6.

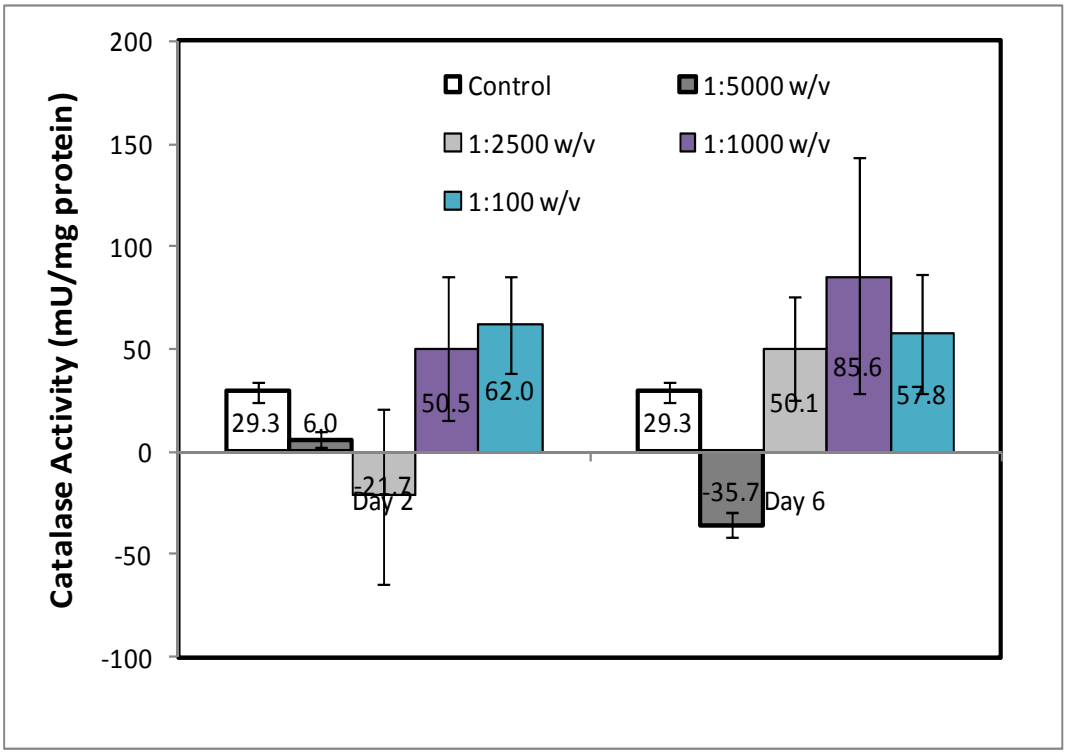

Figure 8. Effect of ALKA-V6 at various concentrations on catalase activity.

CAT is responsible for converting the hydrogen peroxide ROS to water and oxygen; it is a very efficient enzyme because it quenches hydrogen peroxide regardless of hydrogen peroxide concentration. Deficiency of CAT is observed in several types of cancer and increased activity is related to lower vigor in cancer cells. We investigated the ability of various concentrations of

ALKA-V6 on increasing the expression and activity of CAT. In our studies, we diluted ALKA-V6 in DDI water 1:100; 1:1000, 1:2500 and 1:5000 times and measured sustained effect of ALKA-V6 on CAT activity for 6 days. It was observed that both on day 2 and day 6 at 1:100; 1:1000 dilutions an increase in CAT activity occurred, with higher levels observed at 1:100 dilution. On day 6 even at 1:2500 dilution increased activity in CAT was seen. (Figure 8). The decrease in CAT activity with other dilutions is perhaps because of decreased hydrogen peroxide production due to lower ROS production and SOD activity.

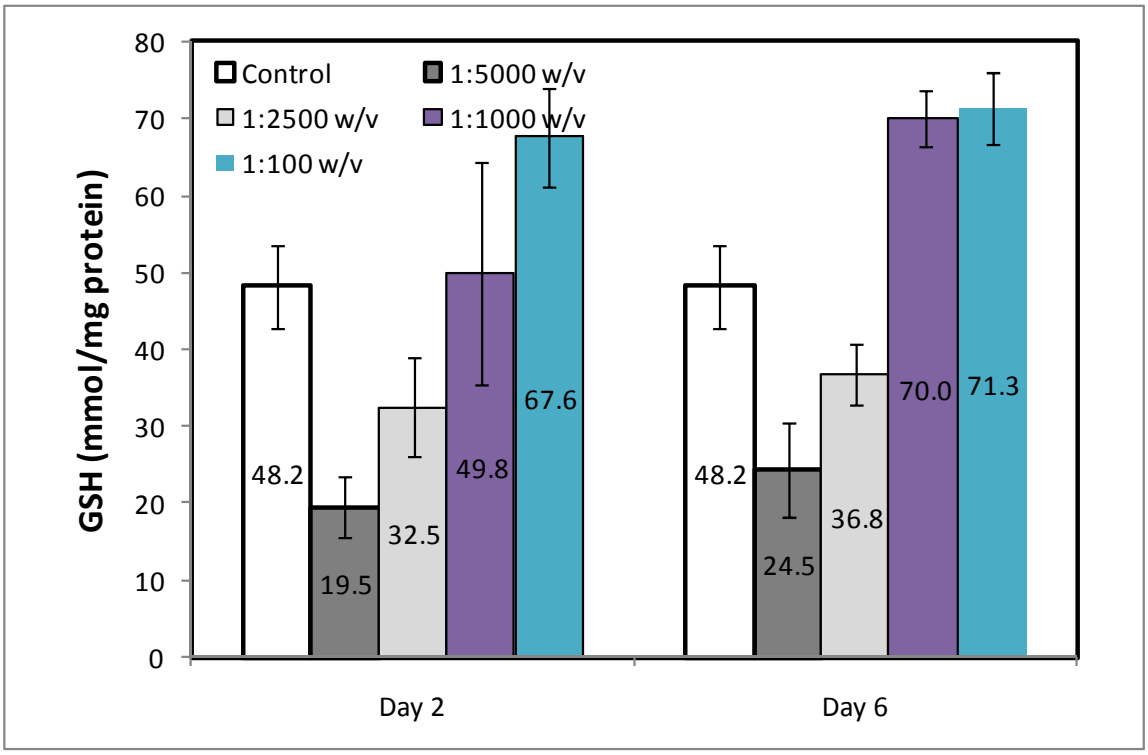

Figure 9. Effect of ALKA-V6 at various concentrations on reduced glutathione levels.

