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**DATA & FINDINGS**

Instead of using an actual soybean as the treatment, we decided to use soy protein, a processed soy product. We believe that processed soy products are more directly applicable to our experiment because it is more commonly consumed. The mass spectrometer analysis was performed to ensure that the basic isoflavones are present in our soy protein; compounds in the soy protein were separated by size and charge using liquid chromatography. Then, these molecules are directed to a mass spectrometer to measure the molecular weight of each molecule. We found the molecular weights of the soy isoflavones genestein, diadzein, and glycitein, and analyzed the soy protein in the mass spectrometer to determine if those molecular weights were present.

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| **Fig F. Mass Spectrometer Analysis of Soy Protein. The first graph shows the overall analysis of chemicals found in the soy protein. The three graphs below it depict the chemicals found in the soy protein at 19.27 minutes, 17.24 minutes, and 16.99 minutes.** |

The peaks in the printout above indicate the molecular mass and amount of a certain molecule by time. The first graph shows an overview of all the peaks, or masses of the molecules found within the soy protein. As there are many peaks in this graph, the mass spectrometer analysis suggests that there are many other compounds in the soy protein besides isoflavones. The other graphs show the peaks of molecules found within the soy protein at specific times. The second graph shows a peak at 270.8 to 271.8 Daltons, which matches genesteinís molecular weight of 271 Daltons. This molecule was read by the spectrometer at 19.27 minutes, with a cumulative molecular weight of 126,997,464 Daltons. The third graph shows a peak at 254.8 to 255.8 Daltons, which matches diadzeinís molecular weight of 255 Daltons. This molecule was read at 17.24 minutes, and the total amount of this molecule was 121,177,570 Daltons. The fourth graph shows a peak at 454.5 to 455.5 Daltons, which is slightly less than glyciteinís molecular weight of 447 Daltons. However, the smaller peaks to the left of the main peak could be parts of the glycitein molecule that were separated during the analysis. These molecules were read at 19.27 minutes, with a cumulative amount of 29,162,478 Daltons. As the peaks in the soy protein analysis matched the molecular weights of genestein, diadzein and glycitein, the mass spectrometer analysis confirmed that the isoflavones are present in the soy protein.

In each of our two trials, cell proliferation was measured in two human breast cancer cell lines, MCF-7 and MDA-MB-231, in response to soy and diadzein. Data was collected at four time points spanning 96 hours. For each time point, there were 8 samples of each treatment. Over the two trials, there were 16 samples for each treatment and time point. However, due to procedural errors, the data from the first trial were invalid and inexplicable. We unfortunately did not have the opportunity to repeat the experiment to collect more data. Therefore, our study only makes use of the data collected from the second trial, reducing the sample size for each treatment group to 8.

To measure proliferation, the MTS cell proliferation assay was implemented. The assay utilizes Owen's reagent [(3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, MTS], which can be reduced by metabolically active cells into a colored product. The amount of colored product is directly proportional to the amount of cell proliferation, and the amount of colored product is determined by light absorbance at 490 nm using a spectrophotometer. Higher absorbance suggests a greater amount of cell proliferation. Cell proliferation was measured every 24 hours and compared by observing the percent proliferation relative to control. These results are shown in the graphs and tables below:

**Fig 1. Average Percent Proliferation of MCF-7 (estrogen positive) treated with soy relative to control over 96 hours. Estradiol, the positive control, is expected to stimulate cell proliferation in estrogen-receptor positive MCF-7. The control with no treatment is the negative control because of the absence of substances that could affect cell proliferation.**

**Table 1. Average Percent Proliferation of MCF-7 (estrogen positive) treated with soy relative to control over 96 hours**

A significant difference in the amount of proliferation can be observed for MCF-7 treated with various concentrations of soy and the control. Beginning at 24 hours, the percent proliferation increased for all treatments, and peaked at 48 hours. From 48 hours to 72 hours, the percent proliferation decreased for all treatments, and increased again from 72 hours to 96 hours. Initially at 24 hours, all treatments exhibited relatively minimal difference in percent proliferation relative to the control. 0.006 µg/mL and 0.3 µg/mL exhibited the lowest percent proliferation at 48 hours and 72 hours respectively. At 96 hours, the soy treatment concentrated at 0.6 µg/mL exhibited the lowest percent proliferation. Meanwhile, all treatments exhibited lower percent proliferation than the control from 24 hours to 72 hours; but the estradiol control exhibited the greatest percent proliferation at 96 hours.

