

Sorafenib and 2-Deoxyglucose: The Future of Hepatocellular Carcinoma Therapy

THESIS

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By

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## Abstract

Sorafenib therapy has been shown to have only a small clinical benefit for liver cancer patients. There is an urgent need to develop new therapeutic strategies for the treatment of advanced stage HCC. In this report, we screened several repurposed therapeutics in order to identify synergistic drug combinations. We demonstrate that the combination of 2-deoxy-glucose and sorafenib drastically inhibit HCC cell viability in Hep3B, Huh7 and sorafenib resistant Huh7 cells. Cell cycle analysis revealed that this therapeutic combination induced complete G<sub>0</sub>/G<sub>1</sub> arrested HCC cells. Our studies suggest that this cell-cycle arrest is due to the depletion of cellular ATP. Overall, this report provides strong evidence for the clinical potential of sorafenib + 2-deoxyglucose combination therapy.

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## Fields of Study

Biomedical Sciences Graduate Program

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## Chapter 1: Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in men worldwide. Due to late diagnosis and lack of effective drugs for treatment, HCC is the 2nd highest cause of male death from cancer.<sup>1</sup> Only a small proportion of HCC patients are diagnosed at an early stage, which enables the use of curative treatments such as tumor resection or liver transplant. However, most patients go undiagnosed until the disease has progressed to an advanced stage where there is little hope. Sorafenib, a multi-kinase inhibitor, is currently the only approved drug used in treating such patients.<sup>2</sup> Unfortunately, the average overall survival of patients treated with sorafenib is only extended by 2.8 months compared to untreated patients.<sup>3</sup> Although sorafenib treatment was shown to extend the overall survival of HCC patients, only 2% of patients displayed partial response to therapy based on RECIST criteria (Response Evaluation Criteria in Solid Tumors).<sup>3</sup> This low response rate is attributed to HCC tumors having an intrinsic resistance to sorafenib toxicity.<sup>4</sup> Since there are no other FDA approved therapies for advanced HCC patients, we sought to develop novel therapeutic strategies to sensitize HCC tumors to sorafenib toxicity. This approach to HCC therapy is highly significant as it may lead to the development of novel treatments to extend the survival of HCC patients. The mechanisms which mediate sorafenib resistance remain relatively unknown.<sup>4</sup> A handful of studies have demonstrated that a variety of mechanisms are involved in

maintaining sorafenib resistance; these mechanisms include CD44 overexpression,<sup>5</sup> activation of PI3K/AKT signaling<sup>6</sup> and increased MAPK14 activity.<sup>7</sup> Another group of studies has linked sorafenib sensitivity to cellular metabolism and glycolysis.<sup>8,9</sup> These studies are partially interesting because sorafenib therapy has been shown to inhibit oxidative phosphorylation and enhance glycolysis in a subset of HCC cell lines.<sup>10</sup>

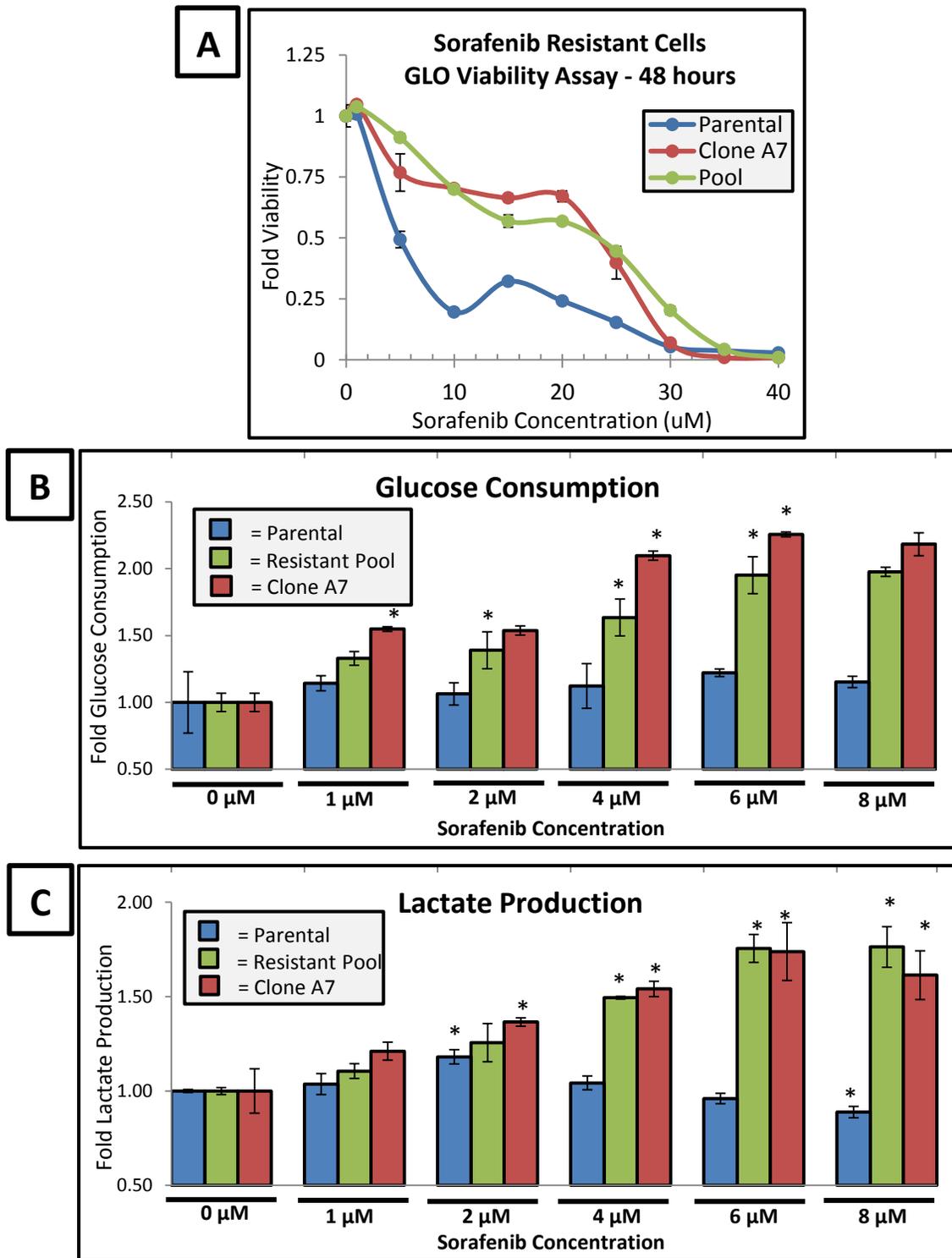
In order to further study the underlying mechanisms of sorafenib resistance, our laboratory has developed sorafenib resistant HCC cell lines. In this report, we demonstrate that sorafenib resistant HCC cells demonstrate markedly higher rates of glycolysis than parental HCC cells when treated with sorafenib. We hypothesized that high glycolytic rates are essential for cells to maintain sorafenib resistance and that suppressing glycolysis will sensitize resistant HCC cells to sorafenib toxicity. We initially examined the combination of several anti-glycolytic and sorafenib in our resistant cell lines. However, only one anti-glycolytic drug displayed synergy with sorafenib, 2-deoxyglucose (2DG). 2DG is an analog of glucose which inhibits glycolysis<sup>11,12</sup>. In this article, we demonstrate that this novel therapeutic combination has immense clinical potential for human HCC patients.