Secondary antioxidant defense molecules protect against oxidation by quenching ROS. Secondary defense encompasses small antioxidant molecules, including glutathione, vitamins E

and C, coenzyme Q, uric acid, and carotenoids (12). Small antioxidant molecules are found in the diet. Glutathione (GSH) has been described for a long time just as a defensive reagent against the action of toxic xenobiotics (drugs, pollutants, carcinogens). 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. Lower levels or deficiency is responsible for several types of cancer. We investigated the ability of various concentrations of ALKA-V6 on increasing the expression and activity of an important antioxidant molecule, GSH. In our studies we diluted ALKA-V6 in DDI water 1:100; 1:1000, 1:2500and 1:5000 times and measured sustained effect of ALKA-V6 on GSH levels for 6 days. It was observed that both on day 2 and day 6 at 1:100; 1:1000 dilutions increase in CAT activity was observed, with higher levels observed at 1:100 dilution (Figure 9). On day 2, ALKA-V6 at 1:100 dilution increased the concentration of GSH by 67 mmol whereas, an increase in GSH levels to 70 mmol was seen at 1:100; 1:1000 by day 6. The decrease in GSH levels similar to CAT levels with other dilutions is perhaps because decreased hydrogen peroxide production due to lower ROS production and SOD activity.

EVALUATION OF ANTI-RETROVIRAL BENEFITS OF ALKA-V6

Acquired immunodeficiency syndrome (AIDS), caused by human immunodeficiency virus (HIV), is an immunosuppressive disease that results in life-threatening opportunistic infections and malignancies. Despite continuous advances made in anti-retroviral therapy, AIDS has become the leading cause of death in Africa and fourth worldwide; the number of people with HIV is increasing at an alarming rate in India and Southeast Asia. Two major types of HIV have been identified so far, HIV-1 and HIV-2. HIV-1 is the cause of the worldwide epidemic and is most commonly referred to as HIV. It is a highly variable virus, which mutates readily. Several therapeutic drugs have been developed to control the onset of AIDS in the carriers of this virus, such as reverse transcriptase inhibitors (ZDV and AZT) and protease inhibitors that suppress HIV replication. While the infections in developed countries have been suppressed with these drugs, there are several limitations which have prevented successful management of retro-viral diseases worldwide. These limitations include high cost and serious side effects such as inhibition of hematopoietic function and development of resistant strains of HIV. Therefore, it is of urgent need to develop new and safer categories of therapeutic strategies that will manage this important public health concern.

ALKA-V6 is a modified value-added silicon-based compound developed by Cisne Enterprises Inc. Recent research with this compound has shown that it has several health enhancing effects in humans and animals. Research conducted by several scientists at universities and private companies have shown that this compound has very high antimicrobial effect and can reduce the risk of infections and poisoning associated with several pathogens in both humans and animals. There are also numerous testimonials obtained by the founders of the company where, they have

documented alleviation of symptoms associated with several viral diseases such as influenza, herpes, warts and HIV.

METHODS:

Nitric Oxide Production

Nitric oxide production was measured using a modified Griess assay for the detection of total nitrites. Briefly, 100 µl of whole cell extract was transferred to a microplate followed by addition of 100 µl of vanadium chloride (0.08 g/10 mL 0.1 M HCl) and 100 µl Griess reagent. Alternatively, 50 µl sulfanilamide and 50 µl N-(1-Naphthyl) ethylendiamine dihydrochloride (NEDD) can be substituted for Griess reagent in the reaction. The microplate incubated for 30 minutes at 37^{o}C and absorbance was measured at 540 nm using the Bioteck EL 808 (Houston, TX). The concentration of nitric oxide was determined by calculating the % change based on a linear standard curve equation: [Conc (umol/L) = (A540-0.0344)/0.0057)].

Reverse transcriptase (RT) assay:

The effect of different concentrations of ALKA-V6 on reverse transcription was tested using a non-radioactive HIV-RT colorimetric ELISA kit from Roche Diagnostics, Germany. The protocol outlined in the kit was followed using 2 ng of enzyme in a well and incubating the reaction for 2 h at 37 °C.

Glycohydrolase enzyme assays:

Glycohydrolase enzymes are found in the eukaryotic host cell's Golgi apparatus and are responsible for glycosylation of proteins. Inhibition of the glycohydrolase enzymes has been found to decrease the infectivity of the HIV virion, as the HIV envelope proteins are highly

glycosylated during the life cycle of the virus. α-Glucosidase has been found to be partly responsible for the glycosylation of HIV gp120. To measure the inhibition of the glycohydrolase enzymes; α-glucosidase (Sigma, MO, USA), β-glucosidase (Sigma, MO, USA) and β-glucuronidase (Roche Diagnostics, Germany) were used with their corresponding substrates ρ-nitrophenyl-α-d-glucopyranose, ρ-nitrophenyl-β-d-glucopyranose and ρ-nitrophenyl-β-d-glucuronide (Sigma, MO, USA) in a colorimetric 96-well microtiter plate-based assay, determining the amount of ρ-nitrophenol released. The method described by Collins et al., 1997 and Collins et al., 1997 was followed with modifications. Briefly, substrates and enzymes were dissolved in their appropriate 50 mM buffers (2-morpholinoethanesulphonic acid monohydrate (Mes)–NaOH (Sigma, MO, USA), pH 6.5, for α-glucosidase and β-glucuronidase and sodium acetate, pH 5.6, for β-glucosidase). The final assay volume was 200 μl and contained 2 mM substrate, 0.25 μg enzyme, and the crude extract at 0.2 mg/ml. The reaction was allowed to proceed for 15 min at 25 °C before termination with 60 μl 2 M glycine–NaOH, pH 10, and measurement of absorbance at 412 nm.

