



Natural Immune Systems Inc

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Report for:

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Report 123-002. Exploratory pilot work on tumor cells in vitro.

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Research Director

Report 123-002. Exploratory pilot work on tumor cells in vitro.

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Executive Summary

Infinitum Health has collected positive testimonials on the product Infinimin® Ultravitamin from patients with diverse health problems, including cancer. As a first step to begin to understand the possible scientific mechanisms behind these observed clinical benefits, the effects of Infinimin® Ultravitamin on the viability of four different cancer cell lines was studied:

- Brain (glioblastoma);
- Skin (melanoma);
- Lung (carcinoma);
- Prostate (carcinoma).

When treating these cancer cells with an aqueous extract of Infinimin® Ultravitamin, a reduction in the viability of some cancer cell lines was seen. Glioblastoma and Prostate carcinoma cells were the most sensitive to treatment, showing a dose-dependent decrease in cellular viability. Only a very minor reduction of cellular viability was seen for the melanoma and lung carcinoma cells.

In the case of Infinimin® Ultravitamin prepared with ethanol, a slight enhancement in cell viability and/or mitochondrial function was seen. This increase may reflect direct effects on mitochondria and not necessarily an increase in cell numbers or viability. If the enhancement is not cell growth, but rather increased cellular energy production under conditions of stress, this could potentially be of benefits to healthy cells as well, especially under inflammatory conditions.

Next steps will need to confirm viability of healthy cells under similar conditions, as well as pilot testing to examine support of anti-cancer immune defense mechanisms.

Report 123-002. Exploratory pilot work on tumor cells in vitro.

Purpose

To perform a screening test for induction of cell death in four selected cancer cell lines.

Background

Infinitum Health has developed an herbal formula called Infinimin® Ultravitamin. Patents have been filed, and investors are actively being sought to support the necessary research to document the biological and clinical effects of the product.

Infinitum Health has collected very positive testimonials from cancer patients, patients with viral illness, fertility problems, weight problems, elevated cholesterol, and other diverse health problems. This broad array of health improvements suggests that the product may have beneficial effects at a foundational level of human health.

Infinitum Health needs 'proof-of-concept' data to help attract investors and to help grow the company. A series of projects are planned. This project represents the first step in this process.

Work Performed

Test Products and product handling

The following test products were compared in this project:

Test product	Source	Lot#
Infinimin® Ultravitamin in PBS	Infinitum Health LLC	AAOCI
Infinimin® Ultravitamin in ethanol	Infinitum Health LLC	AAOCI

The plant-based test product was prepared for addition to cell cultures in vitro in the following manner:

- 0.5g dry product was added to 5ml of phosphate-buffered saline (PBS);
- 0.5g dry product was added to 5mL 95% ethanol (EtOH).

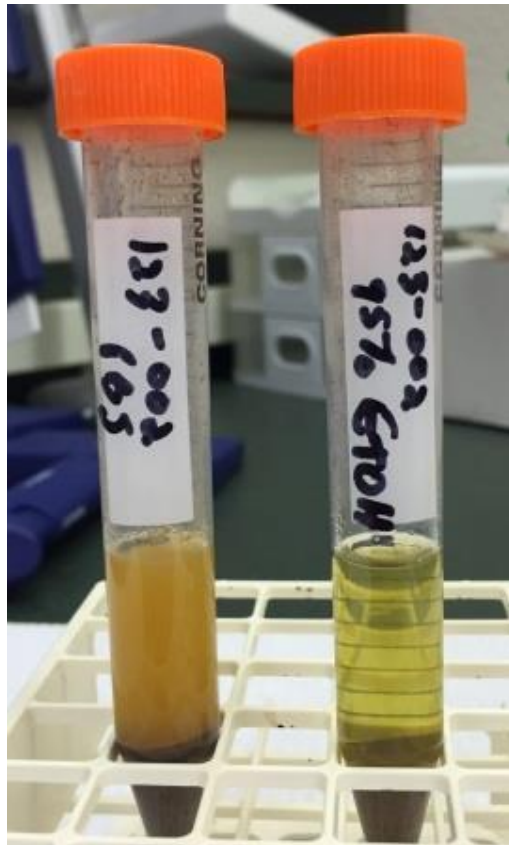


Figure 1. Product stocks following 1 hour agitation with phosphate-buffered saline (left) or 95% ethanol (right). Products were sterile filtered prior to addition to cell cultures at a 1:50 ratio. This ensured that the starting concentration of ethanol in the cell cultures treated with Infinimin Ethanol was less than 2%.