## Chapter 2: Results

### Establishment of Sorafenib Resistant HCC Cell Lines

In order to study sorafenib resistance, sorafenib resistant cell lines were generated from the human HCC cell line Huh7. In brief, parental Huh7 cells were exposed to continuously increasing doses of sorafenib to induce resistance. From the pooled fraction of resistant cells, individual resistant clones exhibiting high degrees of resistance were isolated. These cell lines demonstrated a remarkable resistance to sorafenib toxicity; the IC<sub>50</sub> dose for the resistant cells was about 4-5 times higher than that of the parental cells (Figure 1A).

There have been several recent studies linking sorafenib toxicity and resistance to glycolytic flux. One study demonstrated that exposure of rat hepatocolangiocarcinoma cells to sorafenib induces increased rates of glycolysis.<sup>8</sup> Another study demonstrated that increased glycolytic utilization has a strong correlation with sorafenib resistance across several HCC cell lines.<sup>9</sup> Taken together, we sought to investigate the glycolytic flux of sorafenib resistant cells exposed to sorafenib. Interestingly, the resistant cells demonstrated a large increase in glucose consumption and lactate production when exposed to increasing concentrations of sorafenib (Figure 1 B,C). However, parental Huh7 cells show minimal change in glucose consumption and lactate production upon sorafenib exposure. Based on these observations, we hypothesized that increased



**Figure 1:** Establishment of sorafenib resistant HCC cell lines

glycolytic flux is a key mechanism for the resistance of HCC cells to sorafenib induced toxicity. This lead us to believe that combination of sorafenib with therapeutics that inhibit glycolysis could sensitize cells to sorafenib toxicity.

### *In Vitro* Screening of Anti-Glycolytic Agents

In order to determine if the inhibition of glycolysis could sensitize HCC cells to sorafenib toxicity, we first sought to identify viable anti-glycolytic therapeutics. In order to accelerate the future clinical trial process of successful therapeutic combinations identified in this study, drug repurposing methodology was employed; drugs which were FDA approved or have undergone clinical trials were prioritized over experimental therapeutics. Table 1 contains a list of the repurposed anti-glycolytic drugs selected for this study. Each therapeutic was used alone and in combination with sorafenib in order to generate dose-dependent viability curves in parental and resistant Huh7 cells (data not shown). This initial screening demonstrated that the anti-glycolytic agent 2-Deoxyglucose (2DG) significantly potentiates sorafenib toxicity. Combination of sorafenib with gossypol, imatinib and lonindamine showed little or no synergy (data not shown).

