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Honokoil Treatment on Glioblastoma Cells

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SENIOR THESIS APPROVAL

This Honors thesis entitled

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Abstract and Hypothetical Basis

Glioblastoma is a malignant brain tumor without effective treatment options available because of its resistance to chemotherapy and radiation. This specific type of cancer is difficult to treat because the cancer stem cells that are not actively growing. These cells are dormant, which means they will not react to treatment because they are not dividing, and it is these cells that result in the high prevalence of relapse. Honokiol is a Chinese magnolia species that is known for its anti-inflammatory, anti-proliferative, and proapoptotic effects which make it an optimal candidate for glioma cell treatment. Honokiol was used in this experiment to treat U-87 glioblastoma cells in cell culture to compare to control U-87 cells that were not being treated with honokiol. Honokiol reduced the expression of EGFR which along with an increase in Bax expression which promoted apoptosis amongst the cancer cells and inhibited tumor growth. Altogether, these results indicate that honokiol is an effective treatment for reducing tumorigenic potentials and will hopefully be useful in management of glioblastoma.

Background

Glioblastoma is the most aggressive form of brain cancer, and it is the most common brain malignancy. Glioma stem cell-like cells are fast-growing and develop from astrocytes and oligodendrocytes. These glial cells support the health of nerve cells within the brain by supplying nutrients to the brain and providing the brain's neurons with nutrients. Glioblastoma can occur in the brain or spinal cord, but it is most commonly found in the frontal lobe and temporal lobe of the brain. Once diagnosed, patients have an average survival time of 12-18 months where only 25% survive more than one year (MD Anderson Cancer Center). This is because there is

currently no cure for glioblastoma because of the rapid rate of metastasis within the brain. The severity of these tumors is caused by the inevitable recurrence of GBM, and the advancements in the past years have not significantly increased the overall survival of patients with this disease (AANS). The tumor forms branches that spread all over the brain making it nearly impossible to remove the whole tumor. High levels of metastasis and recurrence contribute to the extreme rates of mortality. Medications are difficult to deliver to the brain due to the blood brain barrier which is designed to prevent toxins from entering the central nervous system. Radiation and chemotherapy are used to slow the growth of the tumor but are unlikely to result in a prolonged remission (Johns Hopkins Medicine).

The epidermal growth factor receptor (EGFR) induces proliferation within the cell, and EGFR dysregulation has been reported to initiate tumor metastasis and a very poor prognosis. This dysregulation is seen in the fast-growing glioblastoma tumor, and in human gliomas, EGFR is over expressed and mutated (Fan, 2018). The epidermal growth factor receptor signaling network is often a target for therapeutic intervention of GBM by focusing efforts on inhibiting the receptor with different treatments that can reduce the expression of EGFR.

Honokiol is a traditional Chinese herbal medicine that has been extracted from the bark of the Magnolia species. This herbal medicine is found to have anti-inflammatory, anti-proliferative, and proapoptotic effects in a wide range of human cells (Fried, 2009). These findings have increased interest in using honokiol as a chemotherapeutic treatment. This drug has two major mechanisms of action. It blocks signaling in tumors with defective p53 function and induces cyclophilin D which causes death in cells with wild-type p53. The tumor suppressor, p53, plays a central role in required resistance to EGFR. With honokiol blocking signals and

inducing apoptosis in tumors with defective p53, this can reduce the expression of EGFR which is needed to be able to treat glioblastoma (Hatanpaa, 2010). These different positive effects on the body have the potential to reduce the effects of carcinogenic cell lines through slowing the rate of metastasis.

A clonogenic assay was run to analyze the proliferative rates of glioblastoma cells treated with and without honokiol treatment. This study was done to determine if honokiol would reduce the rate of proliferation in the cells by running the assay to observe the amount of colonies on each well plate after ten days. In order to determine if the mean of colonies on the plates with the treatment was different than the mean of the colonies on the plates without the treatment, a t-test was run in the program R to determine if there was a significant difference. When this study was run, the hypothesis was that the mean of the colonies on plates that had been treated with honokiol would be lower than the mean of the colonies on plates that were not treated with the drug. The hypothesis indicated that there would be a significant difference, and the null hypothesis would be rejected. If this hypothesis were true, this would determine that the treatment of this drug on glioblastoma cells has the potential to reduce tumorigenic effects, suggesting hopes for honokiol to be useful in the management of glioblastoma.

