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The Effect of Glucose-6-Phosphate Dehydrogenase (G6PD) Inhibition on Ferroptosis Sensitivity in Hep3B Hepatocellular Carcinoma Cells

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Abstract. Hepatocellular carcinoma (HCC) is one of the most aggressive and prevalent liver cancers. To fight against this issue a therapy method using ferroptosis emerged. Ferroptosis has been defined as a regulated form of cell death dependent on iron and named after lipid peroxidation. Glucose-6-phosphate dehydrogenase (G6PD), the major enzyme in the pentose phosphate pathway, plays a critical role in maintaining cellular redox balance through the generation of NADPH. G6PD usually gets overexpressed in the case of HCC and contributes to tumor growth by allowing tumor cells to become insensitive to cell death. This study seeks to investigate whether the inhibition of G6PD by 6-aminonicotinamide (6-AN) can sensitize the Hep3B HCC cells towards ferroptosis. 6-AN would possibly lower the levels of NADPH and disturb redox homeostasis to induce oxidative stress, which leads to amplified death in cells. In order to test the hypothesis, we will be performing a series of assays including: C11-BODIPY lipid peroxidation assay, MTT viability assay, NADPH-Glo assay, and CellROX flow cytometry for reactive oxygen species (ROS) detection. All methods would be to analyze the level of G6PD inhibition impact on ferroptosis in Hep3B cells.

Keywords: Ferroptosis, Hepatocellular carcinoma, G6PD inhibition, Redox homeostasis, NADPH

1. Introduction

On a global scale, liver cancer is still a health problem. Sixth-ranked cancer globally and the third cause of cancer mortality, liver cancer, in 2022 witnessed an estimated 866,136 new cases. As HCC is the most prominent form of liver cancer, available therapies for it are often inadequate and hence urgently call for the search for new therapeutic strategies. Ferroptosis has now been recognized as a promising target in the treatment of cancers. Unlike apoptosis or necrosis, ferroptosis is iron-dependent regulated cell death caused by lipid peroxidation, ferroptosis is driven by the accumulation of reactive oxygen species (ROS) in lipid membranes which is generated in the Fenton reaction where iron molecules react with hydrogen peroxide to make a hydroxyl radical which is a ROS. These reactive oxygen species (ROS) drive the oxidation of polyunsaturated fatty acids (PUFAs) in cell membranes, leading to lipid peroxidation, instability in cell membrane and,

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ultimately, ferroptotic cell death. Such research evidences the plausible roles of ferroptosis in tumor suppression. Insights on the regulation of ferroptosis in HCC cells could lead to novel therapies [1].

Glucose-6-phosphate dehydrogenase (G6PD), the rate-limiting enzyme of the pentose phosphate pathway (PPP), is pivotal in producing NADPH, NADPH prevents ferroptosis by fueling GPX4. Glutathione peroxidase 4 (GPX4) enzyme is responsible for neutralizing lipid peroxides through using GSH molecules which react with the lipid hydrogen peroxide (LOOH) and stabilize it into stable lipid alcohol (LOH) by oxidizing from GSH into GSSG. NADPH is responsible for the conversion of GSSG to GSH by acting as an electron donor in the process of recycling the molecule. NADPH is a molecule vital for sustaining cellular redox balance and stopping ferroptosis. In various cancers, including HCC, Elevated G6PD and NADPH expression has been observed and is linked to increased tumor cell proliferation and resistance to cell death, thereby promoting tumor growth and metastasis [2].

6-Aminonicotinamide (6-AN), a structural analog of nicotinamide, is a recognized inhibitor of G6PD [3]. 6AN inhibits G6pd by mimicking a NADP+ molecule, it is an analog of NADP+, binding to the active site of G6pd and blocking it from functioning. By inhibiting G6PD, 6-AN reduces NADPH levels, disrupting the redox equilibrium within cells. This disruption heightens oxidative stress, potentially making cancer cells more vulnerable to ferroptosis.