The synergetic combination of sorafenib and 2DG was demonstrated in the following cell lines: parental Huh7 cells, sorafenib resistant pool, sorafenib resistant clone A7 and Hep3B (Figure 2). Interestingly, 2DG treatment alone had very low toxicity, however, the combination of sorafenib and 2DG drastically inhibited cell growth. The degree of synergy between sorafenib and 2DG was quantified using the widely accepted Chou-Talalay combination index method.<sup>13</sup> A combination index value

**Table 1: Repurposed Anti-Glycolytic Therapeutics**

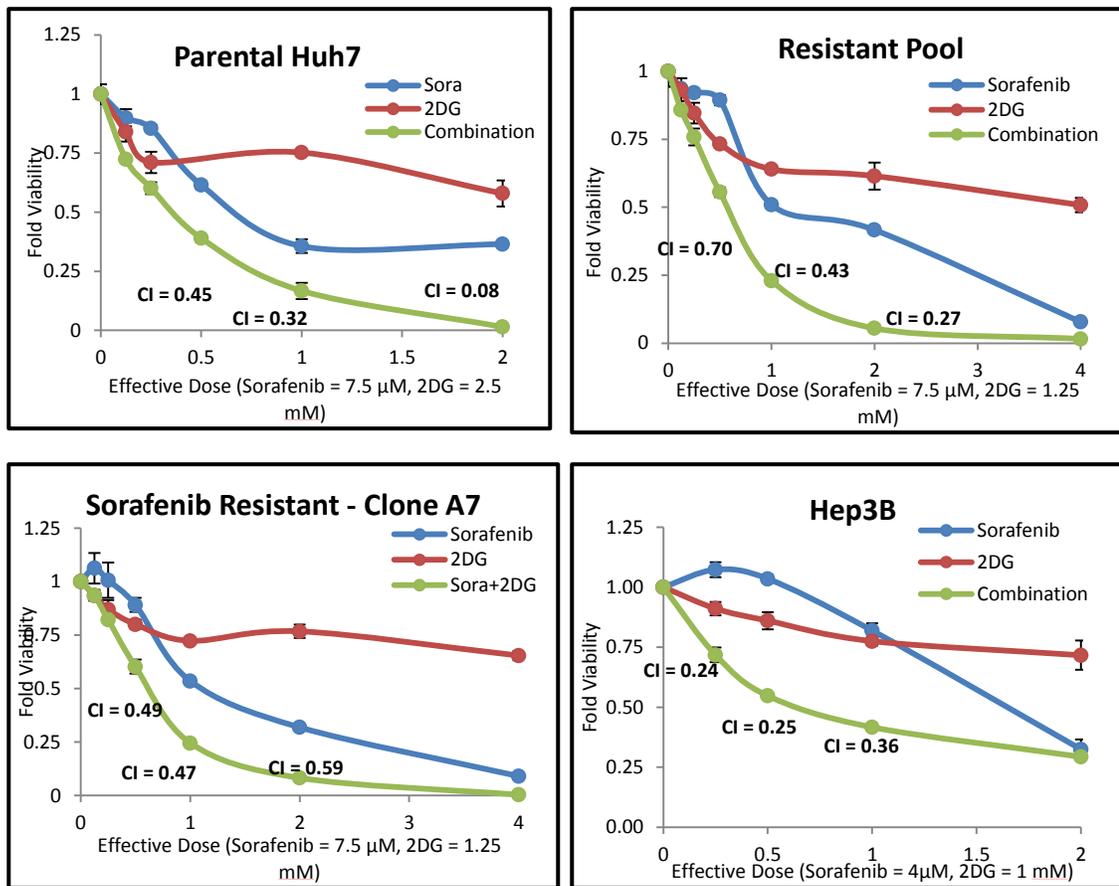
<b>Drug Name</b>	<b>Anti-glycolytic Mechanism</b>	<b>Development Stage</b>	<b>Clinical Use</b>	<b>Clinical Trial Citation</b>
<i>2-Deoxyglucose</i>	Glucose Analog: Hexokinase Inhibition	Phase II	Prostate Cancer	14
<i>Lonidamine</i>	Hexokinase Inhibition	Phase III	Prostate Hyperplasia	15
<i>Gossypol</i>	GAPDH Inhibition	Phase II/III	Lung Cancer	16
<i>Imatinib</i>	Hexokinase and G6PD Inhibition	FDA Approved	CML	-

of less than 1 indicates that the drugs are acting synergistically; a lower CI value indicates a greater degree of synergy. Several of the key CI values for the combination of sorafenib and 2DG were less than 1, quantitatively demonstrating synergy. These experiments demonstrate that the combination of sorafenib and 2DG may be a more effective than sorafenib alone

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## Combination of Sorafenib and 2DG Inhibits Colony Growth

In order to further validate the synergistic combination of sorafenib and 2DG, colony formation assays were performed in parental and resistant huh7 cell lines (Figure 3 A, B, C). Sorafenib treatment alone showed a dose-dependent inhibition of colony formation while 2DG had little effect on colony formation in all cell lines. However, the



