The effects of natural compounds on proliferation of human prostate cancer cells

Kira Radinsky
Halilah 6/16, Nesher
20306
Israel
kirar@012.net.il

Ilan Shahin
5865 Shalom Ave.
Montreal, Quebec
Canada
H4W 3A5
makeweb@hotmail.com

Mentor: Nachshon Maya
Technion; Israel Institute of Technology
Department of Food Engineering and Biotechnology

Abstract
Cruciferous vegetables contain glucobrassicin which, during metabolism, yields indole-3-carbinol (I3C), 3,3'-diindolylmethane (DIM) and ascorbigen (ASC). The anti-carcinogenic effects of I3C and DIM were exhibited in human breast cancer cells. The objectives of this study were to examine the potential effects of I3C, DIM and ASC on the proliferation and induction of apoptosis in human prostate cancer cell lines. Our results indicate that the indole derivatives suppress the growth of these cells in a dose-dependent manner, by inducing apoptosis. It appears that these indolic compounds may offer effective means against prostate cancer.

1. Introduction

Prostate Cancer is a tumor that grows in the prostate, the gland that releases a substance which turns semen into a liquid. It is the most common cancer in American men [1].

For the average American man, the odds of being diagnosed with prostate cancer in his lifetime are approximately 1 in 6. The median age of diagnosis of prostate cancer is 71 and 69 in white men and African-American men, respectively. African-American men have a substantially higher incidence of prostate cancer compared with aged-matched white Americans. Prostate cancer is the second-leading cause of cancer deaths in U.S. men, with 31,900 deaths estimated in 2000 alone. Overall, prostate cancer deaths represent approximately 11% of all cancer deaths in the United States [3].

The cause of prostate cancer is unknown. Hormones may control the gland and may contribute to this type of cancer. The best-established risk factors include: advanced age, ethnic background, family history of cancer and a diet rich in fat. So far, prostate cancer has not been linked to common cancer-causing substances in the environment. Men who have had a vesectomy, use tobacco or have been exposed to cadmium may also be at an increased risk. It is not certain whether viruses, chronic infections, or sexual practices are also responsible for the development of the disease [4, 5].

Depending on the grade and stage of the cancer, treatments can range from passive monitoring to more drastic measures. Surgical solutions are common, such as cryosurgery to freeze cancer cells or surgical removal of the prostate, seminal vesicles or testes to block testosterone production. External or implanted radiation to the prostate and pelvis and chemically blocking the production of testosterone remain viable options as well [1].

The two most common treatments for prostate cancer today are androgen withdrawal and chemotherapy. Androgen withdrawal involves blocking off the testes, thus reducing the level of androgen, which is a class of male hormones responsible for secondary male characteristics such as a deep voice and facial hair [6]. Unfortunately, this treatment is not always successful. Chemotherapy is the treatment for cancer with drugs designed to selectively kill faster-growing tumor cells. However, prostate cancer does not respond well to chemotherapy.

Apoptosis, or programmed cell death, has recently been brought to light as a viable solution to prostate cancer and others. By selectively targeting the cancer cells and inducing their self-destruction, cancer growth can be slowed, and even regressed [2]. Apoptosis is characterized by cell shrinkage, fragmentation into membrane-bound apoptotic bodies, DNA fragmentation and rapid phagocytosis by neighboring cells [7, 8]. Apoptosis is detected by testing for DNA fragmentation through electrophoresis of dead cells in agarose gel.

A number of studies have demonstrated a decreased incidence of various cancers (including prostate cancer) in humans consuming large amounts of Cruciferous vegetables such as broccoli, cauliflower and Brussel Sprouts. These vegetables contain glucobrassicin (GB), which undergoes hydrolysis by myrosinase upon crushing, or by cooking. The main hydrolysis product of GB is I3C. In a low pH environment, I3C is converted into many polymeric products, among which are 3,3'-diindolylmethane (DIM) and ascorbigen (ASC) [10]. Figure 1 shows the chemical structure of the indole compounds [11].