**Fig 2. Average Percent Proliferation of MCF-7 (estrogen positive) treated with diadzein relative to control over 96 hours. Estradiol, the positive control, is expected to stimulate cell proliferation in estrogen-receptor positive MCF-7. The control with no treatment is the negative control because of the absence of substances that could affect cell proliferation.**

**Table 2. Average Percent Proliferation of MCF-7 (estrogen positive) treated with diadzein relative to control over 96 hours**

MCF-7 treated with the diadzein concentrations of 0.05 µg/mL, 0.005 µg/mL, and 0.001 µg/mL exhibited the same growth curve as the MCF-7 treated with soy, increasing in proliferation from 24 to 48 hours, decreasing from 48 to 72 hours, and increasing again from 72 to 96 hours. However, the cells treated with concentrations 0.1 µg/mL and 0.01 µg/mL increased from 24 to 48 hours, and gradually decreased from 48 to 96 hours. All treatments exhibited lower percent proliferation than the control from 24 hours to 72 hours, but the diadzein concentrations at 0.05 µg/mL and 0.001 µg/mL resulted in the greatest percent proliferation at 96 hours. Again at 24 hours, all treatments exhibited relatively minimal difference in percent proliferation relative to the control. Meanwhile the treatment with diadzein concentration 0.001 µg/mL exhibited the lowest percent proliferation at 48 hours, and the concentration 0.005 µg/mL exhibited the lowest percent proliferation at 72 hours. Ultimately at the end of 96 hours, the concentration at 0.1 µg/mL exhibited the lowest percent proliferation.

**Fig 3. Average Percent Proliferation of MDA-MB-231 (estrogen negative) treated with soy relative to control over 96 hours. MDA-MB-231 was a control to examine the effects of soy on estrogen-receptor negative cells.**

**Table 3. Average Percent Proliferation of MDA-MB-231 (estrogen negative) treated with soy relative to control over 96 hours**

Although differences in the amount of proliferation were observed for MDA-MB-231 treated with various concentrations of soy relative to the control, such differences were not statistically significant. In general, the treatments decreased from 24 to 48 hours, increased from 48 to 72 hours, and decreased again from 72 to 96 hours. However, concentration 0.06 µg/mL, 0.03 µg/mL and the estradiol control exhibited a gradual increase over all 96 hours. The diadzein treatment at concentration 0.6 µg/mL exhibited the lowest percent proliferation over all 96 hours. Meanwhile, the control exhibited the highest percent proliferation at 24 and 48 hours, but the diadzein treatment at concentration 0.06 µg/mL exhibited the highest percent proliferation at 72 and 96 hours.

**Fig 4. Average Percent Proliferation of MDA-MB-231 (estrogen negative) treated with diadzein relative to control over 96 hours. MDA-MB-231 was a control to examine the effects of soy on estrogen-receptor negative cells**

**Table 4. Average Percent Proliferation of MDA-MB-231 (estrogen negative) treated with diadzein relative to control over 96 hours**

The treatments show minimal difference compared to the control. There was no discernable trend among the treatments. Concentrations 0.1 µg/mL and 0.001 µg/mL decreased between 24 to 48 hours, increased between 48 and 72 hours, and decreased again between 72 and 96 hours; meanwhile, concentrations 0.005 µg/mL, 0.05 µg/mL, and 0.01 µg/mL exhibited the opposite pattern. The treatment at concentration at 0.1 µg/mL exhibited the lowest percent proliferation at 24 hours, but 0.001 µg/mL exhibited the lowest percent proliferation at 48 hours. At 72 hours and 96 hours, the treatment at concentration 0.05 µg/mL exhibited the lowest percent proliferation. The control with no treatment exhibited the highest percent proliferation at 24 hours, while concentration 0.005 µg/mL, 0.001 µg/mL and 0.01 µg/mL exhibited the highest percent proliferation at 48 hours, 72 hours, and 96 hours respectively.

To analyze the statistical significance of our collected data, we performed stacked ANOVA tests by treatment and hour. In order to perform the ANOVA tests we must assume the population is normal, and that there is equal standard deviation among each treatment. The hypotheses for the test are: 1) The means of each treatment are equal; 2) At least one mean is different from the other treatments. A P-value or probability less than 0.05 will prove the second hypothesis because the probability of obtaining such difference in the treatments is so low that it cannot by random. Therefore, there would be statistical significance in the treatments. In addition, by comparing the 95% confidence intervals, we can observe which treatments are significant. The bars in the 95% confidence intervals show what range the means could deviate by random chance. If these experiments were repeated, there would be a 95% chance of obtaining values within those intervals. Therefore, if the confidence intervals for the treatments do not contain the same values, then it is not a random occurrence that the results were different for each treatment.

**Table 5. P-Values for soy and diadzein treatments on MCF-7 (estrogen-receptor positive) and MDA-MB-231 (estrogen-receptor negative).**

The ANOVA tests for MCF-7 treated with soy and diadzein showed P-values of 0.001 and 0 respectively, meaning that there is close to 0% chance of getting such differences in comparison to the control if the experiment was repeated. This means that there was statistically significant difference among the soy and diadzein treatments. Primarily, the 95% confidence intervals (refer to appendix) indicate that Soy 1 (0.6 µg/mL) and Dia 1 (0.1 µg/mL) are most significant, as those intervals do not overlap with their control intervals.

The ANOVA test for MDA-MB-231 treated with diadzein showed no statistical significance, as the P-value was extremely high at 0.288. This means that there is a 29% chance of getting such differences if the experiment was repeated. The ANOVA test for MDA-MB-231 treated with soy protein also showed no statistical significance, as the P-value was high at 0.148. This means that there is a 15% chance of getting such differences if the experiment were repeated. This data is plausible, as the rates of proliferation were quite similar among all the MDA-MB-231 treatments.