**Figure 2:** The combination of Sorafenib and 2-deoxyglucose displays synergy.

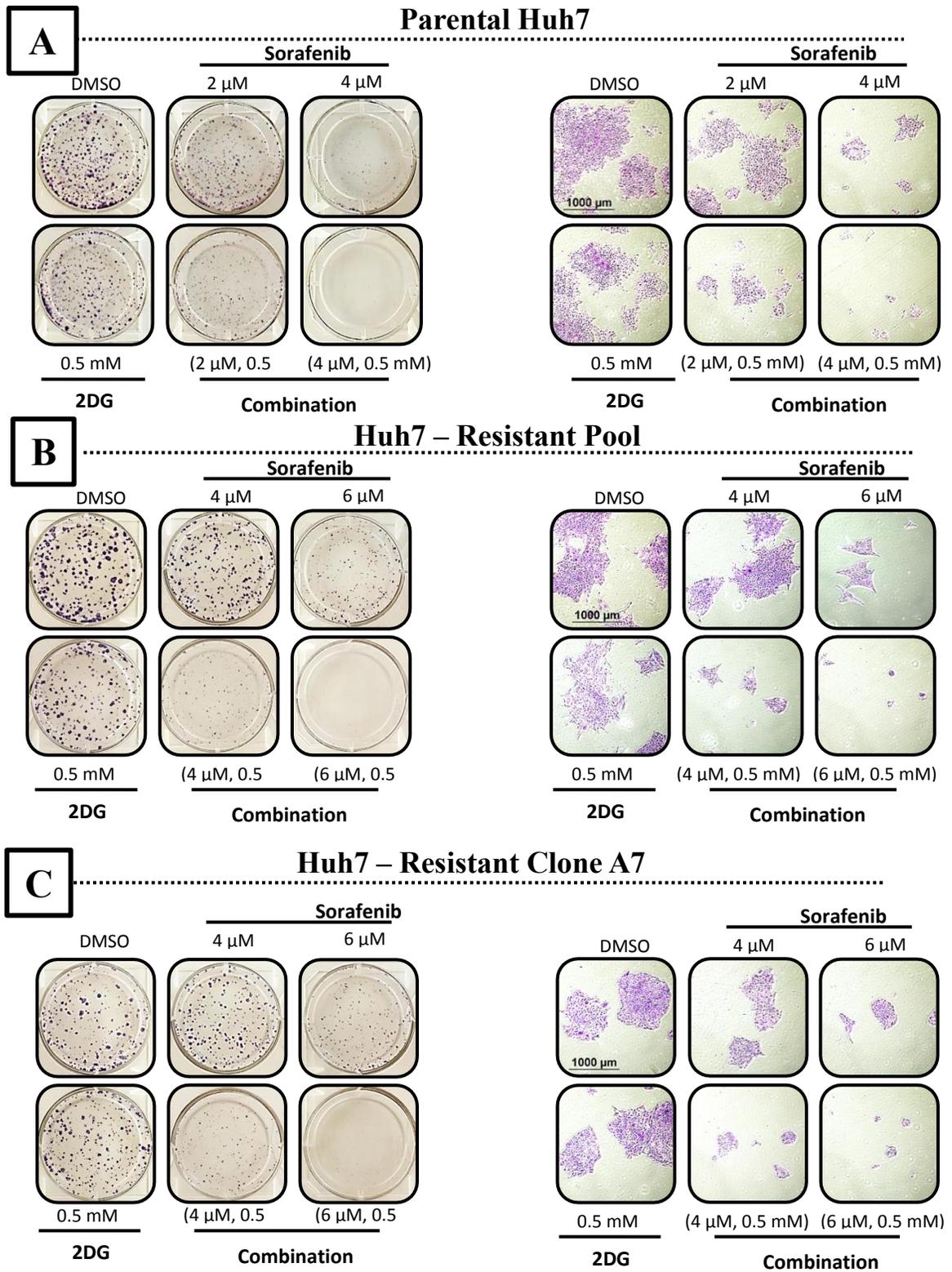
combination of 2DG and sorafenib displayed a drastic inhibition of colony formation compared to independent drug treatments. Further investigation demonstrated that the number of colonies between all treatments was similar; however, the number of cells within each colony was drastically different. Cells treated with a combination of high

dose sorafenib and 2DG formed colonies of only 1-4 cells. These tiny colonies are hardly visible upon visual inspection, but can be seen under microscopic imaging (Figures 3 A, B, C). These data suggest that the combination of sorafenib and 2DG results in a drastic inhibition of cell growth, not cell death.

