Anti-glioblastoma Effects of a Glucose-Independent Benzoylphenoxyacetamide (BPA) Variant

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Abstract

Glioblastomas are notorious for being the most aggressive, lethal, and challenging to treat brain tumors. Current therapies against glioblastoma include surgical resection, followed by a combination of radiotherapy and concurrent treatment with temozolomide (TMZ) for maintenance. Unfortunately, these standard therapies often yield suboptimal results, leading to an average survival time between 12 and 18 months for patients. The limited availability of therapeutic options renders glioblastoma a formidable disease. We have previously documented the potent anti-glioblastoma properties of several synthetic metabolic compounds. These compounds were derived from the chemical structure of a widely used lipid-lowering drug, fenofibrate, and possess a general molecular framework known as benzoylphenoxyacetamide (BPA). Several of these compounds were shown to abruptly halt mitochondrial respiration, and effectively induce apoptosis in low glucose environments. However, extensive in-vitro and in-vivo analyses of these same BPA compounds show that their anti-glioblastoma effects are attenuated in the presence of normal glucose conditions. Based on our findings, we put forth two independent approaches that can potentially counteract glucose-dependent treatment. Firstly, we propose the utilization of a novel drug candidate, PP12, which shares a phenol moiety similar to the active ingredient in Tylenol (acetaminophen). Secondly, we suggest employing glycolytic inhibitors alongside our compounds that have previously been shown to inhibit mitochondrial respiration. Both approaches were tested in this study with the objective of selecting a new glucose-independent BPA variant or a set of synergic compounds for subsequent in-vivo and anti-glioblastoma testing. Out of an initial pool of 300 BPA variants, three compounds were further evaluated for anti-glioblastoma activity in-vitro during this study. In cell culture, all three compounds exhibit cell toxicity toward the glioblastoma cell line LN229. Among them, PP12 and PP211 displayed a substantial increase in cytotoxicity under normal glucose (4.5g/L) conditions suggesting that a separate, non-glucose-related, mechanism is responsible for cell death. Moving forward, PP1 and PP12 will be tested in tandem with several glycolytic inhibitors such as: 2-deoxyglucose (2-DG) and Gnetin H (GH). Research findings indicate that sequential administration of glycolytic inhibitors followed by our newly developed PP compounds enhances cytotoxicity, even in normal glucose environments. In contrast, simultaneous administration of these agents has only mild-additive effects. The hypothesized mechanism behind this heightened cytotoxicity is as follows: pretreatment with 2-DG or GH leads to the inhibition of glycolysis, which in turn triggers a compensatory rise in mitochondrial respiration. Subsequently, the introduction of our PP compound disrupts the functionality of cancer cells' mitochondria, ultimately resulting in cell death. Virtual Screening: (C*ompounds were assessed using computational analysis of the following parameters: solubility (logS), blood–brain partitioning (logBB), and probability of entering the CNS calculated by the Blood Brain Barrier Score (BBB Score) and Central Nervous System—Multiparameter Optimization (MPO-CNS) algorithm*.)

