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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Problem Definition

- Glioblastoma is a highly malignant brain cancer composed of mutated glial cells.[1] In 2015, 67% of patients diagnosed with glioblastoma died within a year.[2]
- New therapeutic targets are needed for glioblastoma treatment.
- Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factors, if activated, induce increased cell growth and proliferation and decreased apoptosis.[3,4]
- Malignant glioblastoma tumors express heightened levels of activated NF-κB.[3] Increased activation of NF-κB pathway may contribute to the invasiveness of glioblastoma tumors.
- 1,2,3,4,6- penta-O-galloyl-beta-D-glucose (PGG) is an antioxidant, antimutagenic compound derived from the root of Paeonia suffruticosa. Previous studies have shown that PGG inhibits the NF-κB pathway in human hepatocellular carcinoma cancer cells.[5]
- The effects of PGG on glioblastoma cells have not been determined.

Hypothesis

Treatment with PGG will inactivate the NF-κB pathway in human glioblastoma cells, causing

a. Decreased cellular viability
b. Decreased cellular proliferation
c. Increased apoptosis
d. Increased cell cycle arrest in the G_0/ G_1 and the G_2/ M phases
e. Decreased levels of activated NF-κB

Experimental Design

CCK-8 Analysis

Control: U87 cells with media alone Treatments: 10, 20, 40, 50, 100, and 200 µM of PGG	Treated for 72 hours CCK-8 toolkit

Clonogenic Assay

Control: U87 cells with media alone Treatments: IC50 and IC60 of PGG	Treated for 72 hours Colonized for 10 days Crystal violet staining

Flow Cytometry

Control: U87 cells with media alone Treatments: IC50 and IC60 of PGG	Treated for 72 hours MUSE Cell Analyzer Apoptosis – Annexin V-FITC and PI staining Cell cycle – PI and RNAse A staining

Western Blot Analysis

Control: U87 cells with media alone Treatment: IC50 of PGG	Treated for 72 hours NanoDrop, iBlot2 Dry Blotting System Anti-NF-κB p65 antibody

Results

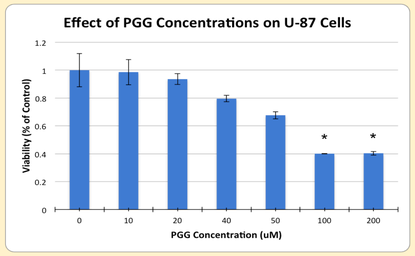

Figure 1. Cell viability of U87 cells treated with PGG. A 96-well plate was seeded with 5000 cells/well and treated with various concentrations of PGG for 72 hours. Absorbance at 450 nm was measured for each group (n=3). A two-tailed unpaired T-test reported statistical significance ($p<0.05$) between 0 µM and 100 µM ($P=0.0258$), as well as between 0 µM and 200 µM ($P=0.0268$). Error bars represent standard deviation.

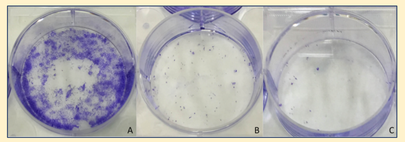

Figure 2. Colonization of PGG-treated U87 cells. Cells were pre-treated for 72 hours with PGG concentrations 0 µM (A), 50 µM (B), or 100 µM (C). A 6-well plate was seeded with 500 treated cells per well, with each group in triplicate (n=3). Colonization occurred for 10 days. Cells were fixed and stained using crystal violet.

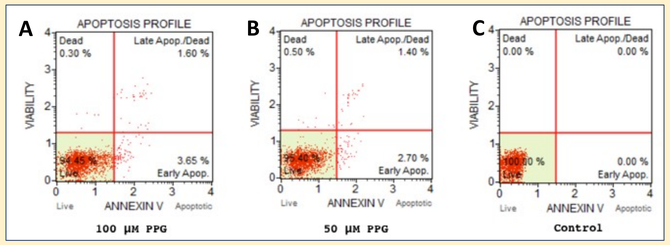

Figure 3. Flow cytometric analysis of apoptotic or necrotic cells of U87-EGFP using Annexin V-FITC. U87 cells were treated in triplicate (n=3) for 72 hours with PGG concentrations of 100 µM (A), 50 µM (B), or 0 µM (C), with 0 µM as the control. Cells were stained with Annexin V-FITC and PI. Gating was performed with the control to remove debris. Cells in the lower left quadrant are viable, and those in the lower right quadrant are in early stage of apoptosis. In the upper two quadrants, cells are not viable. The percentage of the population in each quadrant was recorded.