Protease (PR) assay

The procedure for the fluorometric detection of HIV-PR activity was carried out as described by Au et al., 2000 and Au et al., 2000 using HIV-II PR. The HIV-II PR was obtained from the NIH AIDS Research and Reference Reagent Program, NIAID, NIH, MD, USA. in the form of 100 μg in 100 mM sodium phosphate (pH 8), 50 mM NaCl buffer. A fluorescence resonance energy transfer (FRET) assay using the fluorogenic substrate, DABCYL-γ-Abu-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-EDANS (Bachem, Switzerland), was used to assay HIV-PR. Substrate (10 μM) was added to a 200 μl reaction sample that included 100 nM HIV-II PR, reaction buffer (0.1 M sodium acetate, 1 M NaCl, 1 mM EDTA, 1 mM DTT, 10% DMSO, 1 mg/ml BSA, pH 4.7) and

ALKA-V6 at different concentrations. This was incubated at 37 °C for 2 h. The fluorescence intensity is indicative of protease activity and was measured at an excitation wavelength of 355 nm and emission wavelength of 460 nm.

Anthrone Assay

40µl water (blank), standard (0.05, 0.15, 0.2, 0.25, 0.3 and 0.4), or sample was added to each well of a 96-well microtiter plate. To the wells, 0.1ml anthrone solution (freshly prepared) was added. The plates were mixed well and incubated at 92^{o}C for 3min in a non-shaking water bath. Plates were then transferred to a non-shaking water bath at RT for 5min to stop the reaction and absorbance was read at 600 nm.

Ferric-orcinol assay (Bial's test)

To 200 µl of sample, 200 µl of 10% TCA was added and heated at 100^{o}C for 15 minutes. Tubes were rapidly cooled at 25^{o}C and 1.2 ml of the following reagent: (1.15% w/v ferric ammonium sulfate and 0.2% w/v orcinol in 9.6M HCL) was added and mixed thoroughly. Samples were again heated at 100^{o}C for 20 minutes and cooled to room temperature. Absorbance of the blue-green color was measured at 660 nm.

Sialic Acid Assay Method

To a sample, 0.1ml 0.04M periodic acid was added and thoroughly mixed and incubated in an ice bath for 20min. 1.25ml resorcinol reagent was added mixed and placed in an ice bath for 5min. The solution was heated at 100^{o}C for 15min and cooled in tap water after which 1.25ml of tert-butyl alcohol was added and mixed vigorously to give a single phase solution. The tubes

were then placed in a 37^{o}C water bath for 3min to stabilize the color and absorbance read at 630nm.

Uronic Acid Assay

To 40µl sample containing 200µl concentrated sulfuric acid (96%) w/w containing 120mM sodium tetraborate was carefully added in a microplate and mixed. The plates were incubated at 80^{o}C for 1h and cooled. 40µl m-hydroxydiphenyl reagent (100µl of m-hydroxydiphenyl in dimethyl sulfoxide, 100mg/ml mixed with 4.9 ml 80% sulfuric acid just before use) was added to each sample and mixed. After incubating for 15 min at room temperature, absorbance was read at 540nm.

Heptose Assay

To 50 µl of sample, 50 µl 0.5 N H_2SO_4 was added and vortexed. The mixture was placed in a 100^{o}C waterbath for 8 min and cooled to room temperature. To this 50 µl, H_5IO_6 was added and mixed. This mixture was incubated for 10 min at room temperature and 100 µl arsenite reagent was added and mixed until the yellowish color disappeared. To this 200 µl, thiobarbituric acid reagent was added and incubated at 100^{o}C for 10 min and cooled. 1.5 ml butanol reagent was added and the solution was vortexed. 125 µl of DMSO was then added and absorbance was read at 550 nm.

RESULTS

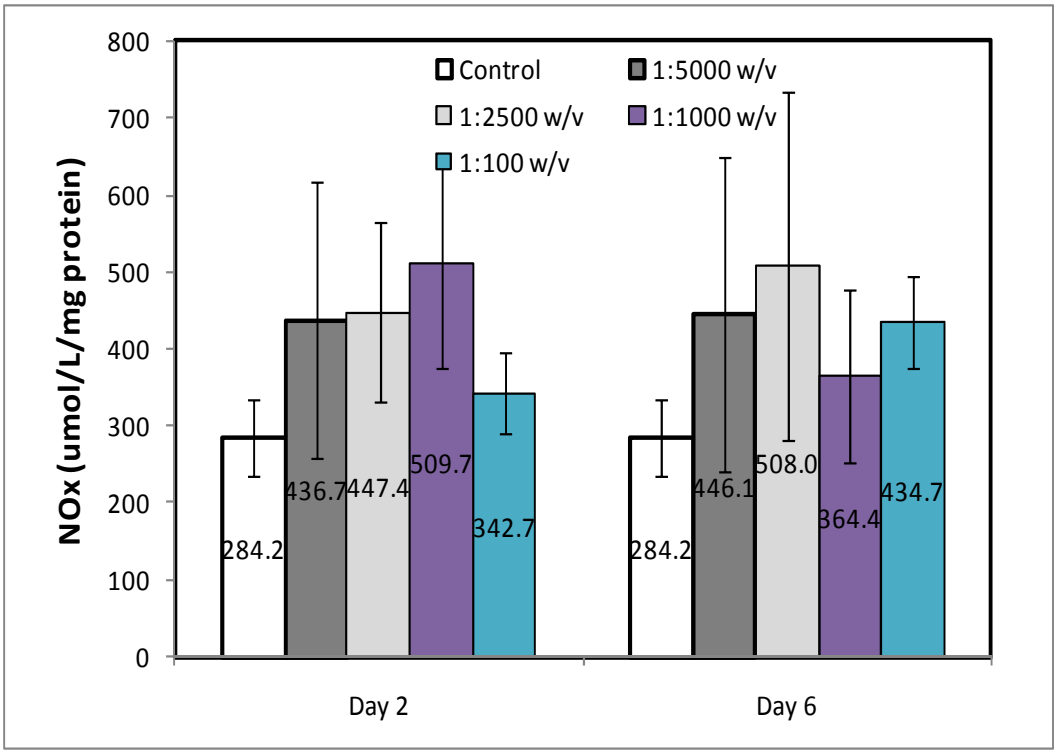

Figure 1. Effect of ALKA-V6 at various concentrations on nitric oxide levels as measured by total nitrates.