The anti-carcinogenic effect of the indole compounds, I3C and DIM has been demonstrated in human breast cancer cells [2].
Recently it has been reported that I3C inhibits the proliferation of PC3, a human prostate cancer cell line leading to apoptosis [17].

These encouraging results prompted our research into prostate cancer, a second type of cancer, which burdens Western society comparably. Our research examined the inhibitory and apoptosis inducing effects of I3C, DIM and ASC on PC3 and DU145 prostate cancer cell lines.

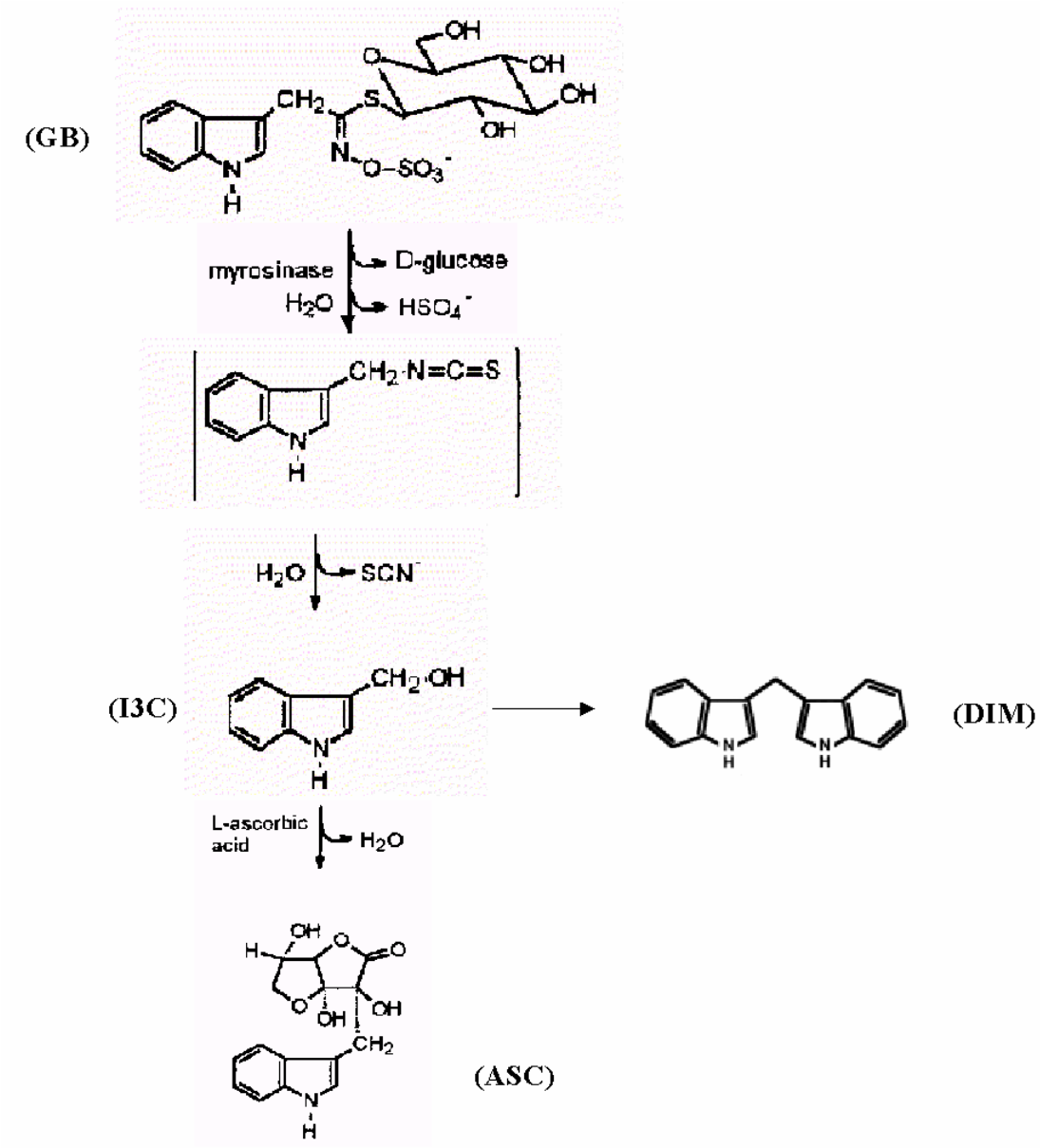

Figure 1 – The derivation and chemical structure of the anti-carcinogenic indole compounds I3C, DIM and ASC from GB is shown chronologically.