Synthesis Strategy

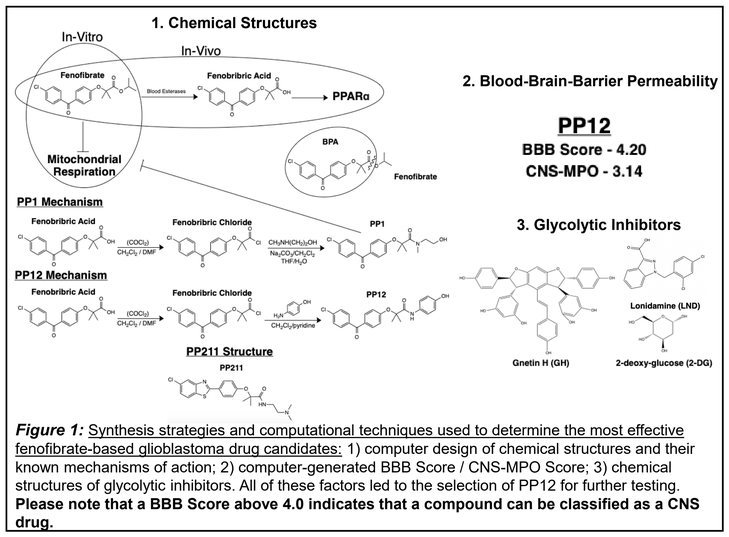
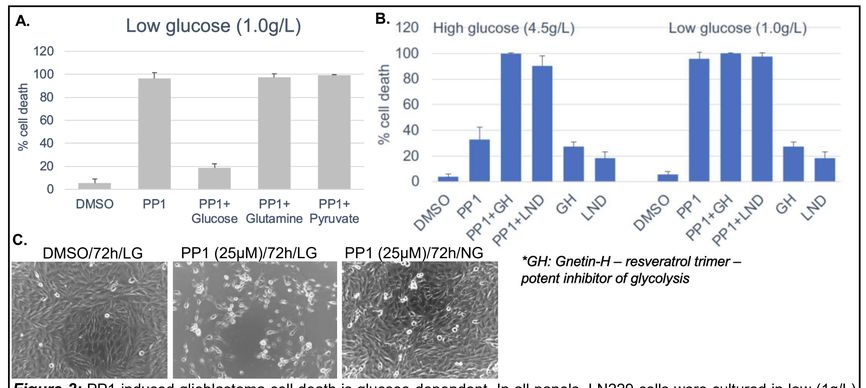


Figure 1: Synthesis strategies and computational techniques used to determine the most effective fenofibrate-based glioblastoma drug candidates: 1) computer design of chemical structures and their known mechanisms of action; 2) computer-generated BBB Score / CNS-MPO Score; 3) chemical structures of glycolytic inhibitors. All of these factors led to the selection of PP12 for further testing. **Please note that a BBB Score above 4.0 indicates that a compound can be classified as a CNS drug.**

Glucose Dependency



**GH: Gnetin-H – resveratrol trimer – potent inhibitor of glycolysis*

Figure 2: PP1-induced glioblastoma cell death is glucose-dependent. In all panels, LN229 cells were cultured in low (1g/L) or normal (4.5g/L) glucose. **Panel A:** 7ADD-based cell survival assay using easyCyte flow cytometry (Millipore). Cell death was evaluated after 72 hours of continuous cell exposure to PP1 (25 μM), PP1+glucose, PP1+glutamine, or PP1+pyruvate. Cells treated with 0.5% DMSO (vehicle) were used as a control. **Please note, the addition of glucose compromises PP1-induced cytotoxicity. Panel B:** Effects of glycolysis inhibitors (**GH and **LND) on PP1-induced glioblastoma cytotoxicity. The cells were treated with PP1 (25μM), GH (10μM), LND (200μM), PP1+GH, PP1+LND, and DMSO (control). In panels A and B data represent average values with standard deviation (n=3) *Indicate values significantly different from PP1 (P<0.05). **Panel C:** Pictures of 0.5 % DMSO and PP1 (25μM) induced cytotoxicity.