Materials and Methods

During the summer of 2020, the research was done at Jones Science Center under Dr. Blake Johnson observing the effects of honokiol on glioblastoma cells. In the research, the response variable, which was quantitative, was how many colonies could be found in each well plate. The explanatory variable, which was qualitative, was the treatment with honokiol or the

control treatment with no honokiol. The first step of the research was cell preparation. Two 6-well plates were used for the experiment. Three wells on each plate were treated with honokiol, and the other three wells were control. There were six control wells and six drug treated wells in total with the two plates. The U87 GBM cell line was grown, split into different plates, and allowed to reach a confluency of 80%. The medium was removed and the cells were rinsed with 10 milliliters of PBS. Four milliliters of 0.25% trypsin was added to the cells, and they were incubated at thirty-seven degrees Celsius for 1-5 minutes until the cells appeared round. Ten milliliters of medium were added with 10% FBS, and the cells were detached with pipetting. The cells were counted using a hemocytometer and seeded into 6-well plates. The second step was the assay setup. After the cells were plated, they incubated for a few hours in a CO2 incubator at thirty-seven degrees Celsius to allow them to attach to the plate. The cells were then treated with 50 micromolars of honokiol or the placebo. The cells were incubated in a CO2 incubator at thirty-seven degrees Celsius for 10 days. The third step was fixation and staining. After the 10 days and the control plates had formed colonies with a substantially good size, the medium from each plate was removed, and the cells were rinsed with 10 milliliters of PBS. The PBS was removed and 2-3 milliliters of fixation solution were added. The plates were left at room temperature for 5 minutes, and the fixation solution was removed. Crystal violet solution of 0.5% was added and incubated at room temperature for 2 hours. Ten milliliters of medium were added with 10% FBS, and the cells were detached by pipetting. The crystal violet was removed carefully, and the plates were immersed in tap water to rinse off the crystal violet. The dishes were air-dried on a table cloth at room temperature for three days. In order to analyze the data, the number of colonies were counted with a stereomicroscope (Yang, 2012). Once the data was

received and a table was made, a t-test was run to determine if there was a significant difference in the means. A Shapiro-Wilk was run to test the normality of the data and made a box plot to be able to clearly compare the differences between the two groups with and without the treatment. Using the package ggplot2 in the statistical programming language R, a plot was created to show the overlap and difference in between the two means. The null hypothesis of a t-test assumes that no difference exists between the means, but the alternative hypothesis for a t-test states that some difference exists between the means of the different variables. For this experiment, the null hypothesis would be that there was no difference between the mean of the colonies treated with drug or without it. The alternative hypothesis for this study would be that there was a difference between the mean of colonies with the drug honokiol and without the drug.

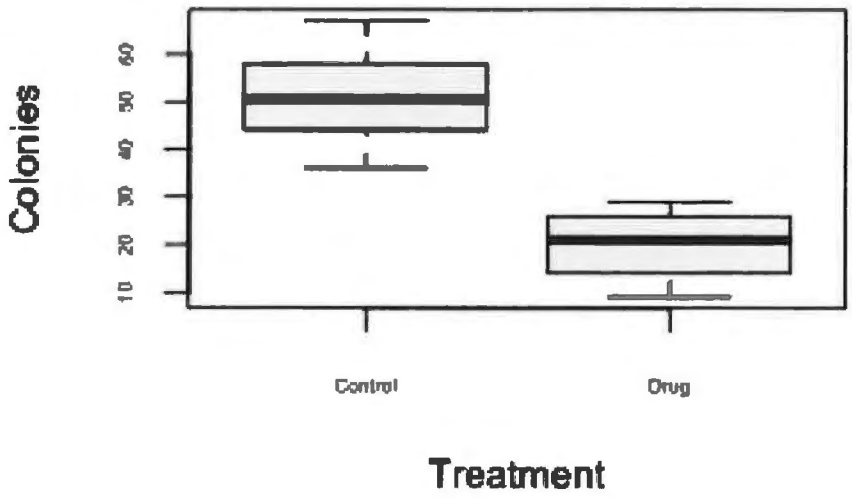
Results

Once the data was collected from the clonogenic assay, the colonies were counted, and the numbers were compiled into a table to analyze the data. The table consisted of two columns including the treatment and amount of colonies. Under the treatment column, it was indicated whether the drug honokiol had been counted or the control. The colonies column indicated how many colonies were counted on each plate. One colony consisted of fifty glioblastoma cells or more.