2. Materials and Methods

2.1. Chemicals

Indole-3-carbinol was purchased from Sigma (Israel). 3,3' diindolylmethane and ascorbigen were purchased from Designed Nutritional Products (USA). All indole derivatives were dissolved in a DMSO solution. Cell culture media and reagents were obtained from Biological Industries, Beit Haemek, Israel.

2.2. Cell Culture

DU145 and PC3 cell lines were maintained in RPMI and F-12 mediums, respectively, containing 10% fetal calf serum, 100 μg/ml of streptomycin and 100 U/ml of penicillin. DU145 medium contained also 100 U/ml of insulin. Cells were grown at 37°C, in humidified air containing 5% CO_2.

2.3. Assay for growth inhibition

We investigated the effect of the indole compounds on cell growth inhibition using the MTT assay. This assay quantifies viable cells by observing the reduction of tetrazolium salt, MTT, to formazan crystals by the cells. Based on the absorbance of the cell samples after the test is carried out, cell viability can be measured [12, 13]. Cells were plated with nutritional medium in 96 well plates (2000 and 5000 cells/well for DU145 and PC3 cells). After 24 hours, cells were treated with a different concentration of the indole compounds, each concentration in 8 repititions. The plates were incubated with the indole derivatives for 24, 48 and 72 hours. At the end of each treatment, cells were washed with PBS solution. Then, 100 μl of fresh medium and 50 μl from a stock solution of MTT (3mg/ml PBS) were added to each well. After 4 hours of incubation at 37^0C, the medium was discarded and 100 μl of DMSO solution were added to each well, in order to dissolve the crystals that were formed. After a 30 minute period, the absorbance of the samples was measured by an Elisa reader. The absorbance data was converted to a cell viabilty percentage.

2.4. Analysis of DNA Fragmentation

Cells were treated with DIM at a concentration of 75 μM for 24-72 hours, or I3C at concentrations of 200 μM in DU145 cells and 400 μM in PC3 cells, for 24-96 hours. At the end of each treatment, cellular DNA was extracted by proteinase K digestion as described previously (O'Connor et al., 1991). Briefly, 10^6 cells were peletted and re-suspended in 1ml of lysis buffer (1 mM EDTA, 0.5% sodium lauryl sarcosine, 0.1 M NaCl, 50 mM Tris HCl, 200 μg/ml of proteinase K, pH 7.5). The mixture was incubated overnight at 55^0C and then at 37^0C for 30 minutes with 0.25 mg/ml of RNase (Sigma, Israel). DNA was extracted by phenol:chloroform:isoamyl alcohol (25:24:1) mixture, precipitated in ethanol and loaded on a 1.5% Agarose gel containing ethidium bromide (0.6 μg/ml), at 80 Volts. The gels were then photographed under UV light.

3. Results

3.1 Growth inhibition of DU145 and PC3 cell lines by I3C, DIM and ASC.

The MTT test provided evidence that I3C, DIM and ASC inhibit the growth of DU145 and PC3 prostate cancer cell lines. Results were measured relative to the control experiment, and are thus expressed as a percent of the control data. In searching for the most effective and potent of the three indole compounds, the concentration required to inhibit cell growth by 50% (IC50) was determined for the 48 hour trials of all three compound treatments. Our experiment showed that the IC50's for DU145 cells were 190, 150 and 1900μM for I3C, DIM and ASC, respectively. For PC3 cells, the IC50's were reached at concentrations of 225, 65, and 1950 μM for I3C, DIM and ASC respectively. Figure 2 shows the number of living cells (as percentage of the controls) versus indole concentrations. Each graph has three lines, each representing either the 24, 48 or 72 hour trial time. Figures 2A-2C and 2D-2F show the experimental data of the DU145 and PC3 cell treatments with the indole derivatives, respectively.