The two samples were allowed to sit at room temperature for an hour under gentle agitation, and were then sterile-filtered through a 0.22 micron cellulose-acetate syringe filter. These filtrates were the 1:1 stock solutions, corresponding to 100g/L product. Serial dilutions were prepared in PBS. Adding each dilution to cell cultures results in a further 10-fold dilution. It was ensured that the product prepared in ethanol was diluted at least 50-fold when added to cells, to bring the ethanol to below 2%. This protocol was followed for preparation of product fresh on each test day, so that prepared extracts were used on the same day of preparation.

Cell viability of cancer cell lines

Viability testing was performed using the MTT screening assay. For the particular purpose of testing effects of Infinimin® Ultravitamin in cancer cells, the MTT assay is a meaningful starting point, and helps serve as a foundation for future work.

In the MTT bioassay, chemical reactions triggers a specific color development based on cellular functions:

- When a reduction in color is measured, this is linked to a reduced cellular viability, either as a result of direct killing, or inhibition of mitochondrial function leading to cell death.
- When an increase in color is measured, this has two possible explanations: 1) Increased cell numbers (growth); 2) increased mitochondrial function (energy production).

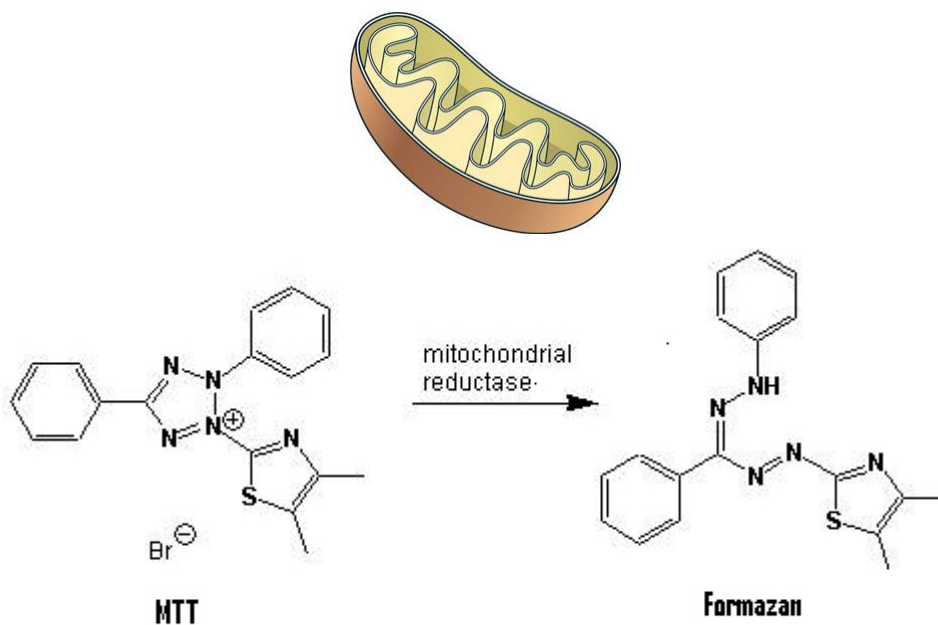


Figure 2. Diagram of MTT assay. The MTT assay utilizes a dye that changes color dependent on mitochondrial function, which is directly related to cellular metabolic activity and viability. Healthy cells metabolize the MTT dye and turn the cultures purple. This assay is commonly used for cytotoxicity testing of compounds. Mitochondria graphic taken from an article in "The Scientist".

The MTT assay is frequently used as a first step when starting work on the biological effects of complex natural products and is a very recognized assay used in determining cellular viability and metabolism. The MTT assay utilizes a dye that changes color dependent on mitochondrial function. It is a colorimetric assay for measuring the activity of enzymes that reduce MTT (or similar dyes such as XTT, MTS, and WSTs) to formazan, resulting in a purple color. This application allows assessment of metabolic activity of cells in culture. The assay can also be used to screen for cytotoxicity of potential medicinal agents and toxic materials, since those agents would stimulate or inhibit cellular metabolic activity.

Changes in metabolic activity can trigger changes in MTT results while the number of viable cells is actually constant. Therefore, while the MTT assay is often used in screening for cellular viability, and works well in showing reduced viability and cellular function, it does not lead to conclusive data on cell viability in cases where an increase is observed. With this caveat in mind, it is a great screening tool for evaluation of overall cellular metabolic activity, determining the total number of cells, cell viability and level of cellular metabolism.