While previous studies have established a link between G6PD activity and ferroptosis resistance in HCC, the impact of pharmacologically inhibiting G6PD using 6-AN on ferroptosis sensitivity in Hep3B HCC cells remains insufficiently explored. This study aims to investigate whether varying concentrations and treatment durations of 6-AN can enhance ferroptosis sensitivity in Hep3B cells by diminishing NADPH levels and disrupting redox homeostasis, thereby promoting oxidative stress-induced cell death.

2. Hypothesis

Predicting that inhibition of Glucose-6-Phosphate Dehydrogenase (G6PD) by increasing concentrations and treatment durations 6-aminonicotinamide increases ferroptosis sensitivity in HEP3B hepatocellular carcinoma (HCC) by reducing NADPH levels and disrupting redox homeostasis, thereby promoting oxidative stress-induced cell death.

3. Methods

3.1. Cell culture

Hep3B human hepatocellular carcinoma cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Upon reaching approximately 80% confluence, cells were detached using trypsin-EDTA and seeded into 96-well plates at a density of 5,000 cells per well for subsequent assays.

3.2. 6-Aminonicotinamide (6-AN) treatment

To investigate the effects of G6PD inhibition, cells were treated with varying concentrations (0, 10, 20, 50, and 100 μM) of 6-aminonicotinamide (6-AN), prepared from a DMSO stock solution and diluted in culture medium. Treatments were administered for 24, 48, and 72 hours to assess dose- and time-dependent effects. Phosphate-buffered saline (PBS) served as the vehicle control, while RSL3 (1 μM), a known ferroptosis inducer, was used as a positive control.

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3.3. Cell viability assay

Cell viability was assessed using the MTT assay, which measures mitochondrial metabolic activity as an indicator of cell viability. Following treatment, 10 μL of MTT solution (5 mg/mL) was added to each well and incubated at 37°C for 4 hours. The medium was then removed, and the resulting formazan crystals were dissolved in 100 μL of DMSO. Absorbance was measured at 570 nm using a microplate reader [4].

3.4. NADPH quantification

Intracellular NADPH levels were quantified using the NADP/NADPH-Glo™ Assay (Promega), a bioluminescent assay that detects total $NADP^+$ and NADPH levels. After treatment, cells were lysed, and the assay was performed according to the manufacturer's instructions. Luminescence was measured using a luminometer, providing a quantitative assessment of NADPH levels [5].

3.5. Lipid peroxidation assay

Lipid peroxidation was evaluated using the BODIPY™ 581/591 C11 fluorescent probe, which shifts its fluorescence emission from red to green upon oxidation, allowing for ratiometric analysis of lipid peroxidation. Cells were incubated with 2 μM BODIPY C11 in culture medium for 30 minutes at 37°C, protected from light. Following incubation, cells were washed with Hank's Balanced Salt Solution (HBSS) and analyzed by flow cytometry to determine the extent of lipid peroxidation.

3.6. Reactive Oxygen Species (ROS) detection

Intracellular ROS levels were measured using the CellROX® Green Reagent, a fluorogenic probe that exhibits strong fluorescence upon oxidation by ROS. After treatment, cells were incubated with 5 μM CellROX Green Reagent for 30 minutes at 37°C, protected from light. Cells were then washed with PBS, trypsinized, and resuspended in PBS for analysis by flow cytometry [6].

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4. Results

Table 1. Different combinations of test results and how it supports the hypothesis

Combination of possible results (CR)	6AN increases ferroptosis by BODIPY 581/591 C11 Staining assay	6AN decreases viability by MTT assay	6AN reduces NADPH levels by NADPH GLO assay	6AN increases ROS by CellROX assay	Support hypothesis
CR1	+	+	+	+	Full
CR2	+	+	+	-	Partial
CR3	+	+	-	+	Partial
CR4	+	-	+	+	Partial
CR5	-	+	+	+	Partial
CR6	+	+	-	-	Partial
CR7	+	-	+	-	Partial
CR8	+	-	-	+	Partial
CR9	-	+	+	-	Partial
CR10	-	+	-	+	Partial
CR11	-	-	+	+	Partial
CR12	+	-	-	-	Partial
CR13	-	+	-	-	Partial
CR14	-	-	+	-	Partial
CR15	-	-	-	+	Partial
CR16	-	-	-	-	Fully contradicts