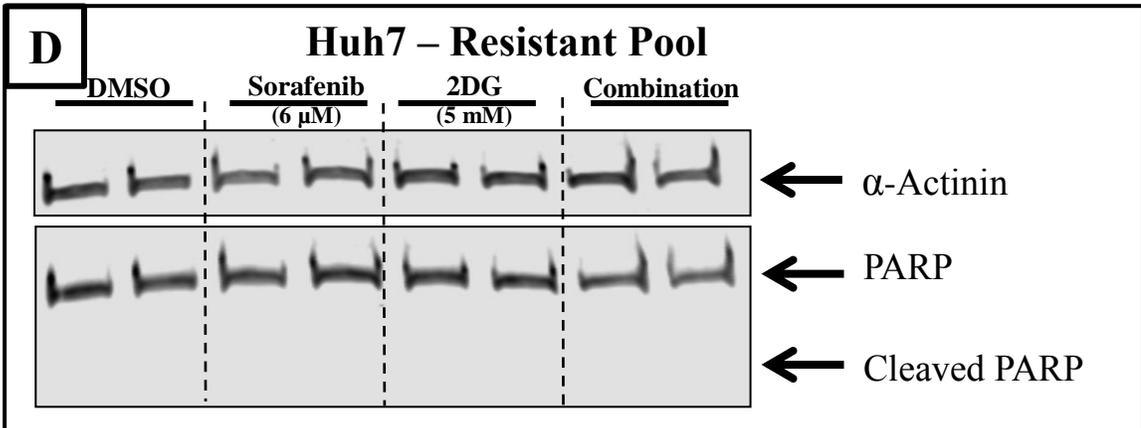
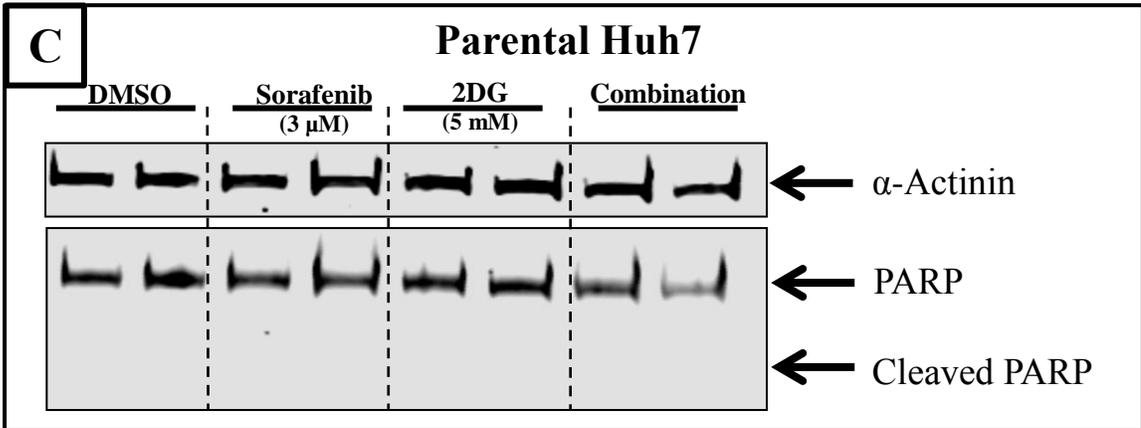
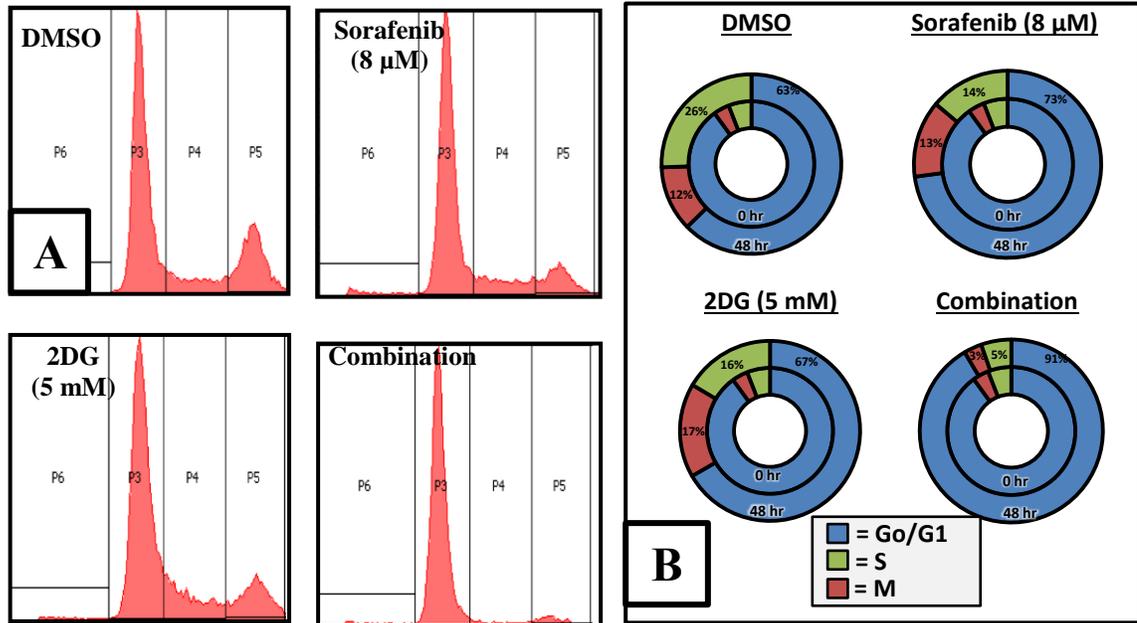
#### Combination Therapy Inhibits Cell Cycle Progression

Next, we sought to investigate the mechanism driving the synergy between sorafenib and 2DG. Based on our initial findings from the colony formation assay, we hypothesized that the combination of sorafenib and 2DG potently induces cell cycle arrest in HCC cells. In order to investigate this hypothesis, we performed a cell cycle analysis of sorafenib resistant Huh7 cells treated with sorafenib alone, 2DG alone or in combination. Cells were synchronized overnight and then treated with sorafenib, 2DG or the combination of both for 48 hours. After treatment, cells were stained with propidium iodide and analyzed via flow cytometry. Cells treated with the combination of sorafenib and 2DG demonstrated complete cell G<sub>0</sub>/G<sub>1</sub> arrest, while independent treatments showed only minor cell cycle arrest (Figure 4 A, B). Additionally, very few apoptotic cells were observed in all of the treatment groups.

