Team Daredevils
Module 4 Report
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In vitro effects of 1,2,3,4,6- penta-O-galloyl-beta-D-glucose on NF-κB activation levels in human glioblastoma cells

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ABSTRACT

PGG, an extract from a Chinese crude medicine, shows promising evidence in inhibiting the in vitro growth on the human hepatocellular carcinoma cell line SK-HEP-1 cells through the inactivation of the NF-kB pathway.[1] This experiment is to understand the possible translation to the treatment of glioblastoma, which induces high level of activation in the NF-kB pathway during the proliferation period.[2] It is hypothesized that the PGG would inactivate the NF-kB pathway, resulting in a reduced cell number in the glioblastoma cell line U87. Four different assays- CCK-8, cell cycle analysis, clonogenic assay analysis and western blot are employed to verify the effect of PGG on the U87. This study is to determine the possible therapeutic advantages using PGG in treating glioblastoma cells.

BACKGROUND

Glioblastoma and NF-κB activation: Glioblastomas are highly malignant tumors that develop in the brain from mutated glial cells and exhibit rapid growth and invasiveness.[3, 4] As tumor size increases, pressure on the brain also increases and can cause headaches, blurred vision, vomiting, or changes in mood.[3, 4] Treatments for glioblastoma include radiation, chemotherapy, and surgery; however, the prognosis for glioblastoma is still poor.[4] Current research is uncovering many signaling pathways that are mutated in glioblastoma, resulting in increased cell proliferation, poor cell cycle regulation, and decreased apoptosis. These mutated pathways may provide new therapeutic targets.[5, 6]

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factors are prolific in glial cells and regulate the expression of enzymes, cytokines, angiogenic factors, and cell cycle regulatory molecules.[2,6,7] Normally, the NF-κB pathway is triggered by

proinflammatory molecules, which begin a cascade of NF-κB activation and translocation to the nucleus.[2] Once in the nucleus, NF-κB induces genes that will increase cell growth and proliferation, decrease apoptosis, and create a proinflammatory environment.[2, 7] Higher expression levels of activated NF-κB are found in malignant glioblastoma tumors. NF-κB activation increases mainly due to the mutation and amplification of signals that trigger the NF-κB pathway and the deletion of genes that create NF-κB inhibitor proteins.[2] Increased activation NF-κB may contribute to uncontrolled accumulation and increased invasion capability of cells.[8, 9] Therefore, the NF-κB pathway should be explored as a potential therapeutic target.

NF-κB inactivation and PGG: 1,2,3,4,6-penta-O-galloyl-beta-d-glucose (PGG) is a gallotannin derived from the root of *Paeonia suffruticosa*.[1] PGG is an antioxidant, antimutagenic compound. It has been shown to inhibit tumor growth in endothelial and liver cancer cells.[1, 10] PGG has specifically been shown to inhibit NF-κB in liver cancer cells. The inactivation of NF-κB reduced cell growth and arrested the cell cycle in the G_0/G_1 phase.[1] Interestingly, apoptosis was not impacted by PGG treatment.[1] Based on the current research and the known activation of NF-κB in glioblastoma, PGG is believed to be a possible therapeutic option.

Hypothesis

It is known that NF-κB, a transcription factor protein which regulates cell proliferation, is activated in the glioblastoma cells. PGG, an antimutagenic compound derived from tree peony, shows evidence to cause inactivation of NF-κB in human hepatocellular carcinoma cell line. Since there are many NF-kB pathways observed in the U87[2], it is hypothesized that PGG can

inhibit cell proliferation of the human glioblastoma cells by inactivating the NF-κB in GMB and the key signaling pathways involved.

SPECIFIC AIMS

Significance

A glioblastoma diagnosis currently has a very grim outlook. The NCI estimates that in 2015 22,850 Americans were diagnosed with glioblastoma and 15,320 of those diagnosed died within the year. [11] Glioblastoma makes up 52% of primary adult brain tumor diagnoses and 17% of the primary and metastatic nervous tissue tumors. [11] The burden of this disease falls on older adults and most often occurs in patients aged 46 to 70 years old. [11] As the aging population in America increases the number of glioblastoma cases each year are expected to increase. Direct medical spending on a glioblastoma patient is between $50,600 and $92,700 per year which is a significant amount of healthcare spending. [12] One of the major needs to improve the glioblastoma prognosis is better therapeutic technologies. The proposed study will be examining the effects of PGG dosing on cell proliferation and survival to determine the potential benefits of PGG as a therapeutic agent. This work is also significant because the NF-κB pathway is present in many other cell types and may help advance scientific knowledge of cancer therapeutics for other types of cancer.

