Thesis

Response of liver cancer-derived cells to starvation in combination with mitochondrial electron transport chain inhibitors

written by

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to earn the

Doktor(in) der gesamten Heilkunde

(Dr. med. univ.)

at the

Medical University of Graz

elaborated at the

Division of Cell Biology, Histology and Embryology

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Graz, 29.08.2023

Statutory Declaration

I declare on my honor, that I have authored this thesis independently and without outside help, that I have not used other than the declared sources, and that I have explicitly marked all material, which has been quoted either literally or by content from the used sources.

Graz, 29.08.2023 ……

(Emir Jagodić eh.)

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List of Abbreviations

ADP = adenosine diphosphate

AMP = adenosine monophosphate

AMPK = adenosine 5´-monophosphate-activated-protein-kinase

ANOVA = analysis of variance

ATP = adenosine triphosphate

Caco-2 = cancer coli 2

cMYC = cellular Myelocystomatosis

CTNNB1 = beta-1 catenin

DMEM = Dulbecco's Modified Eagle Medium

e.g. = exempli gratia

EO9 = EOquin

ETC = electron transport chain

FADH = flavin adenine dinucleotide

FCS = fetal calf serum

Gluc = glucose

Glut = glutamate

GM = growth medium

GSH = glutathione

HBSS = Hanks's Balanced Salt Solution

HBV = hepatitis B virus

HCC = hepatocellular carcinoma

HCT116 = human colon cancer cell line

HCV = hepatitis C virus

Hep3B = hepatoblastoma cell line 3B

HepG2 = hepatoblastoma cell line G2

HIF = hypoxia inducible factor

HuH6 = human hepatoma 6 cells

IRS = insulin receptor substrate

IGF-1R = insulin-like growth factor 1 receptor

LKB1 = liver kinase B1

mtDNA = mitochondrial deoxyribonucleic acid

mIBG = meta-iodobenzylguanidine

mTOR = mammalian target of Rifampicin

NADH = nicotinamide adenine dinucleotide hydrogen

NADPH = nicotinamide adenine dinucleotide phosphate hydrogen

OCR = oxygen consumption rate

OXPHOS = oxidative phosphorylation

PBS = phosphate buffer saline

PDGFR = platelet derived growth factor

Pfn = phenformin

P/S = penicillin-streptomycin

PPP = pentose phosphate pathway

PI3K = phosphoinositide 3-kinase

Raf = rapidly accelerated fibrosarcoma

Ras = rat sarcoma

REDD1 = regulated in development and DNA damage response 1

SM = starvation medium

TCA = tricarboxylic acid cycle

TERT = telomerase reverse transcriptase

TSC2 = tuberous sclerosis complex 2

VEGFR = vascular endothelial growth factor receptor

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Zusammenfassung

Leberkrebs ist an dritter Stelle der krebsbedingten Todesursachen und mit über 900.000 Neuerkrankungen an sechster Stelle der malignen Erkrankungen weltweit. Aufgrund der vielen unterschiedlichen Ätiologien zur Entstehung der am weit verbreitetsten Leberkrebsart, nämlich des Hepatozellulären Karzinoms (HCC) macht eine Therapie oft schwierig. Abhängig vom HCC-Stadium wird nach der S3-Leitlinie eine stadiengerechte Therapie eingeleitet. Falls keine operative Resektion bzw. Transplantation des Organs erfolgen kann, blieb lange Zeit nur eine medikamentöse Therapie mit Sorafenib und im späteren Stadium Regorafenib. Die medikamentöse Therapie ist aufgrund von Resistenzbildungen eine rein palliative Therapie und verlängert das Leben der zu behandelnden Person nur um wenige Monate. Aufgrund des veränderten Metabolismus in Form einer erhöhten Wachstumsrate und somit einem verbundenen gesteigertem Nährstoffbedarf von Krebszellen macht ein Nährstoffentzug sie angreifbarer gegenüber Chemotherapeutika. Eine Kombination von Sorafenib und Nährstoffentzug könnte somit die Behandlung vom HCC beim Menschen verbessern. Die Hypothese lautet, dass durch eine gleichzeitige Unterdrückung der aeroben Glykolyse sowie der oxidativen Phosphorylierung (OXPHOS)), das Wachstum von HCC-Zellen eingeschränkt werden kann. Durch vorangegangene Experimente der Forschungsgruppe Prokesch wurde diese Annahme erhärtet, da man herausfand, dass durch ein eingeschränktes Angebot von Nährstoffen in Form von Fastintervallen die Wirkung von Sorafenib verstärkt wird. Es wurde gezeigt, dass Sorafenib als Multikinase-Inhibitor auch einen Effekt auf die mitochondriale Elektronentransportkette hat und somit die Energiegewinnung über die OXPHOS beeinflusst.