In order to confirm the lack of apoptosis observed in the cell cycle analysis, western blot analysis was utilized to investigate PARP cleavage. Parental and resistant Huh7 cells were treated for 48 hours with sorafenib, 2DG or a combination of both for 48 hours. No PARP cleavage was observed in any of the treatment groups (Figure 4 C, D). These data further confirm that the synergistic combination of sorafenib and 2DG results in a strong inhibition of HCC cell growth, not cell death. However, the molecular mechanisms driving this cell cycle inhibition was still unclear.



**Figure 3:** Colony formation is inhibited by the combination of sorafenib and 2DG

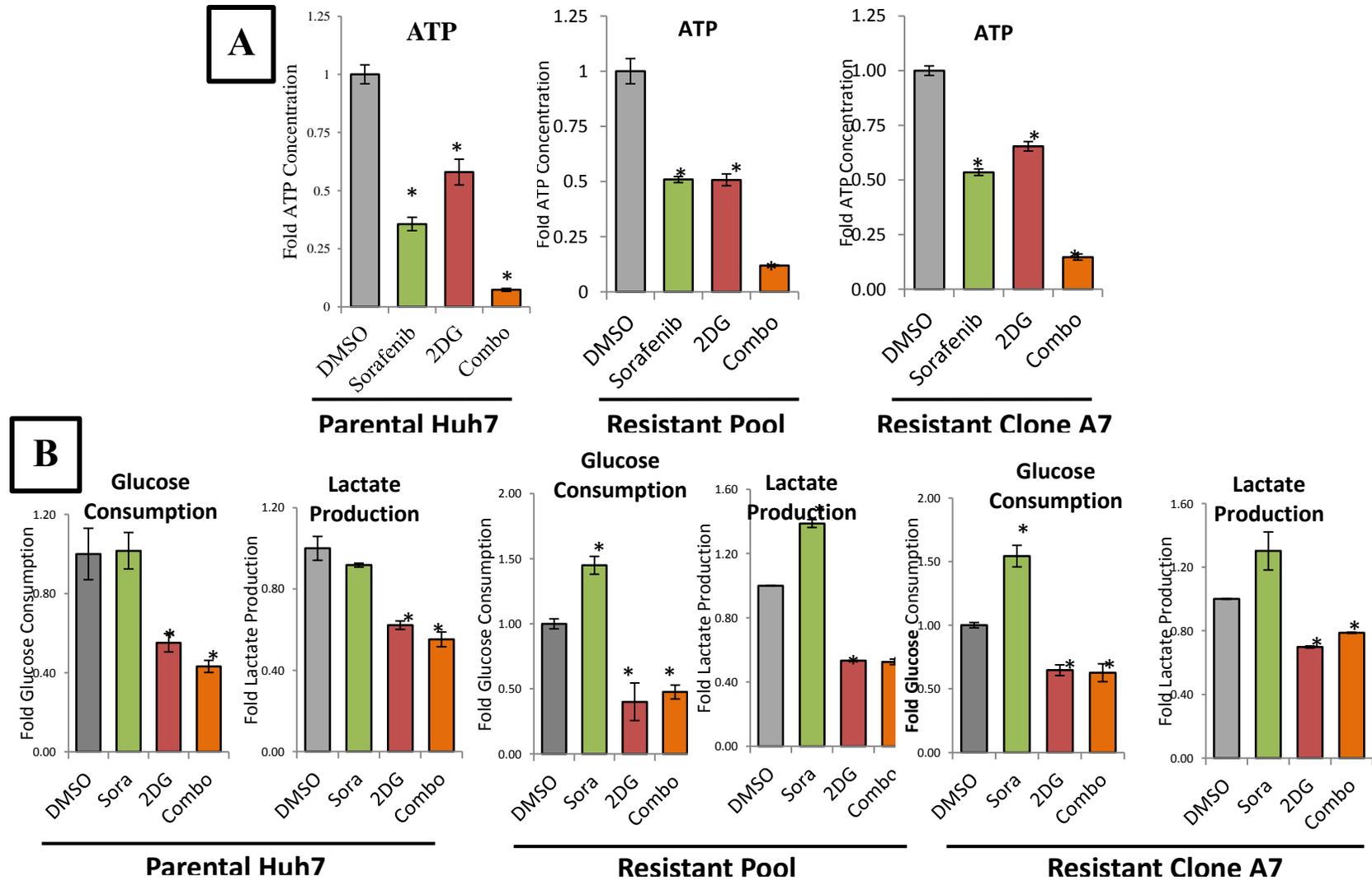


**Figure 4:** The combination of sorafenib and 2DG induces cell cycle arrest in HCC cells

## The Combination of Sorafenib and 2DG Depletes Cellular Energy

We hypothesized that combination therapy induced cell cycle arrest was the result of cellular energy depletion. In order to investigate this potential mechanism, ATP levels were measured in cells treated with sorafenib and 2DG alone and in combination. HCC cells treated with the combination of sorafenib and 2DG contained minimal ATP levels compared to untreated, sorafenib treated and 2DG treated cells (Figure 5A). This drastic reduction of cellular-ATP was observed in parental and sorafenib resistant HCC cells. This extreme depletion of cellular energy could be the primary mechanism driving combination therapy induced cell cycle arrest.