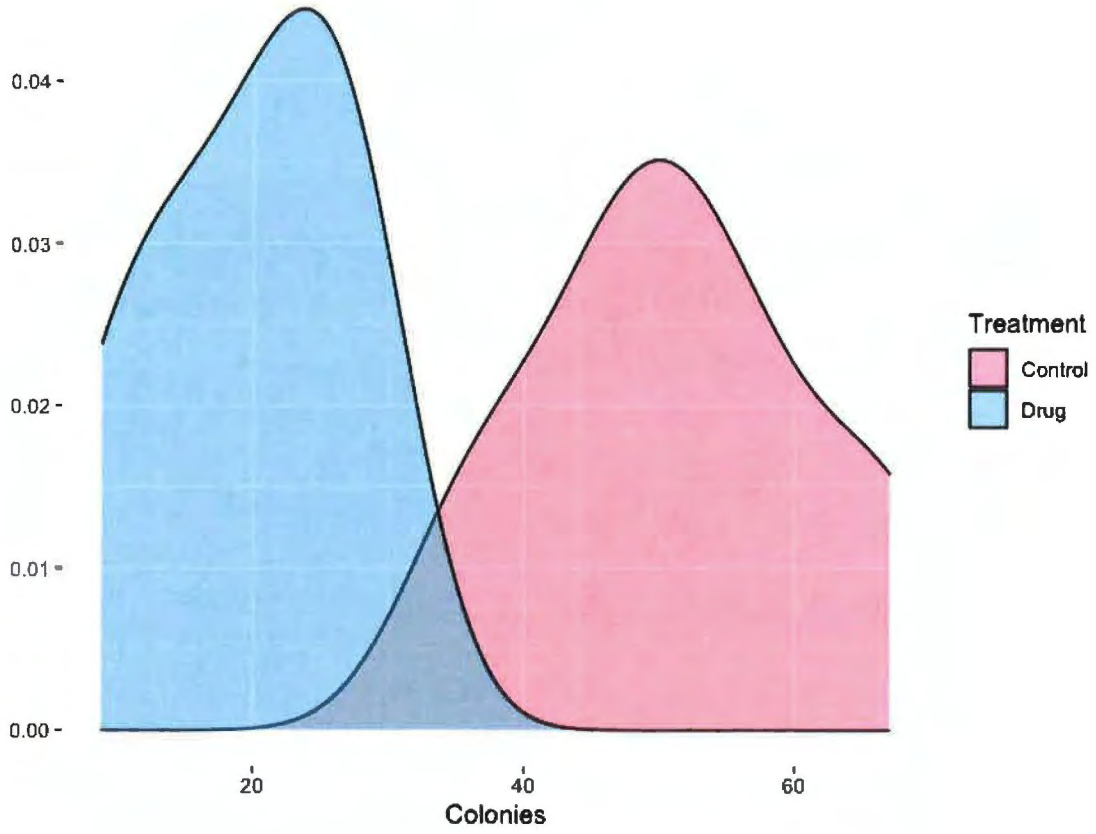
Treatment	Colonies
Drug	26
Drug	19
Drug	29
Drug	9
Drug	14
Drug	23
Control	67
Control	58
Control	36
Control	44
Control	52
Control	49

Table 1. Number of colonies counted. One colony= 50 or more glioblastoma cells. Colonies were counted after ten days of incubated with honokiol drug or as a control. Drug treatment was 50 micromolars of honokiol.

Graph 1 (below). A box plot of the means of the two treatments. The mean of the colonies that were treated with drug is lower than the mean of control colonies.



The Shapiro-Wilk test indicated normality with a p-value of 0.8381. This P-value is greater than 0.05 which indicates that the dataset is normal, and the t-test can be run. After the t-test, the t-value was found to be 5.7632. In the t-test, the degrees of freedom was 8.9329, and the p-value was 0.0002795. The 95% confidence interval from the t-test was 18.81798 and 43.18202. The mean of the control group was 51, and the mean of the drug group was 20. This can be seen in the box plot of the two variables; the control mean is much higher on the graph than the drug mean which indicates the mean value is greater for the control group.