The effects of Infinimin® Ultravitamin on cancer cell viability was tested on the following 4 cell lines:

- A-172, glioblastoma;
- A-375, malignant melanoma;
- A-549, lung;
- DU-145, prostate.

The test product was evaluated across a broad dose range (0.02 to 2 g/L). Testing was performed once.

The standard protocol is to perform multiple repeats of this testing, adhering to typical requirements for generating publication-quality data. However, the intent for this project was as a pilot project, to generate some initial data for marketing and fundraising.

Results

Effects of test product on cellular viability and metabolic activity

Four different cancer cell lines were used to test serial dilutions of test products for their effects on cell viability in 48 hour cultures. Eight 2-fold serial dilutions were tested for each product, starting at 2 g/L. Testing conditions were performed in triplicate and cultures maintained at 37°C, 5% CO₂ for 48 hours. The viability of cells exposed to product dilutions was compared to the viability of untreated cells cultured under the same culture conditions.

Results are described on the following page, and data graphs are presented on the following 4 pages.

Glioblastoma:

- Treatment of Glioblastoma cells with the 6 highest doses of Infinimin PBS led to statistically significant, dose-dependent reductions of cell viability.
- Treatment of Glioblastoma cells with both Infinimin Ethanol and Ethanol alone led to a slight enhancement of cell viability/mitochondrial function at the 6 highest doses. This increase in cell viability was statistically significant and dose dependent. For Infinimin Ethanol, the increase of cell viability/mitochondrial function seen at the 0.125 to 1 g/L dose range was statistically significant from the increase of cell viability resulting from treatment of cultures with Ethanol alone.

Malignant melanoma:

- Treatment of Malignant Melanoma cells with the 4 highest doses of Infinimin PBS led to a slight reduction of cell viability that was significant at the 2g/L concentration.
- Treatment of Malignant Melanoma cells with both Infinimin Ethanol and Ethanol alone led to a slight enhancement of cell viability/mitochondrial function at the 0.25 to 1 g/L concentration range. These increases were statistically significant but appeared to be a response of Malignant Melanoma cells to Ethanol alone.

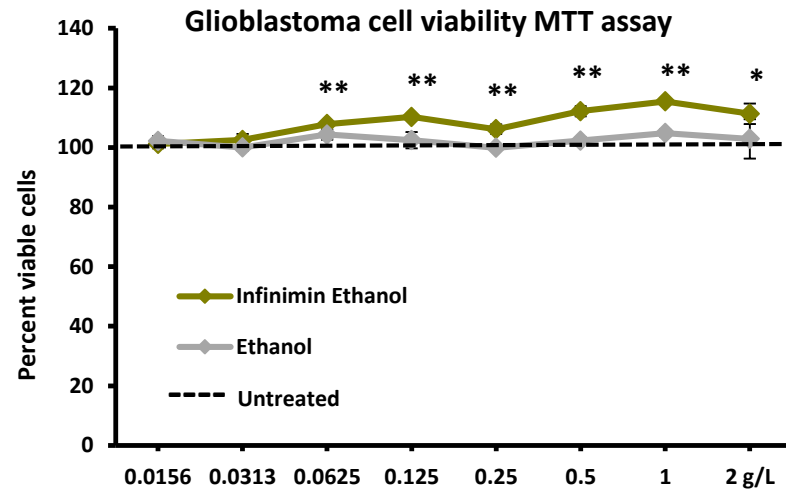
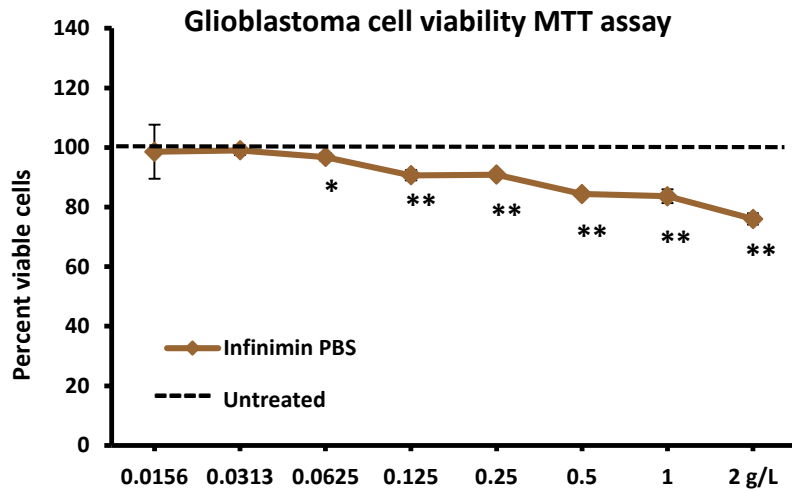
Lung Carcinoma:

- Treatment of Lung Carcinoma cells with the highest dose of Infinimin PBS led to a reduction of cell viability that was statistically significant.
- Treatment of Lung Carcinoma cells with both Infinimin Ethanol and Ethanol led to a slight increase of cell viability/mitochondrial function.

Prostate Carcinoma:

- Treatment of Prostate Carcinoma cells with the 7 highest doses of Infinimin PBS led to reductions of cell viability. This reduction was dose-dependent and statistically significant at the 0.125 and 0.5 to 2 g/L concentrations.
- Treatment of Prostate Carcinoma cells with both Infinimin Ethanol and Ethanol alone led to a slight enhancement of cell viability/mitochondrial function at the 7 highest doses. For Infinimin Ethanol, the increase of cell viability/mitochondrial function seen at the 0.5 to 2 g/L dose range was statistically significant from the increase of cell viability resulting from treatment of Prostate Carcinoma cells with Ethanol alone.

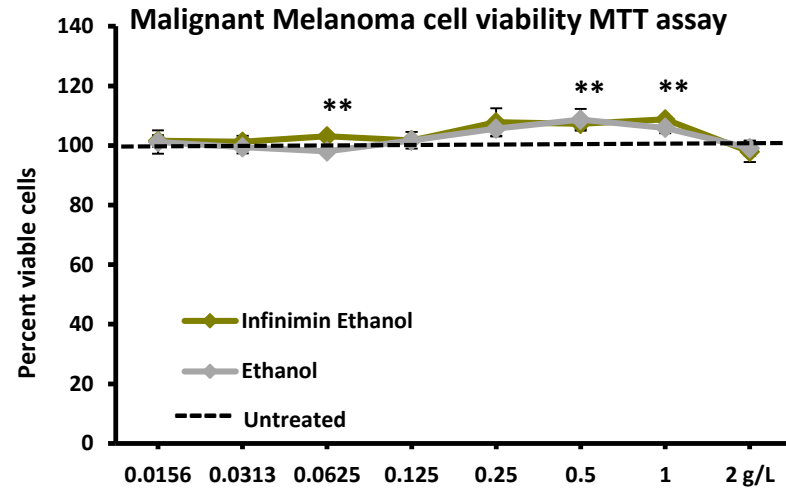
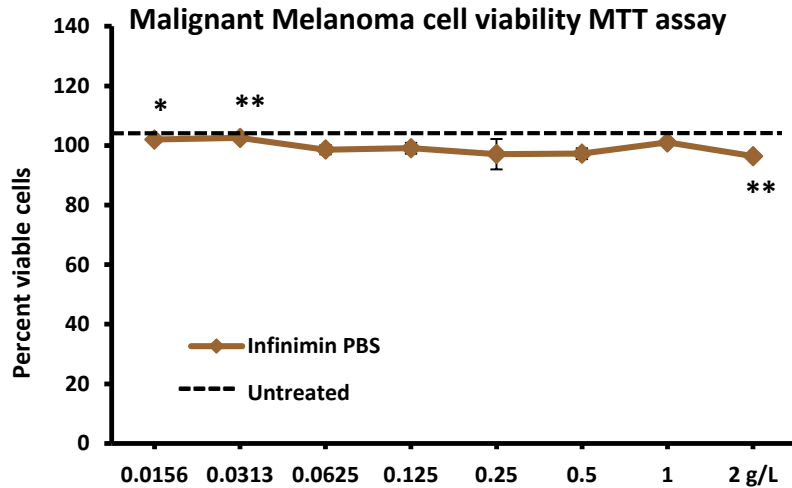
Glioblastoma cells



*** indicates significant ($p < 0.05$), ** highly significant ($p < 0.01$). Dashed line represents the viability of untreated cultures (set at 100%).**

Figure 3. Viability of Glioblastoma cells in 48-hour cultures. Glioblastoma cells were exposed to serial dilutions of products for 48 hours after which time cultures were processed in the colorimetric MTT assay. This assay uses a dye that turns purple as a result of cellular metabolism and reflects the viability of cells. In the graph above, cell viability is expressed as “Percent viable cells” which is determined by comparing the optical density of cultures exposed to products to that of untreated cultures, which serve as the “100% viability” control. Ethanol alone is included as a control for the Infinimin Ethanol product. Statistical significance is indicated for Infinimin PBS and Infinimin Ethanol as compared to the untreated control cultures (dashed line).