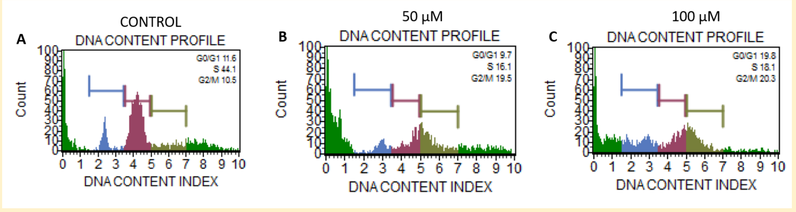

Figure 4. The effect of PGG on cell cycle phase distribution. U87 cells were treated with 0 µM (control), 50 µM or 100 µM of PGG for 72 hours. Each group was performed in triplicate (n=3). Cells were then fixed and stained with PI and RNAse A. Cell cycle distribution was determined through flow cytometry for control (A), 50 µM of PGG (B), or 100 µM of PGG (C).

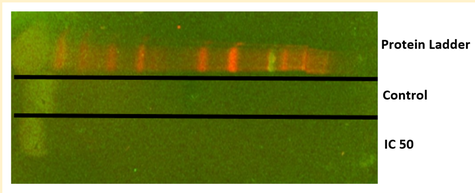

Figure 5. Western blot analysis of the NF-kB protein in U87 cells. U87 cells were treated in duplicate (n=2) for 72 hours with PGG concentrations of 67 µM (IC50) and 0 µM (control). Cells were lysed, and 100 µg of protein per sample was loaded into the gel. The membrane was stained with the primary antibody anti-NF-κB p65 and a secondary antibody. The protein ladder on the top lane indicates that the western blot ran successfully. However, no discernable bands can be seen in the control or in IC50, indicating that NF-kB was not present in the membrane.

Data Analysis

Cell viability:

- Cell viability is first significantly impacted when cells are treated with 100 µM of PGG. Also at 100 µM of PGG, the impact of PGG plateaus at approximately IC60.
- Before plateauing, the cellular viability has a roughly linear relationship with PGG concentration. As the PGG concentration increases, cellular viability decreases.

Cell proliferation:

- Colonies were significantly less for both treatment groups than for the control. Colonies for the control were too numerous to count.
- Based on a manual count, there is an approximately 50% decrease in colonies between 50 µM and 100 µM treatments

Apoptosis:

- Cells in early apoptosis increased as the concentration of PGG increased. However, the percentage of all dead cells remained consistent between the two treatment groups.

Cell cycle distribution:

- The percentage of cells in the S phase of the cell cycle significantly drops with either PGG treatment compared to the control. Likewise, the shift in DNA content indicates that more cells are staying in the G_0/ G_1 and the G_2/ M phases.

Activated NF-κB:

- No fluorescent bands were seen in the membrane when analyzing the Western blot. Therefore, the amount of activated NF-κB is assumed to be below that which produced a visible signal.

Conclusions

- Dose dependent effects of PGG on glioblastoma cell viability were confirmed by the preliminary CCK-8 proliferation assay. The effects of PGG plateaued at concentrations above 100 µM
- Cell cycle distribution via flow cytometry showed that the reduction in proliferation was impacted by cell cycle arrest.
- Apoptosis analysis via flow cytometry confirmed that cell cycle arrest impacted cell proliferation more than apoptosis.
- Long term effects of PGG, revealed by the clonogenic assay, also support the effectiveness of its future development as a treatment.
- NF-κB activation was not present in the IC50 or control samples. Due to the absence of NF-κB fluorescence in the western blot, the mechanism or pathway responsible for cell cycle arrest is unknown.
- This study is the first step towards exploring the possibility of PGG as a treatment for human glioblastoma.

Future Directions

While it is clear that PGG affects cell viability and cell cycle distribution of glioblastoma cells, the specific pathway affected has not been confirmed. Therefore, future studies to be conducted shall aim to:

- Determine what pathway is causing the cellular viability decrease and cell cycle arrest.
- Confirm that NF-κB is highly expressed in an active for in malignant cells.
- Analyze other effects of PGG on glioblastoma cells.
- Repeat this experiment on other cell lines that might be good candidates for PGG treatment.

References

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