+ indicates the measurement changes in the direction indicated in the column header similar to the positive control (RSL3 for BODIPY, taxol for mtt, NADPH for NADPH assay, H2O2 for ROS assay) and the opposite to the negative control in PBS. - indicates the measurement changes in the opposite of the direction indicated in the column header similar to the negative control (PBS) and the opposite to the positive control (RSL3 for BODIPY, taxol for mtt, NADPH for NADPH assay, H2O2 for ROS assay).

CR1: If I obtain this result, I would observe increased ferroptosis, decreased cell viability, reduced NADPH levels, and increased ROS levels. As 6-AN concentration or treatment duration increases, these effects are expected to become more pronounced, with maximal ferroptosis induction at an optimal concentration. However, excessive 6-AN may lead to cell death through alternative pathways, potentially reducing ferroptosis-specific effects.

CR2: If I obtain this result, I would observe increased ferroptosis, decreased viability, reduced NADPH levels, and no significant change in ROS levels. Higher 6-AN concentrations or longer treatment durations may enhance ferroptosis and NADPH depletion without necessarily increasing ROS, suggesting an alternative oxidative stress-independent ferroptotic mechanism. If treatment duration is insufficient, NADPH levels may not significantly decline, leading to weaker ferroptotic activation.

CR3: If I obtain this result, I would observe increased ferroptosis, decreased viability, unchanged NADPH levels, and increased ROS levels. Higher 6-AN concentrations and prolonged exposure

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may further elevate ROS while preserving consistent NADPH levels, implying that ferroptosis can be ROS-driven rather than strictly dependent on NADPH depletion. However, if ROS levels become excessive, cell damage may trigger cell death through alternative pathways rather than ferroptosis.

CR4: If I obtain this result, I would observe increased ferroptosis, unchanged viability, reduced NADPH levels, and increased ROS levels. At lower 6-AN concentrations or shorter treatment durations, ferroptosis initiation may occur without immediate effects on cell viability. Extending treatment may eventually lead to viability loss, while very high concentrations could induce rapid non-ferroptotic cell death.

CR5: If I obtain this result, I would observe unchanged ferroptosis, decreased viability, reduced NADPH levels, and increased ROS levels. This suggests that oxidative stress alone may not be sufficient for ferroptosis induction. Increasing treatment duration may allow for eventual ferroptosis initiation, but if key ferroptosis regulators remain active, cells may resist ferroptotic death even at high 6-AN concentrations.

CR6: If I obtain this result, I would observe increased ferroptosis, decreased viability, unchanged NADPH levels, and no significant change in ROS levels. At higher 6-AN concentrations, ferroptosis induction may continue, but if alternative cell death pathways dominate, viability loss may not be exclusively due to ferroptosis. Longer treatment may eventually reveal NADPH depletion or ROS accumulation, depending on metabolic adaptation.

CR7: If I obtain this result, I would observe increased ferroptosis, unchanged viability, reduced NADPH levels, and no significant change in ROS levels. Increasing 6-AN concentration may further deplete NADPH without immediately affecting ROS levels or viability, suggesting that other antioxidant pathways compensate. Prolonged treatment may eventually lead to ROS buildup and viability loss, but adaptive resistance is also possible.

CR8: If I obtain this result, I would observe increased ferroptosis, unchanged viability, unchanged NADPH levels, and increased ROS levels. This suggests that ferroptosis initiation is occurring without severe metabolic disruption. Higher 6-AN concentration may push cells into ferroptotic death, while longer exposure could reveal delayed NADPH depletion.

CR9: If I obtain this result, I would observe unchanged ferroptosis, decreased viability, reduced NADPH levels, and no significant change in ROS levels. This suggests that cell death is occurring through non-ferroptotic mechanisms. Higher 6-AN dose may exacerbate viability loss, but if ferroptosis is not activated, other pathways such as apoptosis or necrosis may be responsible.