The immune system uses nitric oxide for fighting viral, bacterial and parasitic infections. Nitric oxide is a reactive nitrogen species produced from the conversion of L-arginine to citrulline via nitric oxide synthase (NOS). Nitric oxide is highly reactive due to its reaction with other ROS to form peroxynitrite; it is also a vasodilator and therefore is involved in the control of blood pressure via the stimulation of guanylate cyclase. Three forms of NOS exist, including neuronal NOS-I, endothelial NOS-III, and NOS-II. NOS-I and NOS-III are constitutive, while NOS-II is inducible in macrophages. It is physiologically a very important molecule and plays important role in adhesion molecule/chemokine expression, Leukocyte recruitment for fighting off viral infections. Being a neurotransmitter gas, it plays an important role in long-term potentiation and

in synaptic plasticity, both of which are important for memory development. Additionally, it is also called a endothelium derived relaxation factor (EDRF) and plays an important role in blood pressure reduction and reproductive health. Nitric oxide (NOx) also decreases proliferation of tumours and is also associated with learning, memory, sleeping, feeling pain, and, probably, depression. In our studies, we diluted ALKA-V6 in DDI water 1:100; 1:1000, 1:2500and 1:5000 times and measured sustained effect of ALKA-V6 on NOx levels for 6 days. ALKA-V6 at all concentrations, increased the NOx levels for the duration of the treatment. On day 2, the levels of NOx were treatment. 437umol, 447umol, 509 mmol and 342 mmol at 1:5000 , 1:2500, 1:1000, 1:100 dilutions respectively (Figure 1). Whereas on day 6, these levels changed to 446umol, 508umol, 364umol, 434umol respectively. Suggesting a sustained effect of ALKA-V6 on NOx levels.

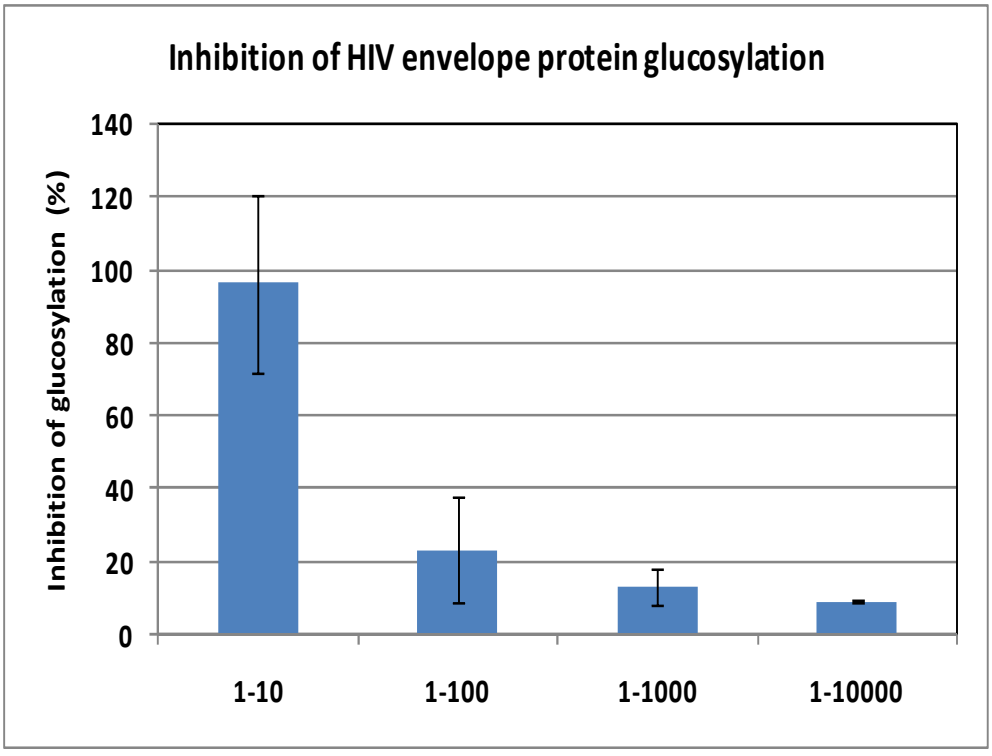

Figure 2: Effect of various concentrations ALKA-V6 on HIV envelop protein glucosylation

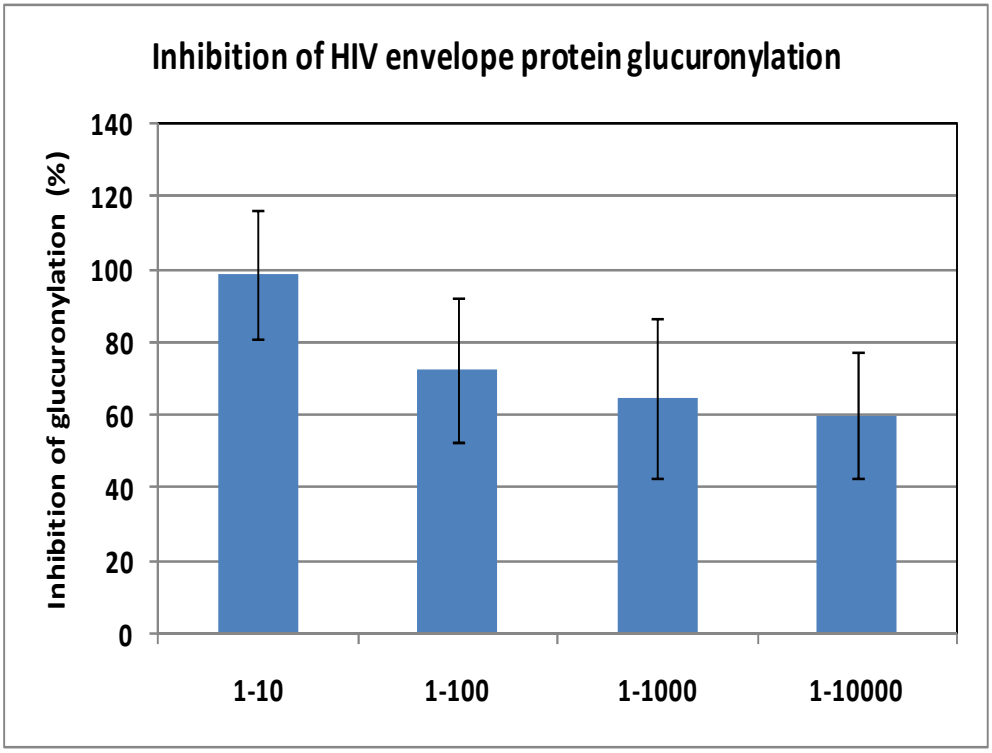

Figure 3: Effect of various concentrations ALKA-V6 on HIV envelop protein glucuronylation