Die Ziele dieser Diplomarbeit waren: (1) Testen von zusätzlichen OXPHOS Inhibitoren in Kombination mit Fast-Intervallen an Leberkrebszelllinien (2) Herausfinden ab welcher Glucosekonzentration die Sensibilisierung auf den Inhibitor aufgehoben wird.

Abstract

Liver cell-derived cancers rank third in terms of cancer-associated deaths, and with over 900.000 new cases per year, the sixth most common cancer worldwide. Due to the many different etiologies that lead to the development of the leading variant of liver neoplasm, hepatocellular carcinoma (HCC), the therapy is often very difficult. Depending on the HCC stage, therapy is initiated according to the S3 guideline. If the organ cannot be resected or transplanted by surgery, drug therapy with sorafenib and, in the later stage, regorafenib were for years the only remaining options. Unfortunately, drug therapy frequently can be considered as palliative since resistance development restricts the extension of patient's life to only a few months.

Due to the rewired metabolism resulting from increased growth rate and thus an increased need for nutrients, cancer cells might be more susceptible to fasting. Previous experiments by the Prokesch research group confirmed this assumption, as they found that fasting increases the effects of sorafenib or even sensitizes resistant liver cancer cells to the drug. Mechanistically, sorafenib, primarily used as a multikinase inhibitor, also inhibits the mitochondrial electron transport chain and oxidative phosphorylation (OXPHOS). Our hypothesis was that by suppressing both arms of the rewired metabolism (aerobic glycolysis and oxidative phosphorylation) the growth of HCC cells can be restricted.

The goals of this thesis were: (1) to test additional OXPHOS inhibitors in combination with fasting in liver cancer cell lines; (2) to investigate whether glucose is the crucial metabolite whose depletion results in the sensitization to the inhibitor.

1 Introduction

1.1 Hepatocellular carcinoma

Liver cancer is, with over 900.000 new cases per year, the sixth most deadly cancer worldwide (1). The data from 2020 shows that of 905.677 with liver cancer diagnosed patients, 830.180 died from the disease. According to this data, liver cancer shows a death rate of 92%. Data from the World Health Organization shows that liver cancer causes 8,3% of all cancer-related deaths globally, which makes it the third most significant cause of such deaths among various types of cancer (1).

With 90% of all cases, hepatocellular carcinoma (HCC) is the most prevalent primary liver tumor (2). The primary etiological factors for developing HCC are: viral hepatitis (hepatitis B and hepatitis C), Aflatoxin B1, and alcoholic liver diseases, such as non-alcoholic fatty liver disease. The development of cirrhosis is a significant stage in the progression of HCC. In the case of hepatitis, the viral genome is integrated into the genome of the host which leads to oncogenesis (2). About 60% of HCC cases are a result of mutation in the TERT (telomerase reverse transcriptase) promoter site of human genome by inserting of viral genome. There are additional known carcinogenic genetic mutations, for examle in *TP53* and beta-1 catenin (CTNNB1) (2). HCC can also be caused by chronic inflammation caused by chronic hepatitis C virus infection or by inreased alcohol intake, followed by fibrosis, necrosis and dysplasia. For simplification we can say there are two ways of HCC genesis: 1) Active modification of the hepatocellular genome via HBV and 2) The inflammatory way which results in cellular stress like in HCV infection or excessive alcohol consumption. Of course, these effects overlap and there is no clean separation line between them (2).