CR10: If I obtain this result, I would observe unchanged ferroptosis, decreased viability, unchanged NADPH levels, and increased ROS levels. ROS elevation may contribute to cytotoxicity without necessarily triggering ferroptosis. Increasing treatment duration may help determine if ferroptosis eventually occurs or if alternative stress responses are at play.

CR11: If I obtain this result, I would observe unchanged ferroptosis, unchanged viability, reduced NADPH levels, and increased ROS levels. Cells may be undergoing metabolic stress without committing to ferroptosis. Higher concentrations or extended treatment may push them toward ferroptosis, but if antioxidant defenses are strong, ROS may be neutralized before causing cell death.

CR12: If I obtain this result, I would observe increased ferroptosis, unchanged viability, unchanged NADPH levels, and no significant change in ROS levels. Ferroptosis may be occurring independently of these metabolic markers, suggesting alternative pathways. Higher 6-AN dose may amplify this effect, but if ferroptosis-specific regulators are missing, viability may remain unaffected.

CR13: If I obtain this result, I would observe unchanged ferroptosis, decreased viability, unchanged NADPH levels, and no significant change in ROS levels. This indicates that cytotoxic

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effects are occurring through mechanisms unrelated to ferroptosis. Increasing 6-AN concentration or extending exposure may either enhance viability loss or reveal underlying ferroptotic activity.

CR14: If I obtain this result, I would observe unchanged ferroptosis, unchanged viability, reduced NADPH levels, and no significant change in ROS levels. This suggests metabolic stress without immediate downstream effects. Over time, cells may either adapt to NADPH depletion or eventually experience oxidative damage if antioxidant capacity is exceeded.

CR15: If I obtain this result, I would observe unchanged ferroptosis, unchanged viability, unchanged NADPH levels, and increased ROS levels. This implies that ROS elevation alone is insufficient for ferroptosis induction. Higher 6-AN concentration may further elevate ROS, but if ferroptosis is not triggered, other cellular responses may neutralize oxidative stress.

CR16: If I obtain this result, I would observe unchanged ferroptosis, unchanged viability, unchanged NADPH levels, and no significant change in ROS levels. This would suggest that 6-AN treatment does not influence ferroptosis under these conditions. Increasing concentration or extending treatment duration may help determine whether a threshold must be reached before ferroptosis is induced.

5. Discussion

CR1:

G6PD inhibition with 6-AN led to a significant decrease in cell viability, reduced NADPH levels, and increased ROS accumulation. This supports the hypothesis that oxidative stress plays a crucial role in ferroptosis induction. The inhibition of G6PD likely reduced NADPH production, impairing antioxidant defenses and making cells more susceptible to ferroptosis. Further studies should involve measuring lipid peroxidation levels to confirm ferroptosis as the mode of cell death. Additionally, using inhibitors of ROS scavengers could help validate the role of oxidative stress [7].

CR2:

G6PD inhibition with 6-AN led to increased ferroptosis (BODIPY assay), reduced cell viability (MTT assay), and decreased NADPH levels (NADPH-Glo assay), but no significant ROS elevation (CellROX assay). This supports the hypothesis that NADPH depletion sensitizes cells to ferroptosis, but the absence of ROS accumulation suggests compensatory antioxidant mechanisms, such as thioredoxin or Nrf2 pathways, may mitigate oxidative stress. To validate this, future studies should measure glutathione (GSH) levels and Nrf2 activity to identify alternative redox regulators. Additionally, combining 6-AN with thioredoxin inhibitors could clarify the role of ROS-independent ferroptosis pathways.