Virus evades immune system and attaches to our cells using surface sugars. If this process can be inhibited, infection can be prevented. Glycohydrolase enzymes are found in the eukaryotic host cell's Golgi apparatus and are responsible for glycosylation of proteins. Inhibition of the glycohydrolase enzymes has been found to decrease the infectivity of the HIV virion, as the HIV envelope proteins are highly glycosylated during the life cycle of the virus. Alpha-Glucosidase has been found to be partly responsible for the glycosylation of HIV gp120. Inhibitors of glycosylation could have potential therapeutic use by interfering with viral maturation, attachment and evasion. In our studies, we diluted ALKA-V6 in DDI water 1:10; 1:100; 1:1000, and 1:10000 times and measured the effect of ALKA-V6 on viral envelop glucosylation and glucuronylation (Figure 2 and Figure 3 respectively). It was observed that ALKA-V6 reduced glucosylation of the viral envelop protein in a dose dependent manner (Figure 2). At 1:10

dilution, the product completely inhibited the activity if glucohydrolase, with further dilution resulting in a linear decrease in inhibition. The EC50 for inhibition of glucosylation was calculated to be 1:40 dilution. ALKA-V6 also inhibited the glucuronylation of viral protein in a similar dose dependent manner. At 1:10 dilution, the compound caused 98% inhibition in the enzyme activity. Further dilution resulted in a linear decrease in the enzyme activity. At the lowest concentration, the compound still retained glucuronylation inhibition activity of 60% Suggesting the EC50 for this assay was much lower than the concentratons tested in this investigation (Figure 3).

In addition, we also confirmed that the inhibition in viral glycoprotein post translational modification resulted in an actual changes in the viral carbohydrate composition. Using the assays described in the previous section, we determined the composition of various tpes of sugars including ribose, heptose, sialic acid and uronic acid levels upon treatment with different concentrations of ALKA-V6. This is an important parameter to evaluate as different sugars determine the conformation and specificity of interaction with receptor systems. This conformation and specificity can be altered by changing sugar composition which renders the protein not suitable for receptor interaction. This inability to bind to a cell surface receptor prevents attachment to cells for invasion and makes them susceptible for clearance by the immune system. It must be noted that our objective here was to just monitor changes in the sugar composition and not necessarily increase or decrease, as just change in composition is enough to induce non-specificity. Our results indicated a change in the composition of ribose (Figure 5), heptose (Figure 6), sialic acid (Figure 7) and uronic acid (Figure 8) levels upon treatment with different concentrations of ALKA-V6 .