We next sought to determine how independent 2DG treatment and combination treatment with sorafenib affects glycolytic flux in HCC cells. Parental and sorafenib resistant cells were treated with sorafenib, 2DG and a combination of both. After 48 hours, the cell media supernatant was collected and analyzed for glucose and L-lactate concentrations. The results demonstrates that treatment with 2DG and the combination of 2DG + sorafenib drastically decreased glucose consumption and lactate production in all HCC cell lines (Figure 5 B). In the resistant Pool and Clone A7 cell lines, combination therapy prevented sorafenib induced increase in glucose consumption and lactate production (Figure 5B). The attenuation of glycolytic metabolism may be a key mechanism driving the synergy of sorafenib + 2DG combination therapy.



### Chapter 3: Discussion

There is an urgent need to develop novel therapeutic strategies to extend the lives of patients with advanced HCC. Currently, there is only one FDA approved therapy for these patients, sorafenib. However, sorafenib has only been shown to extend the overall survival of HCC patients by 2.8 months compared to untreated patients.<sup>3</sup> This lack of clinical efficacy is attributed to HCC tumors exhibiting an intrinsic resistance to sorafenib.<sup>4</sup> In order to understand the mechanisms driving sorafenib resistance, we developed sorafenib resistant HCC cell lines (Figure 1 A). Our initial studies demonstrated that sorafenib resistant cells display increased rates of glycolytic flux compared to non-resistant parental cells when treated with sorafenib (Figure 1 B, C). Based on this observation, we hypothesized that the combining sorafenib with repurposed anti-glycolytic therapeutics would sensitize HCC cells to sorafenib toxicity. After screening several repurposed anti-glycolytic drug combinations, the combination of sorafenib and 2-deoxyglucose (2DG) was identified as the most synergistic therapeutic combination. The combination of 2DG and sorafenib drastically inhibited cell growth in resistant and non-resistant cells (Figure 2).

Our studies demonstrated that the combination of 2DG and sorafenib drastically reduced colony formation (Figure 3) and potently induced G<sub>0</sub>/G<sub>1</sub> cell cycle arrest in sorafenib resistant HCC cells (Figure 4 A,B). Furthermore, combination therapy did not induce apoptosis in parental or sorafenib resistant HCC cells (Figure 4 C,D). These data

suggest that the primary mechanism driving the synergy of sorafenib and 2DG is cell cycle arrest. However, the exact molecular mechanism of this cell cycle arrest is still unclear.

A previous study by Maher, et.al. has showed that the treatment of osteosarcoma cells with 2DG in hypoxic conditions resulted in cell cycle arrest. The mechanism behind this growth inhibition was attributed to the inhibition of ATP and macromolecule synthesis.<sup>17</sup>

We hypothesized that the depletion of cellular ATP could be the mechanism driving the combination therapy induced cell cycle arrest observed in our studies. However, unlike the study by Maher et.al, our experiments were not conducted under hypoxic conditions.<sup>17</sup> We believe that sorafenib treatment mimics the effects of hypoxia by inhibiting oxidative phosphorylation and stimulating aerobic glycolysis. It has been shown that clinically relevant levels of sorafenib impair mitochondrial function in rat heart cells.<sup>18</sup> Additionally, another study demonstrated that sorafenib treatment hinders oxidative phosphorylation and increases aerobic glycolysis in human HCC cell lines.<sup>10</sup> Together, these data suggest that the synergy observed between sorafenib and 2DG is due to the inhibition of oxidative phosphorylation by sorafenib and the inhibition of glycolytic flux by 2DG which ultimately results in the depletion of cellular energy.

This hypothesis is strengthened by our observation that the combination of 2DG and sorafenib significantly depletes cellular ATP level compared to independent drug treatments and untreated cells. Additionally, we demonstrated that combination therapy with 2DG and sorafenib prevents sorafenib-induced stimulation of glycolytic flux. HCC cells treated the combination of sorafenib and 2DG displayed the equivalent levels of

glucose consumption and lactate production as cells treated with 2DG alone. This suggests that 2DG treatment sets a firm limit on the rate of glycolytic flux of HCC cells.

Although the sorafenib resistant cell lines are a useful tool, they do not accurately model HCC tumors seen in human patients. It is unclear whether the sorafenib induced increase of glycolytic flux observed in our *in vitro* studies also occurs in human patients. A recent study sought to investigate the mechanisms of sorafenib resistances in human patients thorough proteomic analysis of a HCC tumor before and during sorafenib therapy. This study revealed the HCC tumor proteome exhibited a large enrichment of glycolytic enzymes during sorafenib treatment. However, it is unclear whether these changes are due to sorafenib therapy or tumor progression.<sup>19</sup> In order to better model human HCC patients, our future studies will aim to extend the therapeutic combination of sorafenib and 2DG to a subcutaneous xenograft mouse model.

It is noteworthy to mention that Threshold Pharmaceuticals was granted a patent on 2005 for the exclusive right to use 2DG in combination therapy for the treatment of cancer.<sup>20</sup> This patent specifically covers use of 2DG with tyrosine kinase inhibitors. This means that any clinical trial for the combination of 2DG and sorafenib would need to have the exclusive permission and support and Threshold Pharmaceuticals. Typically, utility patents grant intellectual property protection for 20 years. However, this patent recently expired in 2014 because Threshold Pharmaceuticals failed pay the required patent maintenance fees. This means that the therapeutic combination of 2DG and sorafenib can now be utilized by other companies and clinicians without infringing on intellectual property rights.