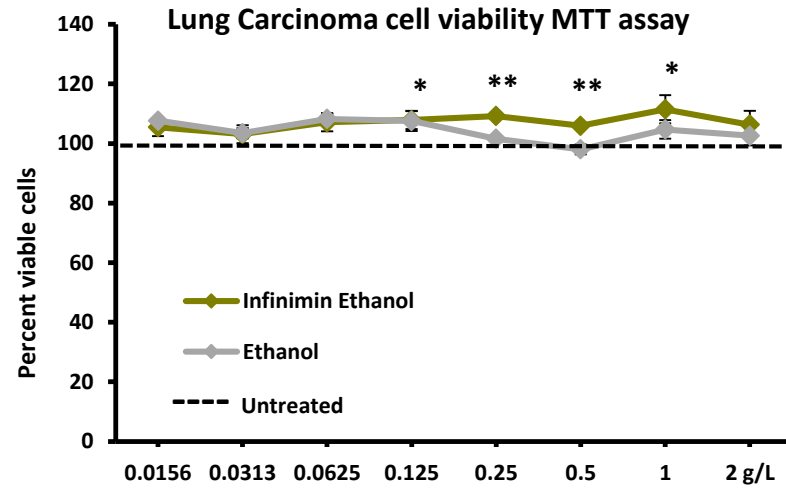
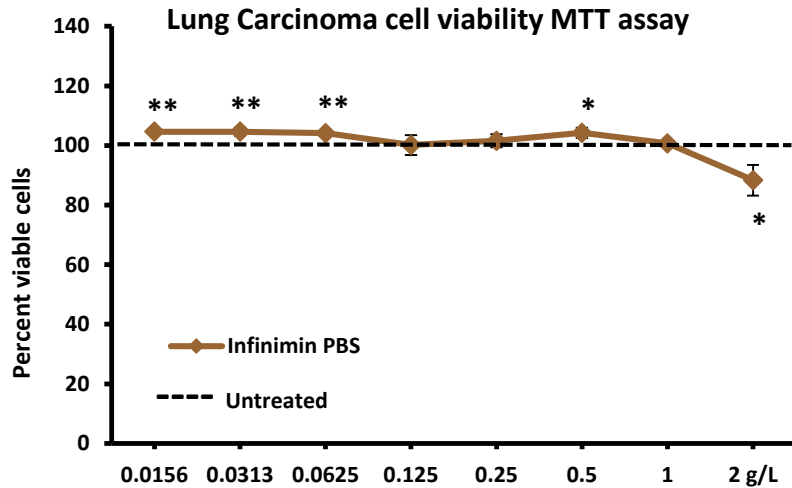
Malignant Melanoma cells



*** indicates significant ($p < 0.05$), ** highly significant ($p < 0.01$). Dashed line represents the viability of untreated cultures (set at 100%).**

Figure 4. Viability of Malignant Melanoma cells in 48 hour cultures. Malignant Melanoma cells were exposed to serial dilutions of products for 48 hours after which time cultures were processed in the colorimetric MTT assay. This assay uses a dye that turns purple as a result of cellular metabolism and reflects the viability of cells. In the graph above, cell viability is expressed as “Percent viable cells” which is determined by comparing the optical density of cultures exposed to products to that of untreated cultures, which serve as the “100% viability” control. Ethanol alone is included as a control for the Infinimin Ethanol product. Statistical significance is indicated for Infinimin PBS and Infinimin Ethanol as compared to the untreated control cultures (dashed line).