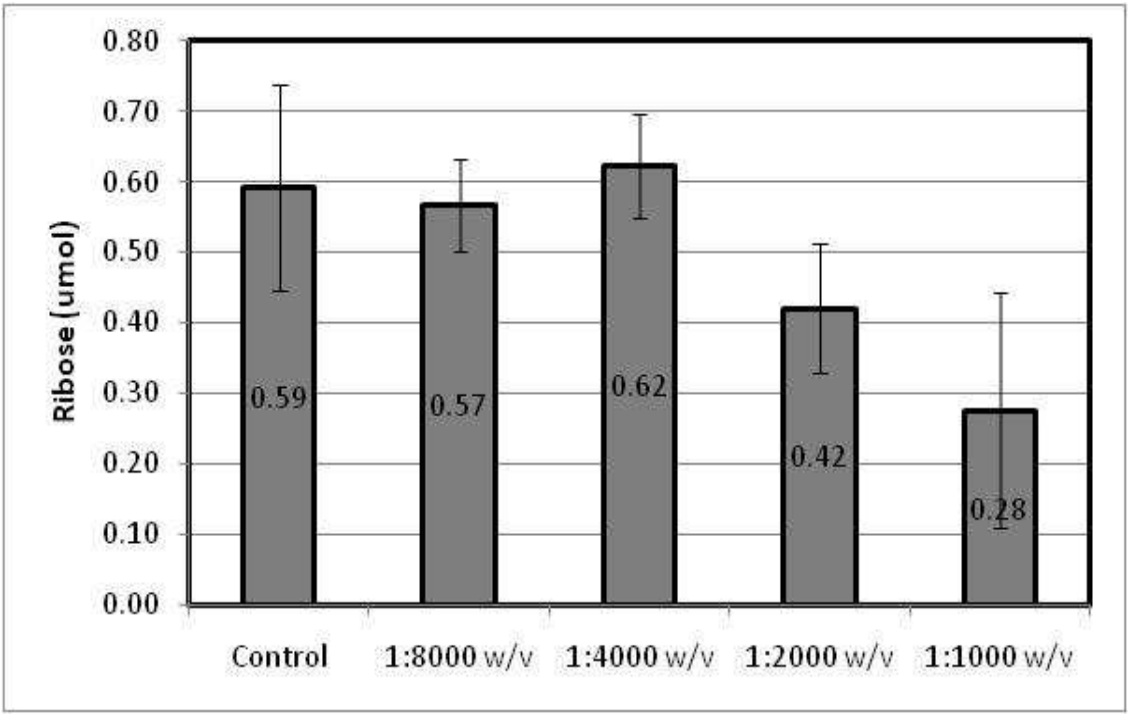

Figure 5: Effect of various concentrations of ALKA-V6 on ribose composition

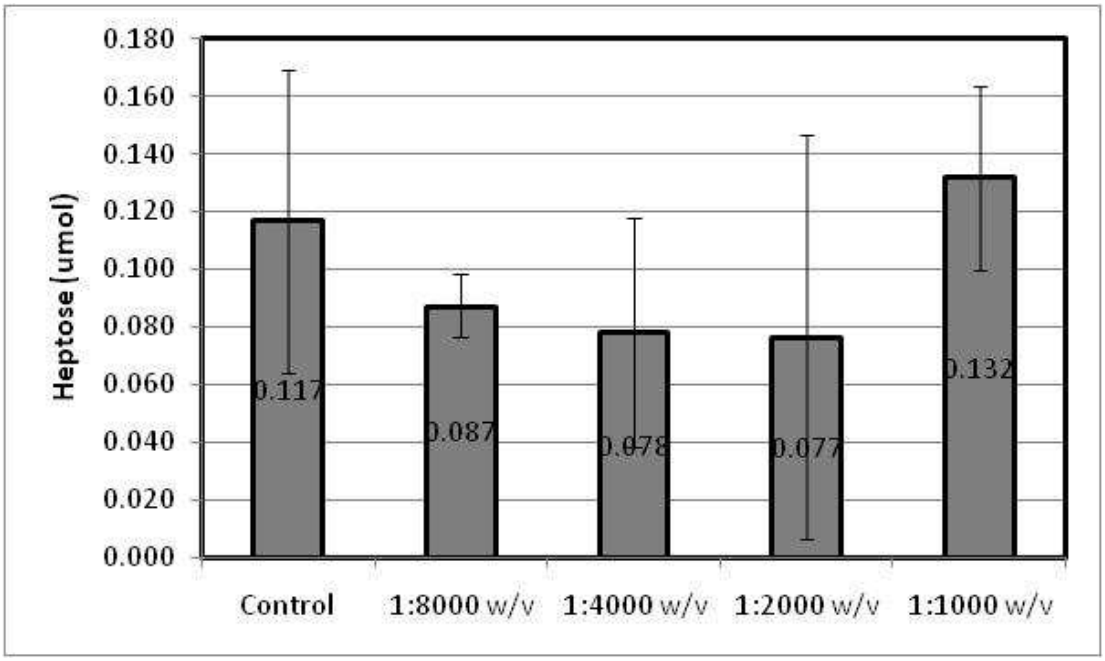

Figure 6: Effect of various concentrations of ALKA-V6 on heptose composition

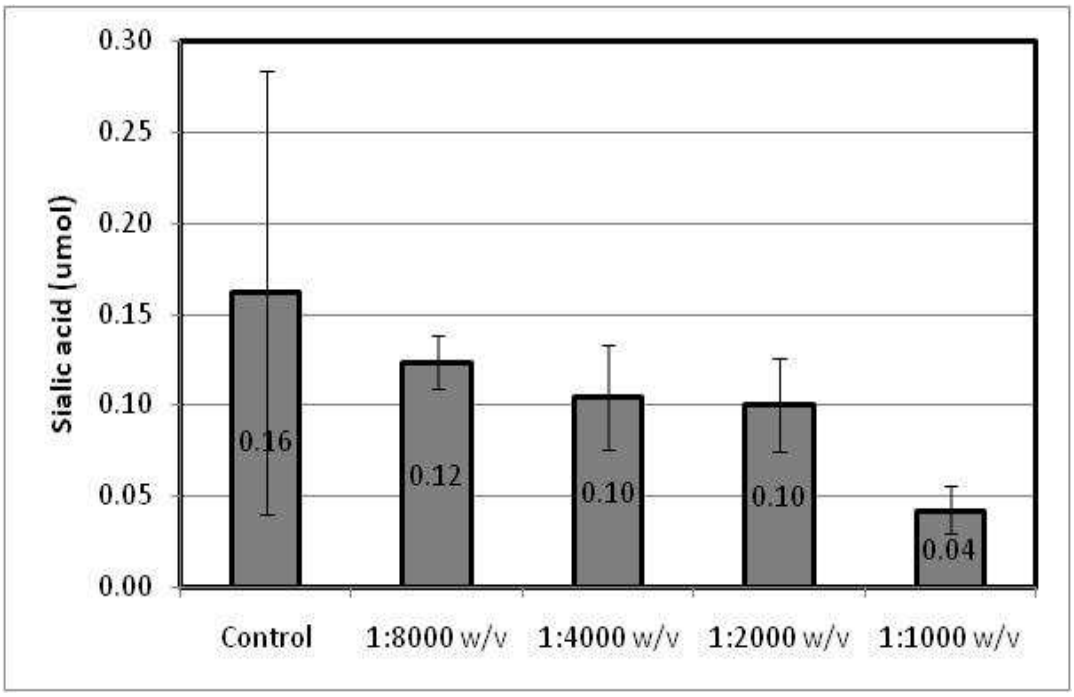

Figure 7: Effect of various concentrations of ALKA-V6 on sialic acid composition

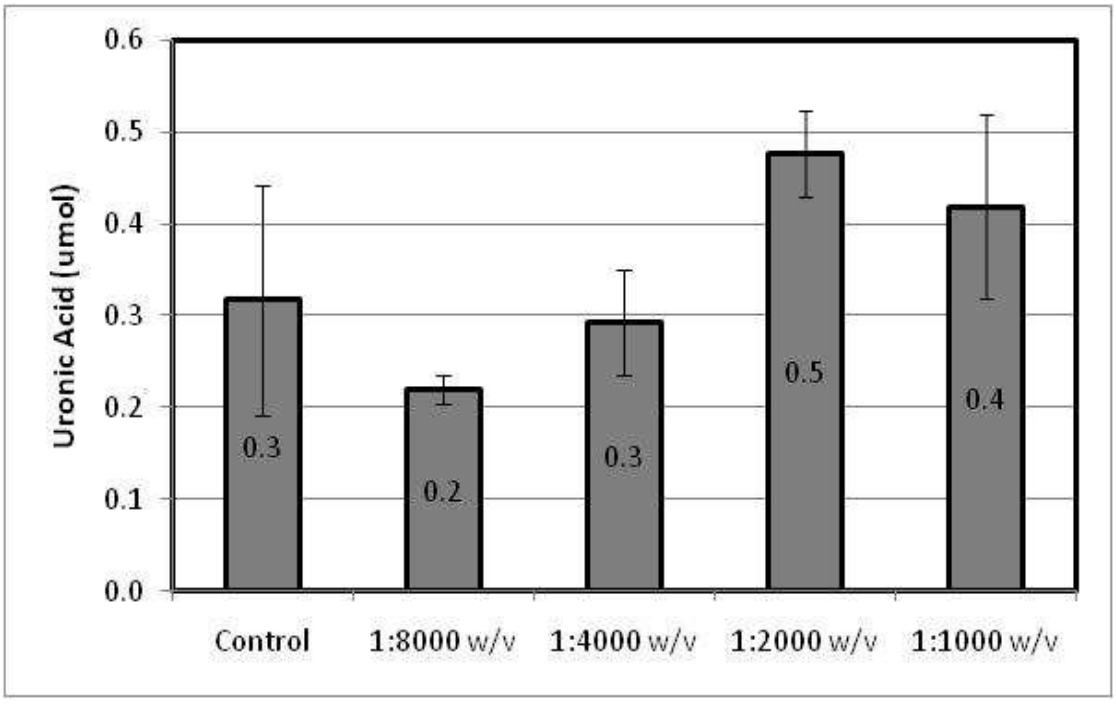

Figure 8: Effect of various concentrations of ALKA-V6 on uronic acid composition

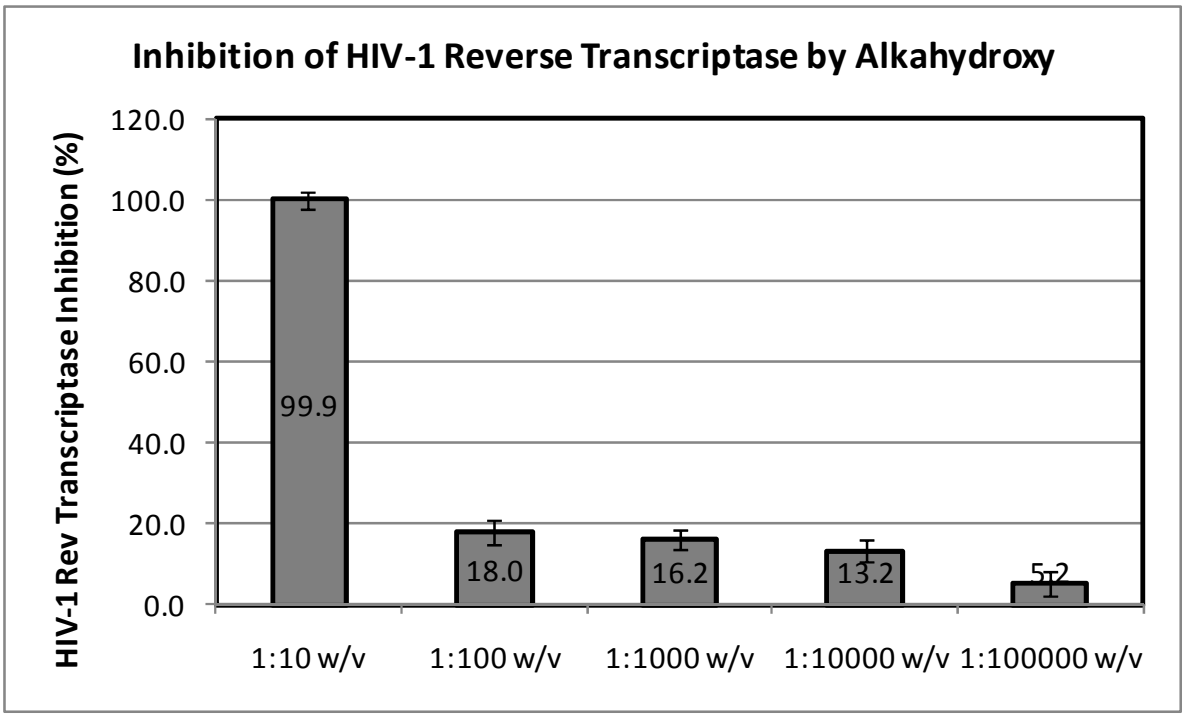

Figure 9: Effect of various concentrations ALKA-V6 on HIV-1 reverse transcriptase activity

Before retroviral infections establish themselves, their nucleic material of RNA needs to be first transcribed into a double stranded DNA by a viral enzyme known as viral reverse transcriptase (RT). Once RT synthesizes DNA, it is ligated into the host genome where it replicates along with the host DNA. When conditions are favorable, the viral DNA is transcribed and translated to make all proteins necessary for the viral assembly. However, without reverse transcriptase, the viral genome couldn't become incorporated into the host cell, and couldn't reproduce. This important step had made anti-RT therapy an important target for developing antiviral drugs for a number of retroviral infections. In our studies, we diluted ALKA-V6 in DDI water 1:10; 1:100; 1:1000, 1:10000 and 1:100000s times and measured the effect of ALKA-V6 on HIV-1 reverse transcriptase activity. Our results showed that at 1:10 dilution, ALKA-V6 completely inhibited the activity of the enzyme resulting in the synthesis of very low levels of HIV-1 DNA (Figure 9).

Upon further dilution, the inhibition activity decreased significantly and at 1:100; 1:1000, 1:10000 the inhibition ranged between 13-18% respectively and 1:100000 the inhibition activity further decreased to 5% compared to the control (Figure 9).