Innovation

This experiment is a novel exploration of the effect of PGG on human glioblastoma cells and will aid in the development of an innovative cancer therapeutic. This experiment will utilize

several different modern technologies: CCK Analysis, Western Blot, Flow Cytometry, and a Clonogenic Assay.

RESEARCH STRATEGY

Aim 1: Establish a profile of the effects of various PGG concentrations on cell proliferation of U87 cells using CCK-8.

Approach and Rationale:

Increased cell proliferation is affected by the NF-κB pathway and is a hallmark trait of cancer.[6] When evaluating the effectiveness of PGG as a therapeutic option, the potential dose-dependent effect of PGG on cell proliferation must first be determined. Once a relationship between cell proliferation and PGG concentration is established, a single concentration of PGG will be used for future experimentation. Cell proliferation will be determined by comparing the cellular density of each trial concentration to that of the control. To estimate the relative living cell density, a CCK-8 analysis will be used. CCK-8 provides a quantitative evaluation of varying cell densities. Using CCK-8 allows for the cells to be used in multiple experimentations because it is a non-toxic assay.

Materials and Methods:

On the first day of the experiment 21 wells of a 96-well plate will be seeded with 5,000 U87 cells in 200μL media per well. The cells will incubate for 24 hours in the 96-well plate before being dosed with PGG in media. PGG concentrations of 0, 10, 20, 40, 50, 100, and 200μM will be prepared in media using serial dilution. After 72 hours of exposure to the PGG, CCK analysis will be run on the cells to determine cell survival at each concentration. For the CCK analysis the

old media will be removed and replaced with 10% CCK and media for 1 hour. The absorbance of each sample at 450 nm will then be recorded.

Expected Outcomes:

We expect that the PGG will have a dose dependant effect on the U87 cells similar to that observed in previous research involving SK-HEP-1 cells. [1] In the previous literature, the concentrations used to treat the cells ranged from 0 to 50μM and the highest concentration only narrowly brought the cell number down to fifty percent of control. In order to calculate the IC50 of PGG on U87 cells we must collect data that includes decrease in cell viability by fifty percent. Since PGG could affect these cells with a different strength than observed in SK-HEP-1 cells, we include higher treatment concentrations in the event that the effect is weaker.

Alternative Approaches:

It is possible that U87 cells are more resilient to PGG than anticipated. In this case, the previously defined concentrations might not be high enough to reach IC50. This would require resetting the experiment with higher concentrations to better show the dose dependent effects of PGG.

Aim 2: Determine the effect of PGG treatment on cell cycle phase distribution and apoptosis using flow cytometry.

Approach and Rationale:

The NF-κB transcription factor regulates genes that affect both the cell cycle and the rate of apoptosis.[6, 7] In other cell lines, higher NF-κB activation levels have been shown to correlate with an increased percentage of cells in the G_0/G_1 phase.[1, 13] NF-κB also controls the expression of several antiapoptotic proteins. Previous research suggests that NF-κB can inhibit the apoptotic

response in some tumor cell types when proapoptotic signals are used as a treatment.[13] Flow cytometry will be performed with a control group that has no PGG treatment applied and a single experimental group that is treated with a PGG concentration shown to significantly impact cell proliferation. Cell cycle phase distribution will be analyzed to determine the response of cell cycle regulatory proteins in U87 cells to PGG treatment. Flow cytometry will also be utilized to determine the effect of PGG on apoptosis.

Materials and Methods:

Two groups of U87 cells will be used for this analysis, one treated with the determined PGG dosage and the other a control. Each group will be incubated for three days and then trypsinized and subjected to cell count, viability, cell cycle phase and apoptosis analyses by Muse™ automated cell analyzer as per manufacturer's instructions. Both cell cycle arrest and apoptosis will be analyzed to determine what is contributing most to decreased cell proliferation.

Expected Outcomes:

According to the previous literature, there is larger proportion of SK-HEP-1 cells accumulated in the G_0/G_1 phase when treated with PGG comparing with the control group, indicating a growth arrest at the G_0/G_1 phase [1]. It is expected that PGG would arrest more cells in G_0/G_1 phase than the control, resulting a higher cell number in the G_0/G_1 phase.

Alternative Approaches:

The standard approach for determining apoptosis involves double staining with PI and Annexin V-FITC and using flow cytometry. Staining positive and negative for different combinations of these two factors distinguishes between early and late stage apoptosis. Alternative methods for determining the presence of apoptosis could involve caspase-3/7 activation, bcl-2 activation, or MAPK activation.

Aim 3:Test the long-term effects of PGG treatment on cell proliferation of U87 cells using a clonogenic assay.