Graph 2. This graph shows the difference between the group means which will be the t-value discussed in the Results section. The graph indicates that the control mean is higher than the drug mean expressing that the null hypothesis will probably be rejected.

Discussion

Before running the research, the hypothesis was that the amount of colonies in the well control well plates lacking the drug honokiol would be greater than the amount of colonies in the treated well plates containing honokiol. This hypothesis would allow for the null hypothesis to be rejected. There would not be a difference between the means of the two different groups of variables, and the alternative hypothesis would not be rejected stating that the means displayed a difference.

When the t-test was run, the p-value was less than 0.05, as indicated in the results section. This p-value concluded that the null hypothesis could be rejected as was predicted when the experiment was being run. I could accept the hypothesis that there was a difference between the means of the two variables which was indicated by the t-test. The t-value, indicated in the results section, measured the size of the difference relative to the variation in the sample data. The larger the t-value is, the greater the evidence against the null hypothesis. With a t-value of 5.8, this was a good indicator that the null hypothesis will be rejected. The t-value is the difference between the means, so if it is small, there is not a large difference in means.

I expected to find that the means would be different, and that the null hypothesis would be rejected. This experiment had been run using honokiol on glioblastoma cells, and a similar outcome was experienced by these scientists where they examined a difference in the means. After I counted the colonies and entered the data into the table, I observed that the numbers for the well plates' colonies that had been treated with drug were much lower than than colonies of the control plates. I knew that the drug honokiol exhibited proapoptitic and anti-tumorigenic effects based on journal articles and previous studies which should have caused the outcome I

predicted. If the drug worked, this would be indicated by a difference in the means which was shown.

The unexpected results from the experiment were the amount of colonies still found in plates treated with honokiol. Although the control was still much higher than the treatment, I was expecting for the drug to have much more potent and apoptotic effects than it did. It had the correct effect of reducing the amount of colonies, but I was hoping for much lower numbers of colonies in the drug treatment wells.

Conclusion and Recommendation for Future Experiments

In the future, I could use a higher dosage of honokiol to see if this would cause a smaller amount of colonies in the well plates. Instead of using 50 micromolars of honokiol on the wells, 100 micromolars could be used to see if this would lessen the amount of colonies and increase the difference between the means even more. The amount of drug has to be determined that will not be too potent that it kills all of the surrounding tissue but just apoptotic enough to where it destroys the tumorigenic potential in tissues. Using honokiol at 50 micromolars is effective to lessen the proliferative rates and increase apoptosis, but it would be interesting to see if this amount could be increased even more.

Glioblastoma is the most aggressive and common form of brain tumor, and it is almost always lethal. Although there are treatments in form of radiation and chemotherapy, these regimens are still not curative and can only provide patients with four more years in the best case scenario. It is critical for new therapeutic treatments to be tested as treatment strategies. Based on the results of this experiment and other experiments like it, honokiol reduces the tumorigenic

potentials of glioblastoma. These observations indicate hope for honokiol to represent a novel therapy for malignant glioma.

Literature Cited

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Appendices

```
> View(honokiol)
> t.test(honokiol$Colonies~honokiol$Treatment)

        Welch Two Sample t-test
```

data: honokiol$Colonies by honokiol$Treatment

t = 5.7632, df = 8.9329, p-value = 0.0002795

alternative hypothesis: true difference in means is not equal to 0

95 percent confidence interval:

18.81798 43.18202

sample estimates:

mean in group Control mean in group Drug

51 20

> shapiro.test(honokiol$Colonies)

Shapiro-Wilk normality test

data: honokiol$Colonies

W = 0.96393, p-value = 0.8381

> boxplot(honokiol$Colonies~honokiol$Treatment)

> boxplot(honokiol$Colonies~honokiol$Treatment,cex.axis=0.5,xlab="Treatment",

+ ylab="Colonies")

+ > library(ggplot2)

+ > ggplot(honokiol,aes(Colonies,fill=Treatment))+geom_density(alpha=0.2)

+ > ggplot(honokiol,aes(Colonies,fill=Treatment))+geom_density(alpha=0.5)