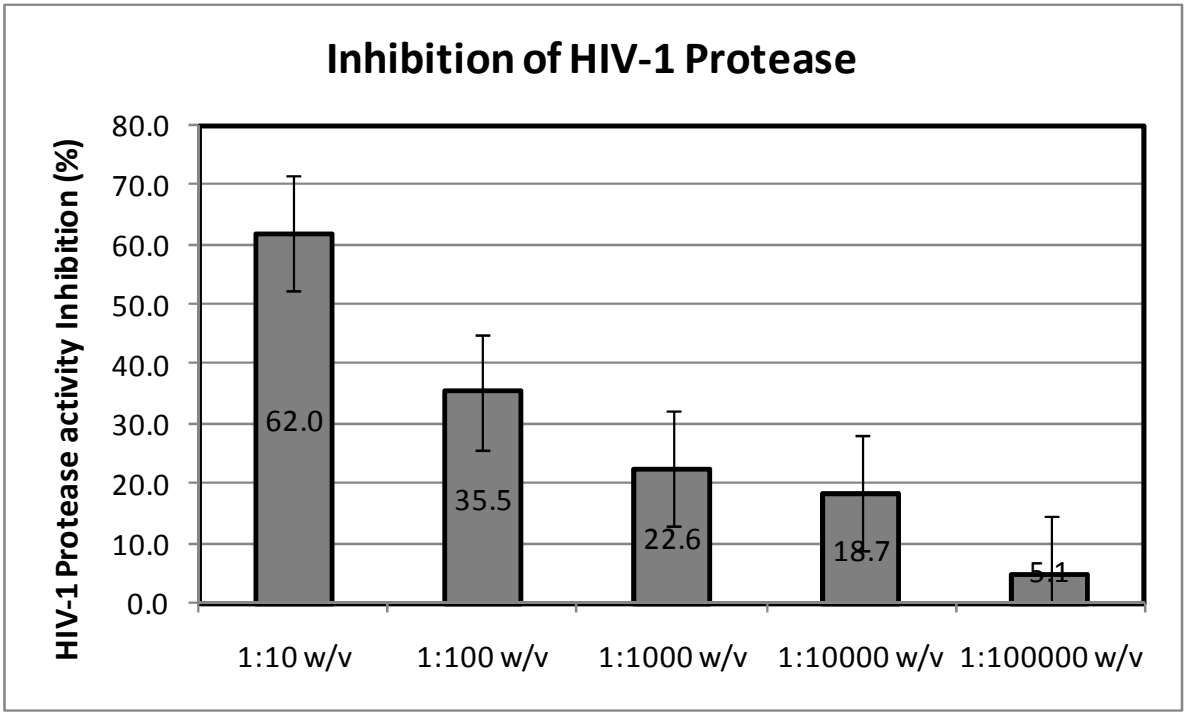

Figure 10: Effect of various concentrations ALKA-V6 on HIV- protease activity

As described above, Viral DNA which is synthesized from reverse transcription is transcribed and translated in the host cell to propagate infection. The viral DNA that is integrated into the host DNA is transcribed and translated into a single polypeptide chain. Different peptides needed for the viral assembly are generated by the post-translational modification of this single polypeptide chain. An important enzyme involved in this processing step is called the viral protease which cleaves the long chain into its individual enzyme components, which then facilitate the production of new viruses. In the absence of this viral protease activity, the viral assembly becomes improbable and therefore is an important target for controlling several types

of retroviral infections. In our studies, we diluted ALKA-V6 in DDI water 1:10; 1:100; 1:1000, 1:10000 and 1:100000s times and measured the effect of ALKA-V6 on HIV-1 protease activity using fluorescence measurements. The compound was able to inhibit the protease activity in a linear dose dependent manner. At the highest concentration, (1:10) the compound was able to decrease the activity of the protease by 62% (Figure 10). Further dilution caused 36%, 22% and 19% decrease in the protease activity. At the lowest concentration of the compound i.e. 1:100000, the inhibition activity was noted to be 5% (Figure 10).

DETERMINATION OF CHEMICAL STRUCTURE OF ALKA-V6 BY MASS SPECTROSCOPIC AND NUCLEAR MAGENETIC RESONANCE ANALYSIS

MS and NMR analysis generated a putative formula of the compound to be (Confidential).. . . .. The formula suggests that ALKA-V6 is not a single compound but a mixture of two different compounds that are in equilibrium with each other. ALKA-V6 appears to be a stoichiometric mixture of

1. . . . . (Confidential)
2. . . . . .(Confidential

(Confidential) . . . . . appears 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. Many of the biological activities of ALKA-V6 could be due to this presence of ALKA-V6 in multiple ionizable forms, giving it the ability to accept and donate electrons and participate in important redox reactions in the body to bring about redox homeostasis.

CONFIDENTIAL INFORMATION

SUMMARY

ALKA-V6 was evaluated in a series of in vitro tests to determine 1) its effects as an anti-cancer product on colon cancer cells; and 2) its effect as an anti-retro-viral product on the HIV virus. Additionally, the elemental and chemical properties of ALKA-V6 were evaluated based on analyses determined at outside laboratories and results provided by CISNE.

ANTI-CANCER EFFECTS:

- ALKA-V6 prevented attachment of cancer cells in a dose-dependent manner
- ALKA-V6 reduced harmful mutations in the DNA and its effects were dose-dependent
- ALKA-V6 induced apoptosis (programmed cell death) of and its effects were time and dose-dependent.
- ALKA-V6 stimulated important antioxidant enzymes in a dose dependent manner

ANTI-RETROVIRAL EFFECTS:

- ALKA-V6 increased nitric oxide dependent anti-viral effects at all concentrations tested
- ALKA-V6 inhibited enzymes important in viral assembly, metabolism and replication
- ALKA-V6 caused changes in the viral carbohydrate composition and metabolism
- ALKA-V6 inhibited the activity of the enzyme responsible for transcribing RNA to DNA in the virus and the effects were dose dependent

ELEMENTAL AND CHEMICAL PROPERTIES:

- ALKA-V6 appears to be a stoichiometric mixture of two compounds that are in equilibrium with each other

- The two ionizable compounds of ALKA-V6 are trimeric sodium silicate $(Na_2SiO_3)_3$ and sodium silicate pentahydrate $(Na_2SiO_3).5H_2O$

CONCLUSIONS AND IMPLICATIONS

ALKA-V6 is a silicon-based alkaline solution with a pH of 13.7. Its elemental and chemical properties give it unique electrochemical and structural characteristics to participate in reactions in colon cancer cells and the HIV-virus that are beneficial. These health promoting properties of ALKA-V6 appear to be directly related to ways it regulates redox processes of biological molecules through different free radical species of oxygen and nitrogen. Changes in redox status appear to lead to unknown ways in which cellular and viral biochemical systems are then modulated.

The unique electrochemical and structural composition of ALKA-V6 indicates that it may provide an alternative basis for control of colon cancer cell growth and HIV virus survival. However, this implication is based solely on in vitro results and end-point in vivo evaluations are imperative.

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