The HCC development is complex and a multistep process wich shows characteristic histological changes (3). At first, the pre-cancerous nodules develop with low-grade dysplasia, followed by formation of high-grade dysplastic nodules, wich again transform into early-stage of HCC (stages 0 and A) and, at the end become more advanced HCC (stage B,C and D) (3).

Because of its difficult early detection, 80% of the HCC diagnoses are made at an inoperable stage (2). The result of these late diagnosis is poor five-year survival of HCC patients. Those with HBV-related HCC have worse prognosis and higher relapse of the

disease. Patients who develop HCC in the background of HCV infection or extensive alcohol intake have a better five-year survival rate (2).

1.1.1 Prevention and management of hepatocellular carcinoma

The first line of prevention of an HBV infection-caused HCC is HBV vaccination, which is anchored in the Austrian vaccination plan and given neonatal (4). There is no vaccination available to prevent HCV infection, so the spread of the virus must be prevented through education and sterile work practices. The most effective strategy to prevent HCC development is to minimize risk factors and maintain a healthy lifestyle. To decrease cirrhosis and fatty liver-related liver injury, people should avoid excessive alcohol consumption and unhealthy eating habits that leads to obesity. Smoking and aflatoxin exposure are established risk factors and should be minimized or in best case avoided. To manage a patient with HCC it is necessary to collect information about tumor extension, patient comorbidities and the severity of liver damage. Resection is potentially curative, but the developing rate of recurrent HCC is, with 70% incidence, very high. Transplantation is, with a five-year post-transplant long-term survival of 80%, the ultimate choice for HCC treatment. For patients with early-stage HCC the percutaneous local ablation treatment is potentially curative. Transarterial embolization and radiotherapy are methods to prevent tumor progression for patients listed for liver transplantation. These treatments are most used for patients with early-stage HCC when the tumor is local, not metastasized and healing is possible. For patients with advanced HCC, sorafenib, lenvatinib and regorafenib were for years the only systemic pharmacological treatment options (4).

Since October 2020, atezolizumab a monoclonal antibody has been applied together with bevacizumab in the medical management of HCC, because of the lower side effects compared to sorafenib (5).

The survival benefits of the systemic pharmacological treatment are low because of rapid resistance development which results in patients survival extension of only 7-11 months (4). Sorafenib acts as a multi-kinase inhibitor and blocks up to 40 kinases, like KIT (mast/stem cell growth factor receptor), vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor (PDGFR) in cancer cells and their environment (4).

Other possible mechanism of action of sorafenib are targeting of the mitochondrial respiration with inhibition of complexes I, II, III and adenosine triphosphate (ATP) synthetase and following mitochondrial dysfunction (6–8). New findings indicate that sorafenib induces ferroptosis and activates HCC cell death (8,9). Further investigation is needed to find additional mechanism of action of sorafenib and to improve the HCC treatment.

1.2 Cancer metabolism

1.2.1 Warburg effect

In the early 1920s, Otto H. Warburg saw in an experiment with tumor tissue slices, the enhanced conversion of glucose to pyruvate followed by lactate formation, even under conditions of adequate oxygen supply (aerobic glycolysis, later referred to as "Warburg Effect") (10,11). Warburg attributed this switch in energy production to mitochondrial respiratory injury, which he thought is the key point in carcinogenesis. Almost 100 years later, we now have clear evidence that mitochondria are not defective in most cancers. To promote growth, proliferation, malignant progression, and long-term survival cancer cells modify their metabolism. Still, the most obvious trait of this modified metabolism is the use of aerobic glycolysis and an increased glucose uptake. The rewired metabolism can be underlined by modified growth factor signaling, through activation of *HIF-1α*-transcription, oncogene activation (e.g., PI3K/Akt/mTOR pathways, cMYC) or mutations in tumor suppressor genes (e.g., LKB1/AMPK pathway). Some of the outcomes of these changes are a) overexpression of glucose transporters and glycolytic enzymes, and faster glycolytic flux, b) high-speed ATP production to cover the higher demand for energy, c) accumulation of lactate which induces tumor progression by increasing acidosis. Nevertheless, we need a better understanding of the Warburg effect to advance the therapies against cancer which center on pharmacological intervention in cell metabolism(10,11).