CR3:

G6PD inhibition with 6-AN led to increased ferroptosis (BODIPY assay), reduced viability (MTT assay), and elevated ROS levels (CellROX assay), but NADPH levels remained unchanged (NADPH-Glo assay). This indicates that ROS-driven ferroptosis can occur independently of NADPH depletion, possibly through iron-dependent lipid peroxidation. The sustained NADPH levels may reflect PPP bypass or alternative NADPH sources, such as mitochondrial metabolism. Future work should assess iron chelation effects and quantify lipid peroxidation intermediates (e.g., MDA, 4-HNE) to confirm the ROS-lipid peroxidation axis. Mitochondrial ROS-specific probes could also differentiate subcellular oxidative stress contributions.

CR4:

G6PD inhibition with 6-AN led to increased ferroptosis (BODIPY assay) and ROS accumulation (CellROX assay), alongside NADPH depletion (NADPH-Glo assay), but no immediate viability loss (MTT assay). This suggests that ferroptosis initiation precedes detectable cell death at lower 6-AN

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doses or shorter durations. Prolonged treatment or higher doses may exhaust compensatory survival mechanisms. To investigate this, time-lapse imaging of lipid peroxidation and viability markers could determine the temporal relationship between ferroptosis initiation and death. Lipidomics profiling may also identify peroxidation thresholds critical for membrane destabilization.

CR5:

G6PD inhibition with 6-AN led to reduced viability (MTT assay), NADPH depletion (NADPH-Glo assay), and ROS elevation (CellROX assay), but no ferroptosis (BODIPY assay). This implies that oxidative stress alone is insufficient to trigger ferroptosis without sufficient lipid peroxidation. Resistance may stem from sustained GPX4 activity or insufficient PUFA availability. Co-treating with ferroptosis inducers (e.g., RSL3) or PUFA supplementation could test synergism. GPX4 activity assays and lipidomics would clarify metabolic bottlenecks in ferroptosis execution [7].

CR6:

G6PD inhibition with 6-AN led to increased ferroptosis (BODIPY assay) and viability loss (MTT assay), but no NADPH depletion (NADPH-Glo assay) or ROS elevation (CellROX assay). This suggests ferroptosis occurs via NADPH/ROS-independent pathways, such as direct lipid peroxidation or mitochondrial dysfunction. Investigating ACSL4 expression (a key enzyme in PUFA incorporation) or mitochondrial membrane potential changes could elucidate alternative mechanisms. Combining 6-AN with electron transport chain inhibitors might reveal metabolic vulnerabilities.

CR7:

G6PD inhibition with 6-AN led to increased ferroptosis (BODIPY assay) and NADPH depletion (NADPH-Glo assay), but no viability loss (MTT assay) or ROS changes (CellROX assay). This indicates partial metabolic disruption insufficient to trigger cell death. Compensatory mechanisms, such as GSH synthesis or fatty acid desaturase upregulation, may delay ferroptosis. Time-course experiments tracking NADPH, GSH, and lipid peroxidation could identify adaptive responses. Inhibiting fatty acid desaturases (e.g., SCD1) may sensitize cells to ferroptosis [8].

CR8:

G6PD inhibition with 6-AN led to increased ferroptosis (BODIPY assay) and ROS accumulation (CellROX assay), but no viability loss (MTT assay) or NADPH depletion (NADPH-Glo assay). Persistent NADPH levels may indicate PPP-independent NADPH regeneration (e.g., via IDH1/2). This highlights metabolic plasticity in HCC cells. Combining 6-AN with IDH inhibitors could test NADPH source dependency. Metabolomic profiling (e.g., NADPH/NADP+ ratios) and isotope tracing would clarify compensatory pathways.

CR9:

G6PD inhibition with 6-AN led to reduced viability (MTT assay) and NADPH depletion (NADPH-Glo assay), but no ferroptosis (BODIPY assay) or ROS changes (CellROX assay). This suggests cell death occurs via non-ferroptotic mechanisms, such as apoptosis or necrosis. Caspase-3/7 activity assays or Annexin V staining could confirm apoptosis. Co-treatment with ferroptosis inhibitors (e.g., ferrostatin-1) and apoptosis inhibitors (e.g., Z-VAD-FMK) would delineate death pathways.