Approach and Rationale:

In order to test the impact of PGG treatment on cellular reproduction, a clonogenic assay will be performed. A clonogenic assay will determine if PGG-treated cells can still undergo unlimited division.[14] If the cells can no longer create colonies, tumor recurrence may be prevented.[14]

Materials and Methods:

A six well plate will be seeded in triplicate with control cells and cells treated with 3 different concentrations of PGG between 10 and 200μM. After treating with the designated treatment concentration the cells will be incubated for ten days with no media changes. Colonies will be fixed, stained, and counted using stereomicroscope to compare the cell's proliferation.

Expected Outcomes:

It is hypothesized that the PGG has an inhibitory effect on the cell proliferation, reducing the number of cell divided within a time range. Therefore, a comparison on the capacity of cell developing colonies can be made by harvesting the cells simultaneously with and without PGG. It is expected that as the concentration of PGG treatment increases, the cell proliferation rate would decrease, resulting in less colonies formed in the higher concentration treatment groups.

Alternative Approaches:

If the colonies are not large enough to be easily counted by day ten, we would extend the duration of this assay beyond ten days until the colonies are of a sufficient size. If the CCK-8 proliferation assay shows that the chosen treatment doses are not sufficient to produce a

significant effect on the survival of cells, we would need to restart the clonogenic assay with higher concentrations so that differentiation can be seen at day ten.

Aim 4:Test the impact of PGG treatment on the activation levels of NF-κB in U87 cells using western blotting.

Approach and Rationale:

NF-κB activation can be identified by staining for the p65 protein complex, which is only present when NF-κB is in its active form.[15] A western blot will be performed with a control group that has no PGG treatment applied and a single experimental group that is treated with a PGG concentration shown to significantly impact cell proliferation. The groups will be incubated with an antibody specific to the p65 complex so that only activated NF-κB will be labeled. Analyzing the fluorescent signal of the western blot will provide a quantitative analysis of the NF-κB activation level. This will confirm that PGG is impacting the NF-κB pathway specifically.

Materials and Methods:

U87 control cells and cells treated with a selected dose of PGG will be used for this experiment. First the Bolt Electrophoresis System will be used for the polyacrylamide gel to load the centrifuged samples onto. Next, the iBlot 2 Dry Blotting system will be used to transfer the gel onto nitrocellulose membrane. The iBind Western System for hybridization will be used. First the iBind card and transferred membrane will be loaded with each sample. Then load the primary antibody, Anti-NF-kB p65, and the secondary antibody, Goat anti-Rabbit IgG (H+L). Then the Western Blot results will be analyzed through various imaging technologies.

Expected Outcomes -

It is hypothesized that PGG inactivates the NF-kB pathway which contributes to cell proliferation. Evidence of this suppression on the NF-kB pathway is also shown in the previous literature on the PGG to SK-HEP-1 cells, with the western blot having less fluorescent signal observed in the higher concentration PGG than the lower concentration groups or the positive control group. [1] Therefore, we expect to see a similar effect of the PGG on the U87 cell line.

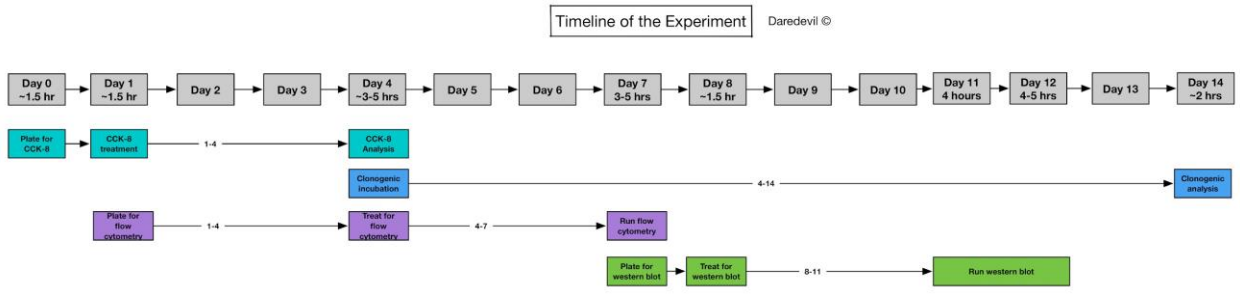
Alternative Approaches:

It is necessary to use the Anti-NF-kB p65 antibody to verify NF-κB activation changed in the treatment groups. To better understand the other mechanisms involved in the change in cell viability, other antibodies could be chosen based on flow cytometry results.

CONCLUSION

In summary, these four experiments will be used to determine the effect of PGG dosing on the activation of the NF-kb transcription factors. This novel experiment will provide insight into the action of PGG on glioblastoma cells that could be translated into a new treatment for glioblastoma patients.

TIME TABLE



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