1.2.2 Impact of fasting on cancer metabolism

Dysregulated metabolic processes in cancer cells contribute to an enhanced uptake of nutrients (12,13). Upregulated metabolic activity has the potential to generate and accumulate reactive oxygen species, thus promoting the development of extra mutations. For example, some cancer cells develop genetic changes in oncogenes such as IGF-1R (IGF-1 receptor), or its following pathways with Ras/Raf (GTP proteins), or in the PI3K/Akt pathway, which all lead to continuous activation of proliferation pathway, mostly unaffected by external factors (12,13). Normal mammalian cells enter a quiescent or low-proliferative state under starvation to utilize all energy resources for defending cells from multiple insults (14). This is the result of low IGF-1 levels, which lead to a reduction of intracellular mitogenic signaling cascades regulated by IGF-1R – the pathways controlled by Akt and Ras, are able to trigger cell cycle arrest by activation of p21 and p53. The stimulation of oncogenic proteins like Ras in cancer cells are able to block the access into the quiescent state during starvation, which can have a negative result on cancer cell viability under starvation (14).

1.2.3 Oxidative phosphorylation

Cells use OXPHOS to generate ATP via the electron transport chain (ETC). The ETC means the electron movement to a line of transmembrane protein complexes I to IV in the inner mitochondrial membrane (15). Electron donors such as succinate, NADH and FADH are required for the functional ETC. As a result of ETC, complexes I, II and IV, transport protons into the intermembrane space out of the mitochondrial matrix. This generates a substantial proton gradient over the membrane, which allows protons to move inside the mitochondrial matrix by crossing ATP synthase or complex V. The proton flow through ATP synthase generates ATP. At least the electron is taken up by oxygen (15).

The downregulation of OXPHOS in some cancers might be associated with genetic changes in mitochondrial DNA (mtDNA) (15). Thirteen subunits of the ETC protein complexes I to V are encoded by the mtDNA. Different cancer variations with OXPHOS downregulation are linked to unfavorable clinical response and typical for infiltrating and spreading tumors. Genome analysis of neoplastic and normal tissue samples showed tumor exclusive alterations in mtDNA. These identified alterations are expected to have an influence on OXPHOS protein activity. Neoplastic cells which contained mtDNA alterations in complex I components showed increased sensitivity to phenformin and

metformin, complex I inhibitors, in comparison to normal cells. Experiments with phenformin and xenografts, also show the anti-proliferative capacity of phenformin (15).

Some cancer types strongly rely on OXPHOS for growing, so these cells can be targeted with OXPHOS inhibitors for future medical treatment. Analysis of 31 cancer cell lines and 16 normal cell types indicated variability between cell types in the amount of ATP synthesis from OXPHOS and glycolysis. Neoplastic cells generate about 83% of their ATP from OXPHOS compared to 80% in normal cells. The mtDNA content may be the reason of variance in ATP synthesis from OXPHOS between different neoplastic cell types. For example, studies with breast cancer indicate upregulated OXPHOS. Histochemical activity tests of the transmembrane protein complexes I, II and IV in neoplastic breast tissue, demonstrated an increased ETC protein activity in neoplastic cells compared to normal breast cells. The activity of these complexes could be inhibited or reduced by sodium azide and metformin. Analysis of gene expression from patients with neoplastic breast cells pointed out that OXPHOS is upregulated on transcriptional level, so OXPHOS may be a promising target in medical therapy of neoplastic breast disease. Because of the ability of tumors to display metabolic flexibility, increased OXPHOS activity does not automatically imply dependency. Neoplastic cells which are able to switch between glycolysis and OXPHOS for ATP synthesis, could be sensitive to OXPHOS inhibitors under starvation (15).