Drug Efficacy

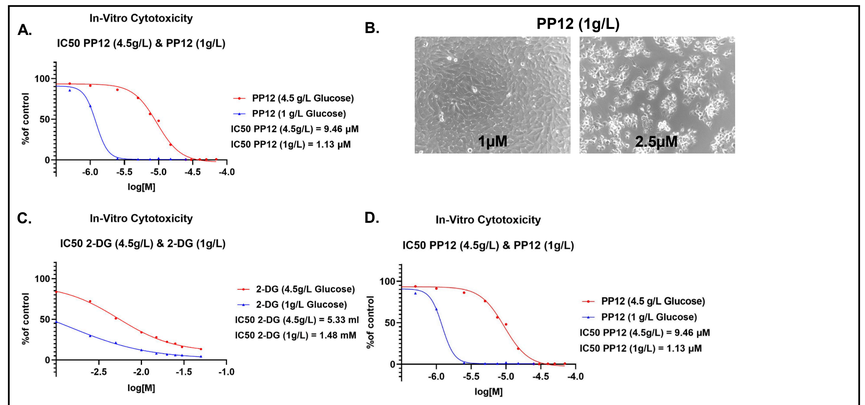


Figure 3: Characterization of PP12 as a new glioblastoma drug candidate using half maximal inhibitory concentration assays (IC50). **Panel A:** PP12 IC50 evaluated with LN229 cells cultured in (4.5g/L) and (1g/L) glucose conditions. **Panel B:** Pictures from 1μM and 2.5μM wells of PP12 (1g/L) IC50 assay. **Panel C:** 2-DG IC50 evaluated with LN229 cells cultured in (4.5g/L) and (1g/L) glucose conditions. **Panel D:** PP12 and 2-DG (5mM) IC50 evaluated with LN229 cells cultured in (4.5g/L) glucose conditions. **Please note that 2-DG was introduced at hour zero, followed by the administration of PP12 after 24 hours.** The most significant cytotoxic effects of 2-DG were observed at a concentration of 5mM, and all assays were terminated at the 72-hour mark.

Metabolic Response

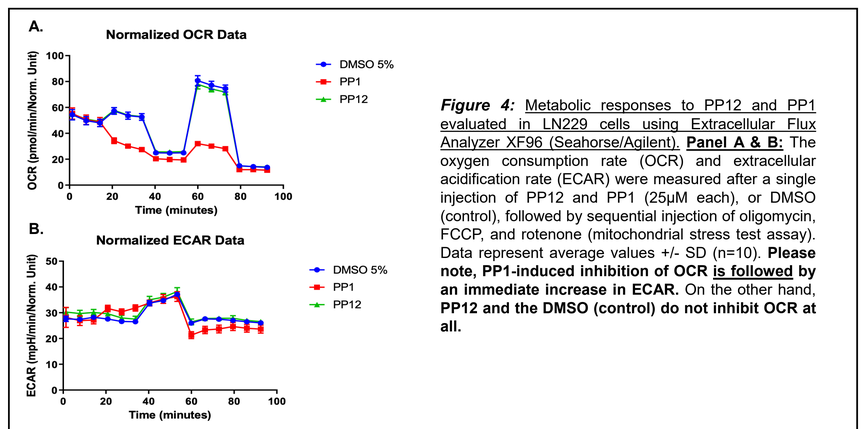


Figure 4: Metabolic responses to PP12 and PP1 evaluated in LN229 cells using Extracellular Flux Analyzer XF96 (Seahorse/Agilent). **Panel A & B:** The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured after a single injection of PP12 and PP1 (25μM each), or DMSO (control), followed by sequential injection of oligomycin, FCCP, and rotenone (mitochondrial stress test assay). Data represent average values +/- SD (n=10). **Please note, PP1-induced inhibition of OCR is followed by an immediate increase in ECAR.** On the other hand, **PP12 and the DMSO (control) do not inhibit OCR at all.**

Toxicity/Pathology

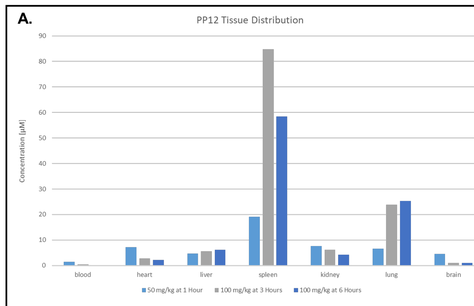
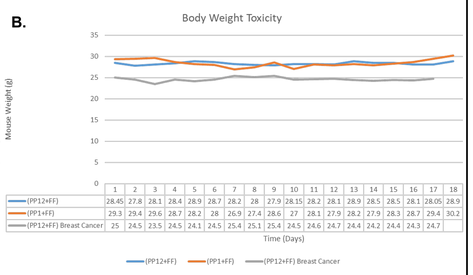