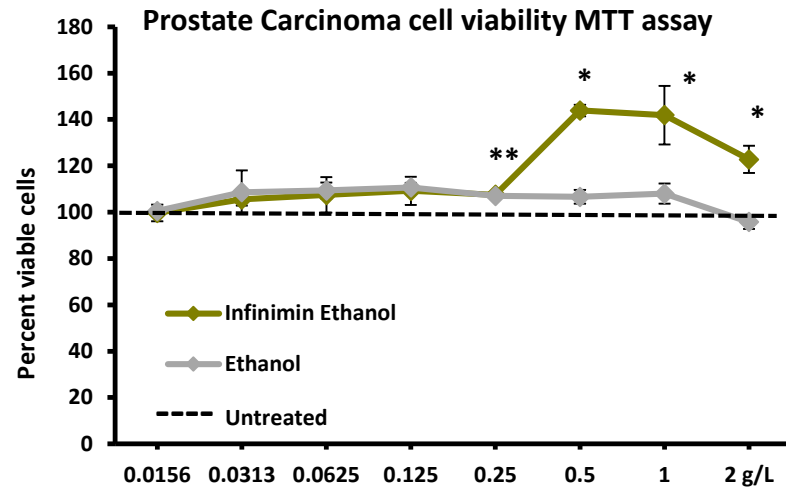
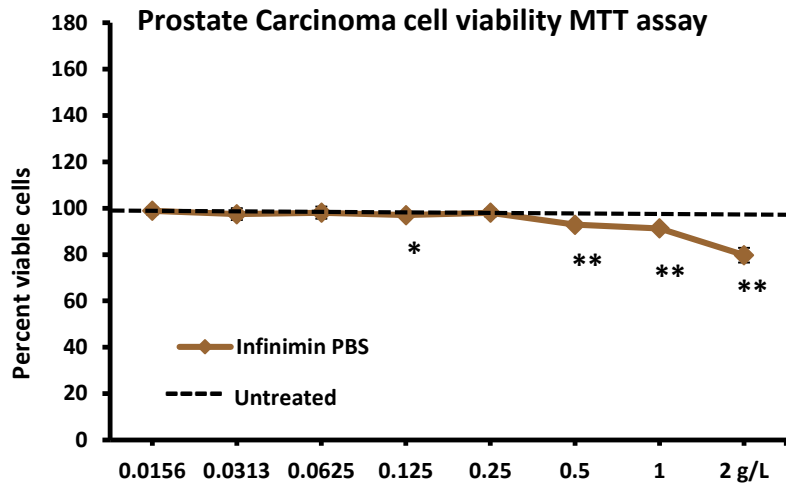
Lung Carcinoma cells



*** indicates significant ($p < 0.05$), ** highly significant ($p < 0.01$). Dashed line represents the viability of untreated cultures (set at 100%).**

Figure 5. Viability of Lung Carcinoma cells in 48 hour cultures. Lung Carcinoma cells were exposed to serial dilutions of products for 48 hours after which time cultures were processed in the colorimetric MTT assay. This assay uses a dye that turns purple as a result of cellular metabolism and reflects the viability of cells. In the graph above, cell viability is expressed as “Percent viable cells” which is determined by comparing the optical density of cultures exposed to products to that of untreated cultures, which serve as the “100% viability” control. Ethanol alone is included as a control for the Infinimin Ethanol product. Statistical significance is indicated for Infinimin PBS and Infinimin Ethanol as compared to the untreated control cultures (dashed line).

Prostate Carcinoma cells



*** indicates significant ($p < 0.05$), ** highly significant ($p < 0.01$). Dashed line represents the viability of untreated cultures (set at 100%).**

Figure 6. Viability of Prostate Carcinoma cells in 48 hour cultures. Prostate Carcinoma cells were exposed to serial dilutions of products for 48 hours after which time cultures were processed in the colorimetric MTT assay. This assay uses a dye that turns purple as a result of cellular metabolism and reflects the viability of cells. In the graph above, cell viability is expressed as “Percent viable cells” which is determined by comparing the optical density of cultures exposed to products to that of untreated cultures, which serve as the “100% viability” control. Ethanol alone is included as a control for the Infinimin Ethanol product. Statistical significance is indicated for Infinimin PBS and Infinimin Ethanol as compared to the untreated control cultures (dashed line).

Conclusions

The data reported here represents the first step to begin to understand the biology behind these clinical benefits, the effects of Infinimin® Ultravitamin on the viability of 4 different cancer cell lines was studied.

A reduction in the viability of all 4 cancer cell lines was seen following treatment with Infinimin (PBS). Glioblastoma and Prostate Carcinoma cells were the most sensitive to treatment, showing a dose-dependent response. Only the highest dose of Infinimin (PBS) reduced the viability of Malignant Melanoma and Lung Carcinoma cells.