1.2.4 Glycolysis

To meet the energy demands of rapid cell proliferation, neoplastic cell upregulate anaerobic glycolysis and increase glucose consumption (16). Cells produce 30-32 ATP molecules per each glucose molecule through the tricarboxylic acid cycle (TCA). Through anaerobic glycolysis, one glucose molecule is metabolized to two lactate molecules, generating two ATP molecules in the process. Because of the low yield of ATP production in anaerobic glycolysis, cancer cells compensate by increasing glucose uptake. Because it generates many intermediates which can also act as signaling molecules, the glycolytic pathway can serve as a potential therapeutic target (16).

Some cancer cells exhibit a glycolytic phenotype, but it remains unclear why (17). Switching the glycolytic phenotype to oxidative phenotype can induce cell death in tumor cells (18). Defective OXPHOS and mitochondrial impairment can underly the rewired metabolism of some cancer cells (17). Compared to mitochondrial OXPHOS, the ATP

output is much lower from glycolysis. Several key factors drive tumor cells to use primarily glycolysis for energy production. One factor is faster glucose consumption and ATP production, which give tumor cells a superiority for metabolism of environmental energy sources. Another factor, is the rapid demand of substrates for biosynthesis of macromolecules, which are necessary for cancer growing (19). Through the intermediate products from glycolysis, the PPP (pentose phosphate pathway) is activated and generates ribose-5-phosphat and NADPH. Ribose-5-phophat and NADPH are critical for the production of nucleic acids and lipids (17). Accumulation of NADPH supports the production of reduced forms of GSH (glutathione). GSH acts as an antioxidant and defends tumor cells from antineoplastic chemicals by maintaining the redox status. Chemoresistant tumor cells upregulate anaerobic glycolysis, which could be a metabolic connection between energy production und resistance (17).

1.3 Inhibitors of oxidative phosphorylation

1.3.1 Metformin

Metformin (1,1-Dimethylbiguanide), a derivate of biguanide, is, because of its safety and low costs, used as first-line drug therapy for patients with type 2 diabetes (20). The anti-diabetic effect of guanidine in animals was found in the early 20th century. In the 20s of the last century scientists synthesized metformin, a much safer derivate for use in humans. Since then, metformin became the most used drug for patients with type 2 diabetes because of its potential to reduce glucose levels in the blood plasma, while having low side effects. Studies showed the antiproliferative effect of metformin on various tumor types (e.g., liver cancer, bone cancer, breast cancer), improved outcome for cardiovascular disease, renal disease, obesity, liver disease and neurodegenerative disease. By inhibition of the mitochondrial complex I, the AMPK (adenosine 5′-monophosphate-activated-protein-kinase) gets stimulated by metformin. Because of the important function of mitochondrial complex I for the electron transport, the inhibition results in decreased ATP production and increased intracellular concentration of adenosine diphosphate (ADP). As a result, cellular levels of adenosine monophosphate (AMP) increase and activate AMPK. There is an indication that metformin activates AMPK also through the lysosomal cascade. The impact of AMPK in metabolic pathways is numerous (e.g., on lipid and glucose metabolism). The suppressing of IGF receptor and insulin signaling by metformin, changes the metabolic homeostasis in cells. Studies showed that metformin by itself change one protein level cellular mechanisms like aging and energy metabolism. All pathways associated with metformin are still not fully understood and need more future investigation (20).

1.3.2 Phenformin

Ciba-Geigy and Grünenthal developed in the late 1950s phenformin (phenformin hydrochloride), a more lipophilic biguanide than metformin (21). The primary use was as an antidiabetic drug until the end of the 1970s, when it was taken off the market, because there have been cases of fatal lactic acidosis. Due to its lipophilic structure, phenformin displays a significant preference for the inner mitochondrial membrane, which obstruct the OXPHOS and trigger lactic acidosis. Phenformin causes 20 times more lactic acidosis than metformin. Overall, in all tested tumor cells (e.g., colon, breast, prostate cancer) phenformin is in comparison to metformin a stronger inhibitor of tumor cell proliferation

in vitro. Because of its lipophilic characteristics phenformin can easily cross the cellular membrane, block complex I, suppress IRS (insulin-receptor-substrate) receptor and stimulates REDD1 protein activity. The result of these mechanisms is activation of AMPK and blocking of the mTOR pathway by tuberous sclerosis complex 2 (TSC2 protein). The results are genetic damage, cell cycle inhibition, suppression of protein synthesis and decreased cell survival (21).