In conclusion, we have demonstrated that the therapeutic combination of sorafenib and 2DG demonstrates remarkable synergy in sorafenib resistant and non-resistant HCC cell lines. The synergy of 2DG with sorafenib was much greater than other repurposed anti-glycolytic therapeutics examined in this study. We observed that mechanism driving this synergy was the drastic inhibition of cell cycle progression. Although the exact molecular mechanism driving this cell cycle inhibition is unclear, we hypothesize it is due to the depletion of cellular energy. Our future studies will extend this synergistic combination to a subcutaneous xenograft mouse model. If successful, this therapeutic combination has the potential to move into human clinical trials. It is clear that independent sorafenib therapy has limited efficacy in human patients. As a research community, we should continue to seek out novel therapeutic strategies to extend the lives of HCC patients.

## Chapter 4: Materials and Methods

### Reagents and Antibodies

Sorafenib (catalog #S-8502) was purchased from LC Laboratories (Woburn, MA). 2-Deoxy-D-glucose (catalog #D6134) was purchased from Sigma Aldrich (St. Louis, MO). Antibodies used for western blotting were purchased from Cell Signaling (Danvers, MA).

### Cell Culture

All cells were maintained in Minimum Essential Media supplemented with L-glutamine (2 mM), 10% FBS, sodium pyruvate (0.11 g/L) and penicillin/streptomycin (100 U/mL). Cell media for sorafenib resistant cell lines was also supplemented with sorafenib (6 $\mu$ M and 0.1% DMSO). Sorafenib was withdrawn from the cell media of resistant Huh7 cells for 5-7 days prior performing all experiments.

The HCC cell lines Huh7 and Hep3B were obtained from the ATCC. In this paper, “parental huh7 cells” refer to the Huh7 cells obtained from the ATCC. Sorafenib resistant “pool” and “clone A7” huh7 cells were generated in our laboratory. In order to generate sorafenib resistant “pool” huh7 cells, parental huh7 cells were grown in MEM media with a low concentration of sorafenib. Media sorafenib concentration was slowly increased to a final concentration of 6  $\mu$ M after several months. Several individual colonies were

isolated from the resistant “pool” huh7 cells. The “clone A7” cell line is derived from one of the individually isolated “pool” colonies.

#### Cell Viability and ATP Assays

Cells were seeded into Eppendorf 96 well plates (~2,000 cells/well) and allowed to attach overnight. Cell media was then changed for media containing sorafenib or other therapeutics + 1% DMSO. After 48 hours of incubation the CellTiter-Glo® was added following the manufacture’s protocol (Promega: Madison, WI). The luminescent supernatant was transferred to an opaque luminometer 96-well plate prior to measuring luminescence. The same procedure was followed for the ATP measurement assay.

#### Glucose Consumption and Lactate Production

Cells were seeded into 6 well plates (50% confluency) and allowed to attached overnight. Cells were then treated for 48 hours with phenol-red free DMEM media containing therapeutics and 1% DMSO. After 4 hours, cell media supernatant was removed and analyzed for glucose and lactate concentrations.

Media glucose concentration was measured using a ReliOn® ULTIMA glucometer (Alameda, CA). Cell media was diluted 1:1 with PBS prior to glucose measurement in order to be within the linear range of the instrument. Glucose concentrations were compared to “fresh” media that was not exposed to cellular metabolism. Glucose consumption was determined by subtracting the cellular glucose concentration from that of the fresh media. It is worth noting that 2-deoxyglucose is detected by the glucometer at the same sensitivity was D-glucose. Therefore, media containing 2-deoxyglucose was

compared to fresh media containing the same initial concentration of 2-deoxyglucose. The glucose consumption measured by these cells is equivalent to D-glucose + 2-deoxyglucose consumption. With this method, it is not possible to distinguish D-glucose consumption from 2-deoxyglucose consumption.

Media lactate concentrations were measured using the L-Lactate Assay kit from ScienCell (Carlsbad, CA). Cell media was diluted 1:30 with the kit assay buffer prior to measurement in order to be within the linear measurement range. The assay was conducted following the manufacturer's recommendations.

#### Colony Formation Assay

Cells were seeded into 6 well plates (2,000-5,000 cells/well) and allowed to attach for 24-48 hours. Cells were then treated with a continuous dose of therapeutics + 1% DMSO for 14 to 18 days. Media was changed every three days. After colonies were of sufficient size, the cells were fixed with 3.7% paraformaldehyde (in PBS). Cells were then stained with a 0.05% crystal violet solution and imaged.

#### Flow Cytometry

Cells were seeded into 6 well plates (50% confluency) and allowed to attach overnight. Cells were then treated for 48 hours with therapeutics and 1% DMSO. After 48 hours, cells were collected via trypsinization and fixed in 75% Ethanol. After washing, cells were stained with a solution PI (0.5 mg/mL) and RNase A (10 mg/mL). Cells were filtered through a 70  $\mu$ M cell strainer immediately prior to flow cytometry.

Flow cytometry was conducted by the Ohio State University Comprehensive Cancer Center Analytical Cytometry Core Facility on a BD LSR II (San Jose, CA).

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