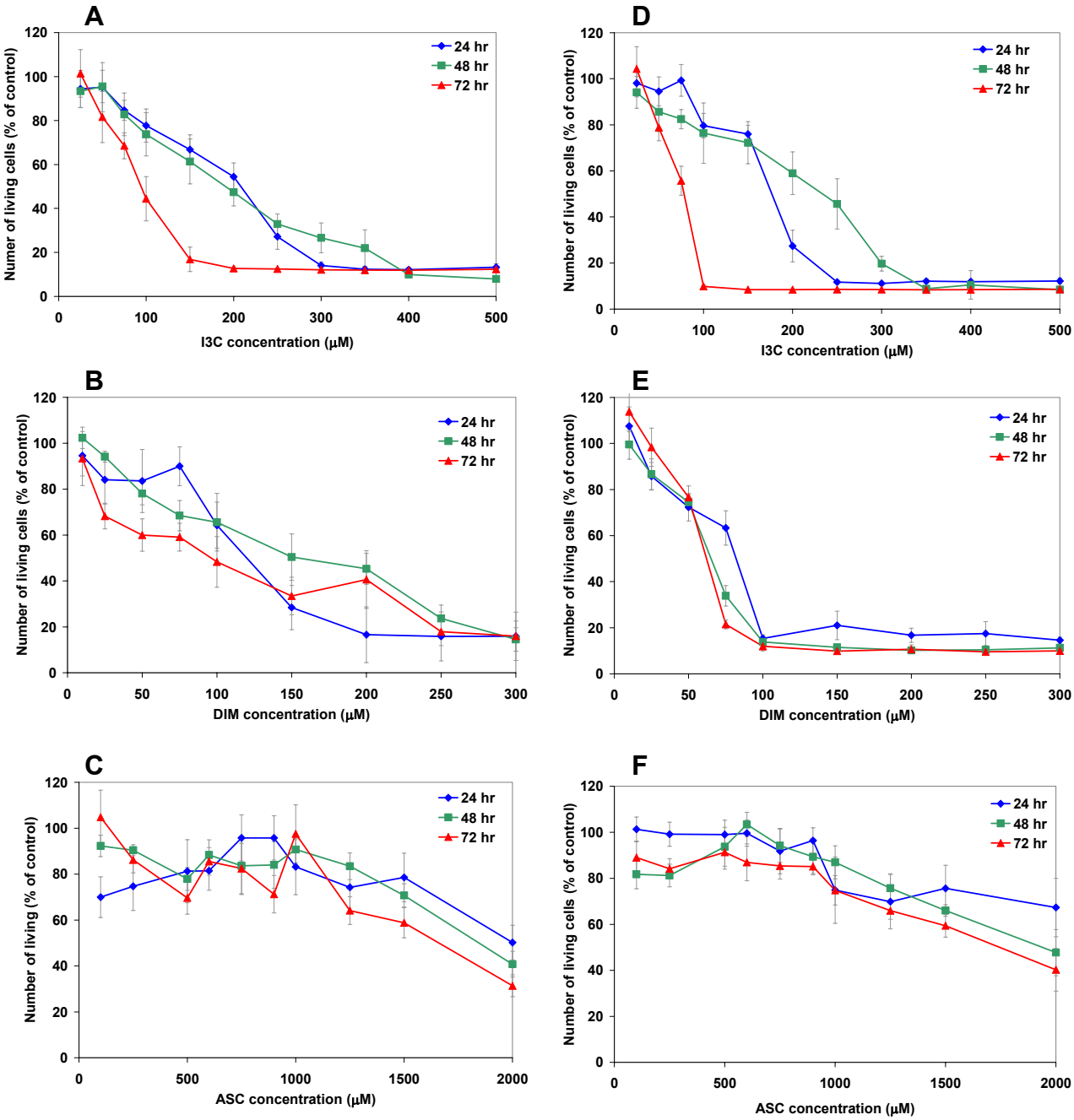

Figure 2: The effect of the indole compounds on the number of living cells relative to the control experiment is shown. Figures 2A-2C show the effect of IC3, DIM and ASC, respectively on DU145 cells. Figures 2D-2F show the effect of the latter compounds on PC3 cells.