1.3.3 Meta-iodobenzylguanidine

Meta-iodobenzylguanidine (mIBG) is an analog of the neurotransmitter norepinephrine with antitumor activity. Clinically, mIBG is used as radioiodinated Iobenguane sulfate as a tumor-targeted radiopharmaceutical in diagnosis and treatment of adrenergic tumors (22).

In the late 1970s Wieland et al developed mIBG for imaging adrenal medullary tissue and associated neoplastic tumors (e.g., pheochromocytomas) (23). The uptake of ^{131}I-mIBG (radiolabeled mIBG) into cells takes place via the uptake-1 system. Norepinephrine transporter is necessary for the uptake-1 system. MIBG is chemical similar to norepinephrine and so able to pass through the norepinephrine transporter into mitochondria and cytoplasm (23).

MIBG inhibits cell growth of human and mouse leukemia, fibrosarcoma, melanoma, and neuroblastoma cell lines (22). It's known that mIBG is a potent affector of endogenous mono-ADP-ribosyl-transferase of erythrocyte membranes (22). By inhibition of the mitochondrial complex I and complex III activity of the ETC could possibly be one of the main antitumor activity mechanism of mIBG (15,24).

1.3.4 IM156

IM156 (HL156A) is chemically similar to metformin and a powerful stimulator of AMPK (25). Through suppression of mitochondrial complex I, IM156 inhibits OXPHOS and enhances cell death. In comparison to Metformin, IM156 is 60 times more potent in cellular AMPK phosphorylation. The main distribution of IM156 is in the lung, liver, heart and kidney, where its effect is not just the AMPK activation than also OXPHOS modulation. Other effects of IM156 are increasing of lipolysis, glycolysis, amino acids and β-oxidation, and decreasing of TCA cycle activity, protein synthesis and free fatty acids. Studies with murine bleomycin model of pulmonary fibrosis in combination with regular intake of IM156 shows a reduction of lung fibrosis and inflammatory cell infiltration (25).

IM156 is more hydrophobic than metformin and phenformin and, therefore, more bioavailable to cells (26). Metformin, phenformin and IM156 treatments on *Myc*-dependent mouse lymphoma cells (*Eμ-Myc* cells) show a greater decreasing of oxygen consumption rate (OCR) by IM156. By greater reduction of cellular ATP production at equal concentrations, IM156 has a stronger impact on OXPHOS than other biguanides. These data suggest that IM156 is a more potent inhibitor of mitochondrial respiration than metformin and phenformin (26).

1.4 Aims of the thesis

Due to the limited therapeutic options for advanced HCC, further therapeutic strategies are necessary. Based on previous data of our workgroup which showed that starvation can sensitize HCC to an OXPHOS inhibitor (27), we wondered if there are other approved drugs which act as OXPHOS inhibitors which could also have an impact on the HCC cell viability under limited glucose availability.

The first aim was to test which concentrations of OXPHOS inhibitors metformin, phenformin, mIBG and IM156 influence the cell viability of HepG2, Hep3B and Huh6 cell lines under growth and starvation medium.

For further adaptation of the treatment, it is important to know which glucose concentration is effectively causing cell sensitization, to obtain the best therapeutic output. For this, our second aim was to apply OXPHOS inhibitors in combination with starvation medium and different glucose concentrations in the glucose add-back experiments.

2 Materials and Methods

2.1 Cell culture

In the table below the experimental used HCC-derived cell lines are represented (*Table 1*).