The results that this data reveals are suggestive of the inhibiting effects of soy on the proliferation of breast cancer cells. The results showed an overall trend in which MCF-7 cells treated with soy exhibited significantly lower percent proliferation relative to the control. This suggests that soy has the ability to suppress the proliferation of breast cancer cells. Furthermore, MCF-7 treated with the highest soy concentration, 0.6 µg/mL, exhibited the lowest percent proliferation at 96 hours. Generally, this group also exhibited lower percent proliferation than most of the other treatments over all 96 hours. This would suggest that higher concentrations soy further suppress the rate of proliferation in cancer cells. Meanwhile, the results indicated that MDA-MB-231 treated with soy had neither discernable trends nor significant difference in proliferation relative to control, suggesting that soy has no effect on breast cancer cells without estrogen receptors.

n addition, the results suggest that diadzein has inhibitive effects on the proliferation of breast cancer cells as well. An overall trend was observed in which MCF-7 cells treated with diadzein exhibited significantly lower percent proliferation relative to the control. It is noted that MCF-7 cells treated with diadzein treatments 0.05 µg/mL and 0.001 µg/mL exhibited greater percent proliferation relative to control. However, because the difference in percent proliferation for these treatments was not significant, the results could have occurred by random chance. Overall, the data suggests that diadzein is an active ingredient in soy’s ability to suppress the proliferation of breast cancer cells. Furthermore, MCF-7 treated with the highest diadzein concentration, 0.1 µg/mL, exhibited the lowest percent proliferation at 96 hours. Generally, this group also exhibited lower percent proliferation than most of the other treatments over all 96 hours. This would suggest that higher concentrations diadzein further suppress the rate of proliferation in cancer cells. On the other hand, MDA-MB-231 treated with diadzein exhibited no significant difference, suggesting that diadzein has no effect on breast cancer cells without estrogen receptors.

**Figure 5. Chemical Structure of Estradiol. *(Image provided by “Oestrogenen”)***

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| **Figure 6. Chemical Structure of diadzein and genestein, isoflavones found in soy. Genestein and diadzein have chemical structures that closely resemble estradiol. *(Image provided by “Oestrogenen”)*** |

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| **Fig 7. Chemical Structure of the Estrogen Receptor. Estradiol binds to the estrogen receptor to stimulate cell proliferation. Because isoflavones structurally resemble estradiol, they can bind to the estrogen receptors as well, thereby competitively inhibiting estradiol from binding to the estrogen receptor. *(Image provided by David Shattuck)*** |

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| **Fig 8. Mechanism of Action in Estrogen Receptors. A: Estrogen receptor; B: Estrogen; C: Estrogen helper proteins; D: Nucleus; E: DNA genetic material. Estrogen and helper proteins bind to the estrogen receptors, which then move to the genetic material to initiate transcription and cell proliferation. *(Image provided by David Shattuck)*** |

Because the results showed that soy and diadzein have significant effects on MCF-7 but not MDA-MB-231, it suggests that soy and diadzein only have an effect on the proliferation of breast cancer cells with estrogen receptors. Isoflavones such as diadzein structurally resemble estradiol, which stimulates cell growth by binding to the estrogen receptors; perhaps the isoflavones in the soy competitively inhibit the estradiol from binding to the estrogen receptors, thus preventing the cancer cells from proliferating. However, the results indicated that estradiol did not stimulate growth in MCF-7; in fact, it appeared that estradiol limited the proliferation of MCF-7 cells. This data could be because the estradiol was degenerated, or the concentration of estradiol was too low to be effective. Nevertheless, because the results indicated that soy and diadzein lower the percent proliferation in MCF-7 cells, the data is still valid, and we can speculate that soy and diadzein suppresses growth in breast cancer cells through the estrogen receptor.

It is interesting to note that all of the MCF-7 treated with soy and some of the MCF-7 treated with diadzein had the trend of increasing in percent proliferation between 24 to 48 hours and 72 to 96 hours, while decreasing in percent proliferation between 48 to 72 hours. Since we replenish the cells with the soy and diadzein treatments at 48 hours, this trend suggests that the soy and diadzein degraded. Degradation would mean the cells were not suppressed as much, allowing the cells to proliferate between 24 to 48 hours and 72 to 96 hours, while growth was suppressed at 48 to 72 hours because the cells were replenished with soy and diadzein treatments at 48 hours. Future experiments should measure proliferation at 0 hours to confirm this idea. Also, the soy protein concentrations were set so that they would contain approximately the same amount diadzein as in the diadzein concentrations. Yet, the results for the two treatments were very different. The discrepancy between the diadzein treatment and the soy treatment may be due to other substances and chemicals in the soy protein. The mass spectrometer analysis showed many other peaks, suggesting that there are chemicals in the soy protein other than isoflavones. These chemicals could stimulate growth or counteract the isoflavonesí inhibiting effect.