3.2 Effect of I3C, DIM and ASC on Apoptosis Induction in DU145 and PC3 cell lines. The significant decrease in viable cells occurring after exposure to I3C and DIM led us to investigate whether the effect of these compounds was mediated through the induction of apoptosis. Following treatment with I3C or DIM, DNA was extracted from the cells and loaded on Agarose gel, as described under Materials and Methods. DNA fragmentation - characteristic of apoptosis – is shown in figure 3. The white areas are DNA; the gradual spread of the DNA is the DNA fragmentation that is a testament to the apoptotic process. DNA fragmentation was observed in DU145 cells, which started after a 48 hour exposure to 75 μM DIM or after a 72 hour exposure to 200 μM I3C. PC3 cells show a fragmented DNA after a 72-hour exposure to 75 μM DIM and 400 μM I3C.

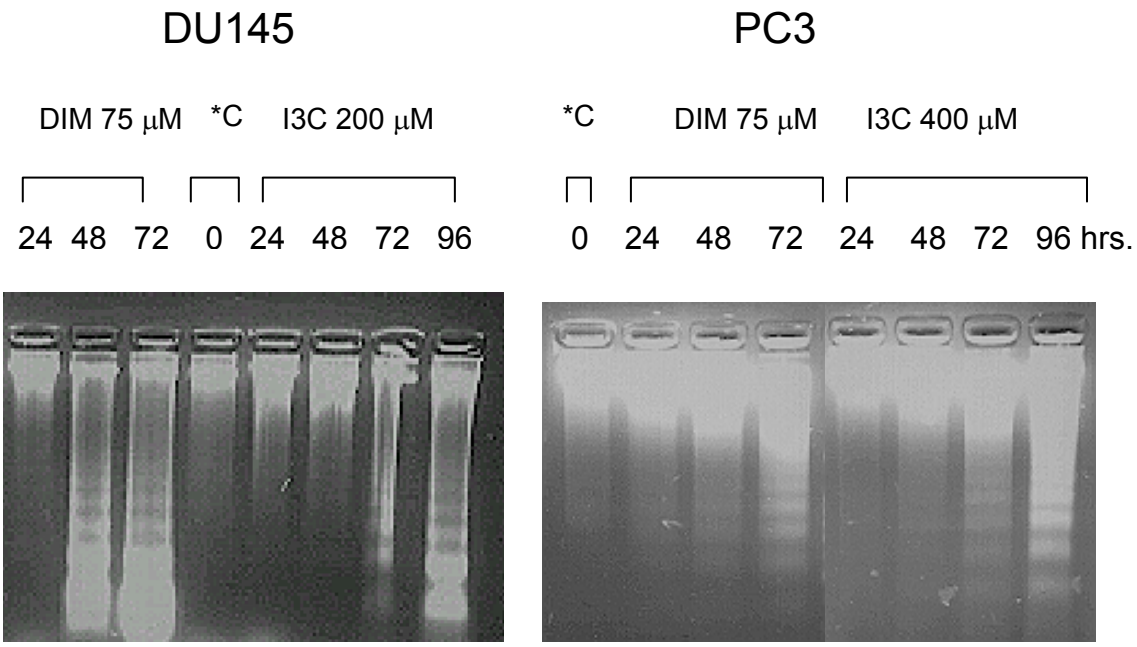

Figure 3 - DNA fragmentation in prostate cancer cells after treatment with I3C and DIM. *C denotes control group

4. Discussion

In this study we have demonstrated that the indole derivatives found in crucifers, I3C, DIM and ASC, cause a decrease in the number of living cells and induce apoptosis in two human prostate cancer cells lines; DU145 and PC3.