Table 1: Cell culture: cell lines

Cell line	Passages	Medium
HepG2	30 - 38	DMEM [+] 4.5 g/L glucose [+] 4 mM L-glutamine [+] 10% FCS [+] 1% P/S
Hep3B	10 - 30	
Huh6	33 - 42	

Once thawed from nitrogen-storage (- 196°C), the HCC-derived cell lines were cultured in growth medium (GM) comprising DMEM (Dulbecco's Modified Eagle Medium, *Gibco*) with 500 U/mL P/S (penicillin-streptomycin, stock: 10.000 U/mL, *Gibco*) ,4 mM L-Glutamine, 4.5 g/L L-Glucose (high glucose), and 10% FCS (fetal bovine serum, *GE Healthcare Life Sciences*) at 37°C in humidified atmosphere with 5% CO_2.

For cell cultivation we used T75 flasks (75 cm^2 cell culture flasks from *Thermo Fisher scientific*). Once the cells reached approximately 80% flask coverage, they were passaged to new culture flasks. To perform this, the GM was aspirated, and the cells were washed using phosphate buffer saline (PBS, *Gibco*). Subsequently, 3 mL of Trypsin-EDTA (10x, *Biowest*) diluted 1:10 in PBS were added to detach the cells, followed by incubation for 5 min at 37°C and 5% CO_2.

By adding medium (twice the amount of Trypsin-EDTA or 6ml), the cells were homogenized by pipetting. The entire cell suspension was then distributed among newly prepared flasks. For adequate cell growth, 2 x 10^6 cells were used per flask, with a total volume of 10-15 mL of cell suspension and GM.

2.2 Viability assay

To perform the viability assay, the following cell numbers were seeded per well in a 96well plate: $2x10^5$ HepG2, $65x10^3$ Hep3B and $40x10^3$Huh6 cells. The seeding was done using 200 µL of GM per well. The cells were than incubated at 37°C and 5% CO_2 for 24 hours.

After an incubation period of 24 hours, the culture medium was removed, and the cells were washed using PBS. Following this, the cells were exposed to various media formulations containing the components listed in *Table 2*, with different compositions. Additionally, the cells were treated with various concentrations of metformin, phenformin, IM156 and mIBG

Table 2: Viability assay: medium compositions

Name	Additives/Concentration	Manufacturer
DMEM high glucose [+] phenol red	[+] D-Glucose (4500 mg/L) [+] L-Glutamine (4mM) [+] Sodium Pyruvate (110 mg/L)	*Gibco*
HBSS++ with Hepes	[+] $CaCl_2$ [+] $MgCl_2$ 10mM Hepes	
DMEM -/-/- [-] phenol red	[-] D-Glucose [-] L-Glutamine [-] Sodium Pyruvate	
sodium pyruvate	100mM	
glutamine	200mM	
FCS	10%	
D-glucose	450 g/L	*Sigma Aldrich*

In the subsequent sections, the growth medium (GM) refers to DMEM supplemented with 1% P/S and 10% FCS, starvation medium 1 (SM 1) corresponds to HBSS supplemented with 0.01 M Hepes (1 M stock, *Gibco*).

Starvation medium 2 (SM 2) represents DMEM-/-/-[-] phenol-red.Depending on the experiment, the cells were cultured in presence of inhibitors (*Table 3*).

Table 3: Viability assay: used inhibitor stock concentrations diluted in DMSO

Name	Stock concentration	Manufacturer
Metformin	200 mM	*MedChemExpress USA*
Phenformin	10 mM	
mIBG	10 mM	
IM156	10 mM	*Selleck Chemicals*

Through the EZ4U assay (*Biomedica Immunoassays)*, cell viability was assessed. EZ4U is a nonradioactive cell proliferation and cytotoxicity assay. The preservation of intact mitochondria and their active mitochondrial reductase in living cells is necessary for the assay. Yellow -colored tetrazolium salt is used as reagent.

The reduction of tetrazolium salt to its red formazan derivate occurs in viable cells. The change in color can be quantified using photometry. After 24 hours of treatment, the media was removed and 200 µL of GM along with 20 µL of EZ4U solution were added to each well. Following two hours, the absorbance was measured using Spark® 10M multimode microplate reader at 492 nm (reference-wavelength 620 nm).