- Statistically significant reductions of cell viability were seen for all 4 cancer cell lines following treatment with Infinimin PBS;
- The decrease in Glioblastoma and Prostate Carcinoma cell viability following treatment with Infinimin PBS was dose-dependent and extended across a broad dose range;
- Treatment of cancer cells with Ethanol alone and Infinimin Ethanol led to slight increases of cell viability as measured in the MTT assay. These increases may reflect increases in mitochondrial number or activity and not necessarily increased cell survival;
- The differences in response of all 4 cancer cell lines to the Infinimin PBS and Infinimin Ethanol products is interesting. The cancer cell lines tested here represent a broad range of cancer cell types (brain, skin, lung and prostate), yet all 4 cancer cell lines had a reduction in cell viability (as indicated by reduced mitochondrial function) when exposed to Infinimin PBS. Conversely, exposure to Infinimin Ethanol at the higher doses led to an increase in viability in 3 of the 4 cancer cell lines (brain, lung and prostate). This result points to a number of possibilities and must be interpreted in the context of the response of the cancer cell lines to Ethanol alone.
 - Treatment of the 4 cancer cell lines with low doses of Ethanol (0.06 to 1.9%) led to increased mitochondrial metabolism. Evidence in mice suggest that this could be due to an increase in mitochondrial numbersⁱⁱ;
 - Treatment of 3 of the 4 cancer cell lines (brain, lung and prostate) with Infinimin Ethanol led to increased mitochondrial metabolism that was higher than Ethanol alone. This suggests that in the context of low dose Ethanol treatment, which is a situation of increased oxidative stressⁱⁱⁱ, ingredients in Infinimin® Ultravitamin that are soluble in ethanol, may support viability under conditions of increased oxidative

stress. This may be a function that is independent of malignancy, and may possibly translate to a protective function of healthy cells under oxidative stress as well.

In this pilot project some cancer cell lines (Glioblastoma and Prostate Carcinoma) were more sensitive to Infinimin® Ultravitamin treatment than others (Malignant Melanoma and Lung Carcinoma). This is not surprising since each cancer cell line possesses different genetic alterations that lead to differences in response to anti-cancer drugs and therapies^{iv,v}.

It is possible that Infinimin® Ultravitamin may have different effects (reduced versus increased viability/mitochondrial function) in susceptible cell types, depending on the culture environment. In the presence of alcohol, cultures may produce more reactive oxygen species and in this context compounds in Infinimin® Ultravitamin may increase cellular metabolism rather than reduce it.

Additional research is needed to determine whether cancer cell lines are responding to the same or different compounds in the Infinitum PBS and Infinitum Ethanol preparations.

Additional research is needed to evaluate whether the effects of Infinitum Ethanol translate to protection of mitochondrial function from free radical stress, and whether this protection is independent of anti-cancer effects; if so, this may be beneficial to healthy cells.

Further work

This initial pilot work is a first step in beginning to understand how consumption of Infinimin® Ultravitamin supports health. In the special case of cancer, the data here suggest that some compounds in Infinimin® Ultravitamin could have a direct effect on reducing the growth of cancer cells. It is more likely however, in the context of the human body, that consumption of Infinimin® Ultravitamin supports the defense and anti-cancer activities of the immune system.

Virally infected and transformed cells are normally recognized by cells of the immune system (natural killer cells and macrophages) and eliminated^{vi}. It is the breakdown on these normal processes that allow cancer to be established^{vii}. Therefore additional work should include studies on the effects of Infinimin® Ultravitamin on activation of the immune system. This would include effects on the activation of and cytokine production by specific immune cell populations as well as free radical formation by inflammatory cells. Based on the ingredients in Infinimin® Ultravitamin, particularly mushroom and seaweed extracts, we would expect to see immune activating effects.

In addition, based on the cancer cell line pilot data reported here, further work should include an investigation of the effects of Infinimin® Ultravitamin on mitochondrial function and protection from apoptosis in normal cells under similar culture conditions.

The strategic plan is to continue with a series of pilot tests, to have the following complete set of pilot data:

- Effect on tumor cell viability/death (this report);
- Immune cell activation (NK cells, NKT cells, T cells, monocytes);
- Cytokine production;
- Effects on free radical formation by inflammatory cells;
- Cellular antioxidant protection and bioavailability;
- Antioxidant capacity;
- Protection of healthy cells from apoptosis;
- Effects on mitochondrial numbers and function.

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