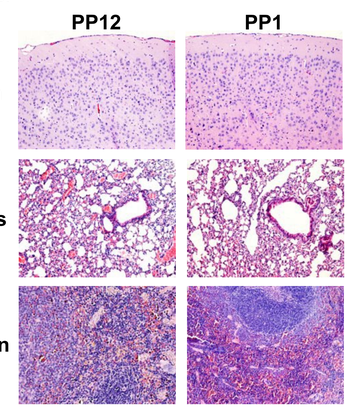
Figure 5: Toxicity and Pathology of PP12. In all panels, immunocompromised nude mice were used to study the toxicological effects of PP12. **Panel A:** Levels of PP12 in the blood, heart, liver, spleen, kidneys, lungs, and brain evaluated by high-performance liquid chromatography (HPLC) protocol developed in our lab. Non-tumor-bearing mice were treated intraperitoneally (IP) with PP12 at 50 (mg/kg/day) or 100 (mg/kg/day). Tissues were extracted after injection at one, three, and six hours. **Please note, the continuous detection of PP12 in the brain, lungs, and spleen at 6 hours. Importantly, nearly 25μM and 60μM of PP12 were detected in the lungs and spleen tissue at 6 hours – concentrations that exceed IC50 of PP12 in normal glucose (4.5g/L) 2.5 and 6 fold respectively.**

Panel B: The body weight of mice was measured every day for 18 consecutive days of treatment with PP12 or PP1 (50 mg/kg/day) and FF (50 mg/kg/day). **Please note that all mice exhibited weight gain or maintained starting weight by the end of the study.** Data represent average values from two mice (n=2).



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
(PP12+FF)	28.45	27.8	28.1	28.4	28.9	28.7	28.2	28	27.9	28.15	28.2	28.1	28.9	28.5	28.5	28.1	28.05	28.9
(PP1+FF)	29.3	29.4	29.6	28.7	28.2	28	26.9	27.4	28.6	27	28.1	27.9	28.2	27.9	28.3	28.7	29.4	30.2
(PP12+FF) Breast Cancer	25	24.5	23.5	24.5	24.1	24.5	25.4	25.1	25.4	24.5	24.6	24.7	24.4	24.2	24.4	24.3	24.7	

C.



Panel C: Histopathological evaluation of the heart, liver, kidneys, spleen, lungs, and brain was performed on the tissues of mice treated for 18 consecutive days. The sections were stained with hematoxylin and eosin (H&E). **Importantly, no evidence of drug-induced disease was found in any of the tissues despite having such high concentrations in the lungs and spleen.** However, in the spleen, a significantly higher number of apoptotic cells and increased extracellular deposits of hemosiderin were detected in the PP12 mouse.

Conclusion

Even though the peak concentration of PP12 in the brain, using IP injection as the delivery method, reaches only 5μM, this remains a potentially relevant therapeutic concentration, considering the IC50 of PP12 + 5mM 2-DG is 5μM in normal glucose conditions and the IC50 of PP12 in low glucose conditions is 1μM. In the future, intra-nasal drug administration techniques will be employed with the aim of enhancing the concentration of PP12 in the brain to target glioblastoma more effectively. Even if intra-nasal drug administration fails to deliver a greater concentration of PP12 to the brain, the drug will still be considered for future patient-derived xenograft glioblastoma efficacy studies. At some point in the future, PP12 is slated to undergo a pre-clinical efficacy study utilizing a xenograft murine model of lung cancer. The decision to conduct a pre-clinical efficacy study of PP12 using a xenograft murine model of lung cancer is primarily based on its recently discovered affinity toward lung tissue and its capability to maintain high concentrations in the lungs for prolonged durations of time. As the mechanism by which this compound induces cell death remains unknown, further testing will be conducted to narrow down the list of potential possibilities. Based on our mitochondrial stress test assay (OCR and ECAR), mitochondrial respiration and glycolysis have been ruled out as potential targets, as PP12 exhibited similar effects to the DMSO (control). In closing, PP12 stands out as a promising novel drug candidate capable of inducing glioblastoma cell death even at low concentrations. Its mechanism of action is not dependent on glucose, and it demonstrates no toxicity within various tissues in murine model studies. Additionally, PP12 showcases improved stability compared to other BPA variants, further solidifying its potential as a valuable therapeutic agent.

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