Our experiment has allowed us to conclude that DIM is the most effective and potent anticancer agent of the three indole compounds we tested. According to the MTT assay, for both DU145 and PC3 cell lines, the IC50 of DIM was consistently lower than the other two indoles. I3C was nearly as effective for DU145 cells, but for PC3 cells, its IC50 was more than two folds.

The MTT test provided evidence that ASC is almost negligible as an anticancer agent. It's IC50 for PC3 was 30 times that of the DIM IC50. While DIM and I3C were very effective, their effectiveness was limited. Throughout our MTT experiments, living-cells percentages remained higher than a threshold of approximately 10%. Figures 2A-2B and 2D-2E show that higher concentrations of the indoles, no matter what the treatment time is, the living-cells percentage stays above this lower limit. We can speculate that this 10% represents the cancer cell population that is immune to indole compound treatment. DIM and I3C treatments for both cell lines show this limit clearly. Unfortunately, neither of the two ASC treatments even came close to reaching this 10% threshold. Further experiments should be conducted to determine if long term treatment will eventually eradicate these immune cells. If the results indicate that these cells are truly immune, then additional complimentary treatment will be necessary to treat patients of prostate cancer. *In vivo* experiments would be the most appropriate method for this type of experiment.

In the present study we investigated whether the effect of the indole derivatives was mediated through the induction of apoptosis. One of the biochemical characteristics of apoptosis is the cleavage of DNA to fragments consisting of 180-200 base pairs and the generation of typical DNA ladder on gel electrophoresis [7]. DNA extracted

from cells treated with I3C or DIM was shown to be fragmented in all cell lines, albeit to a different extent. The electrophoresis experiment was consistent with the MTT assays, reinforcing the superiority of DIM as an anticancer agent. While there is evidence of DNA fragmentation, and thus apoptosis, there is no indication that the cell death caused by the indole compounds as observed in the MTT assays is purely apoptotic. In order to confirm that induction of apoptosis did occur within the cells, additional methods should be conducted as well. Flow cytometry, a general method for rapidly analyzing large number of cells individually using light-scattering, fluorescence and absorbence measurements, may be used in order to measure the number of apoptotic cells out of the entire cell population [16]. Furthermore, a hallmark of the apoptotic process is the cleavage of PARP (poly ADP-ribose polymerase), an enzyme implicated in DNA damage and repair mechanisms. During apoptosis, PARP is cleaved from its precursor having a mass of 116 kDa, to yield an 85 kDa fragment [15]. Results from such independent methods of measuring apoptosis will provide a stronger evidence that indeed apoptosis was induced in the cell lines after treatment with the indole derivatives.

Our experiment has shown that there is much potential for indole compounds as a treatment for prostate cancer. Its anticancer properties were also observed in similar research with breast cancer cells (MCF-7 & T47-D cell lines). The research determined that I3C was very effective against tumor cells [2]. Our study, combined with the study carried out on the breast cancer cells establishes indole compounds as a partial *in vitro* solution to cancer cell proliferation.

Laboratory limitations do not allow us to test the effect of these compounds on regular healthy cells with accuracy. Regular cells do not survive well in an artificial environment, and thus an observed decline in the number of living cells would be meaningless. An *in vivo* study would allow us to see whether high doses of indole compounds have any negative side effects on other cells of the body. To limit the body's exposure to indole compounds should any difficulties arise, localized injections of the indole compounds to the tumor should be considered as an alternative. This however, can only be studied and confirmed through *in vivo* studies.

In conclusion, indole compounds show much effectiveness in inhibiting the growth of prostate cancer cells. The accessibility of these compounds through common foods such as broccoli, cabbage and cauliflower makes indole compounds ingestion a promising treatment, or compliment to a more drastic treatment. Our *in vitro* experiments have been encouraging, but before these compounds can be considered seriously as a treatment for cancer, more experiments have to be carried out on their effectiveness and potency in the environment of a living body. Nonetheless, DIM is the most effective of the three indole compounds we tested, and should be used as a starting point towards a fully developed treatment to prostate cancer, through indole compounds ingestion.

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