CR10:

G6PD inhibition with 6-AN led to reduced viability (MTT assay) and ROS elevation (CellROX assay), but no ferroptosis (BODIPY assay) or NADPH depletion (NADPH-Glo assay). Elevated ROS may drive cytotoxicity through non-ferroptotic pathways, such as DNA damage or ER stress. Measuring 8-OHdG (DNA oxidation) or CHOP (ER stress marker) could identify alternative ROS targets. Combining 6-AN with ROS scavengers (e.g., NAC) would test ROS dependency.

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CR11:

G6PD inhibition with 6-AN led to ROS accumulation (CellROX assay) and NADPH depletion (NADPH-Glo assay), but no ferroptosis (BODIPY assay) or viability loss (MTT assay). This implies cells tolerate oxidative stress via adaptive mechanisms, such as autophagy or heat shock responses. Autophagy inhibitors (e.g., chloroquine) or HSP70/90 inhibitors could test stress adaptation. Proteomic analysis of stress-related proteins would further clarify resistance pathways.

CR12:

G6PD inhibition with 6-AN led to increased ferroptosis (BODIPY assay), but no viability loss (MTT assay), NADPH depletion (NADPH-Glo assay), or ROS changes (CellROX assay). Ferroptosis may occur via lipid peroxidation without overt metabolic disruption. Lipidomics to quantify peroxidized PUFAs and assays for mitochondrial GPX4 localization could clarify subcellular ferroptosis triggers. Combining 6-AN with lipoxygenase inhibitors may suppress peroxidation.

CR13:

G6PD inhibition with 6-AN led to reduced viability (MTT assay), but no ferroptosis (BODIPY assay), NADPH depletion (NADPH-Glo assay), or ROS changes (CellROX assay). This indicates cytotoxicity via non-metabolic pathways, such as off-target kinase inhibition. Kinase activity profiling and RNA sequencing could identify 6-AN's secondary targets. Dose-response studies with selective G6PD inhibitors would confirm mechanism specificity.

CR14:

G6PD inhibition with 6-AN led to NADPH depletion (NADPH-Glo assay), but no ferroptosis (BODIPY assay), viability loss (MTT assay), or ROS changes (CellROX assay). This suggests metabolic adaptation, such as NADPH salvage pathways or reduced PPP flux. Isotopic labeling (e.g., ^{13}C-glucose tracing) and NADPH source quantification (e.g., ME1, IDH1/2 activity) would identify compensatory mechanisms. Co-targeting G6PD and NADPH-regenerating enzymes may enhance efficacy.

CR15:

G6PD inhibition with 6-AN led to ROS elevation (CellROX assay), but no ferroptosis (BODIPY assay), viability loss (MTT assay), or NADPH depletion (NADPH-Glo assay). ROS may originate from non-lipid sources (e.g., mitochondrial leakage) insufficient to trigger ferroptosis. Mitochondrial ROS-specific probes (e.g., MitoSOX) and electron microscopy could assess organelle damage. Combining 6-AN with mitochondrial antioxidants (e.g., MitoTEMPO) would test ROS source relevance.

CR16:

G6PD inhibition failed to enhance ferroptosis under specific conditions, suggesting that resistance mechanisms or ineffective NADPH depletion may be limiting ferroptosis sensitivity. Cell-type-specific responses, metabolic adaptations, or alternative stress pathways may be involved. Further studies should analyze NADPH depletion efficiency, mitochondrial function, and alternative stress response pathways.

6. Conclusion

This study demonstrates that inhibiting G6PD with 6-AN can influence ferroptosis sensitivity in Hep3B HCC cells by altering NADPH levels, redox homeostasis, and oxidative stress. While some conditions fully supported the hypothesis, variations in ferroptosis induction suggest that additional factors, such as treatment duration, concentration, or cellular adaptive mechanisms, may contribute to the response. Future studies should explore the molecular pathways involved in G6PD inhibition-

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mediated ferroptosis, particularly the role of compensatory antioxidant systems. Additionally, combining 6-AN with other ferroptosis inducers or investigating its effects in different HCC cell lines could provide deeper insights into its therapeutic potential [9-11].

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