3 Results

3.1 Dose-response to OXPHOS inhibitors in growth and starvation conditions

3.1.1 Treatment of HCC-derived cell lines with OXPHOS inhibitors in growth and starvation conditions

To determine the effective concentrations of OXPHOS inhibitors (Metformin, Phenformin, IM156 and mIBG), we treated the cells under high glucose conditions (growth medium, GM) and low/no glucose conditions (starvation medium, SM). The rationale behind this experimental set up was to investigate whether starvation will increase the efficacy of OXPHOS inhibitors and which concentrations are needed to reduce the viability below 50% of the starting value. The GM without inhibitors was used as a control, in which cells pertain maximal viability. In first experiments, HBSS (containing 1g/L glucose) was used as starvation medium (SM1), while later experiments DMEM without glucose, glutamine, pyruvate and FCS was used (SM2), to deplete glucose entirely. To interpret if there is an effect on cell viability under SM, we first analyzed how strong the inhibitor acts under GM and chose 50% cell viability in SM as cut off.

Starvation potentiates the effect of Metformin on cell viability

The viability of HepG2 cells remained similar after inhibitor treatment in GM (*Figure 1 A and B*, black bars), indicating that the cells were resistant to metformin.

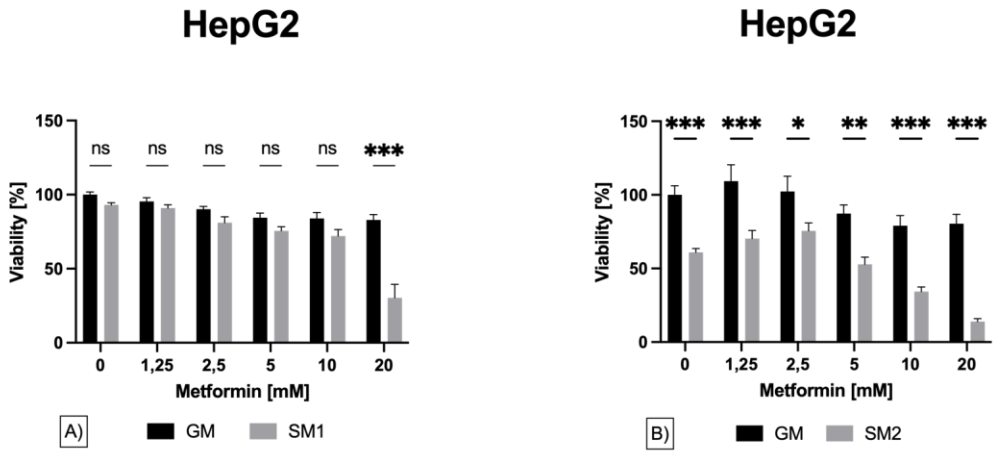

Figure 1: Viability of HepG2 cells measured after 24h treatment with indicated concentrations metformin in growth medium (GM), HBSS + Hepes (SM1) (A) or DMEM -/-/-/- (SM2) (B). Average values and SEM are shown. 2-way-ANOVA was performed. Asterisks (*) indicate significance between growth medium and starvation medium for each inhibitor concentration. *** $p<0.0001$, ** $p<0.001$, * $p<0.1$, ns no significance.

Compared to the control condition (GM, no inhibitor), the addition of 20 mM metformin in SM1 (*Figure 1A*) reduces cell viability by 69.65%. Due to the presence of glucose in HBSS we then used SM2 to achieve glucose-free conditions. Because of the absolute lack of glucose (*Figure 2B*), lower metformin concentrations were sufficient to reduce cell viability: at 5mM (-47.17%), 10mM (-65,74%) and 20mM (86.12%) compared to the control condition (GM, no inhibitor).

The viability of Hep3B and Huh6 cells remained unchanged under GM with and without metformin (*Figure 2 A and B*, black bars), similar to HepG2 cells.

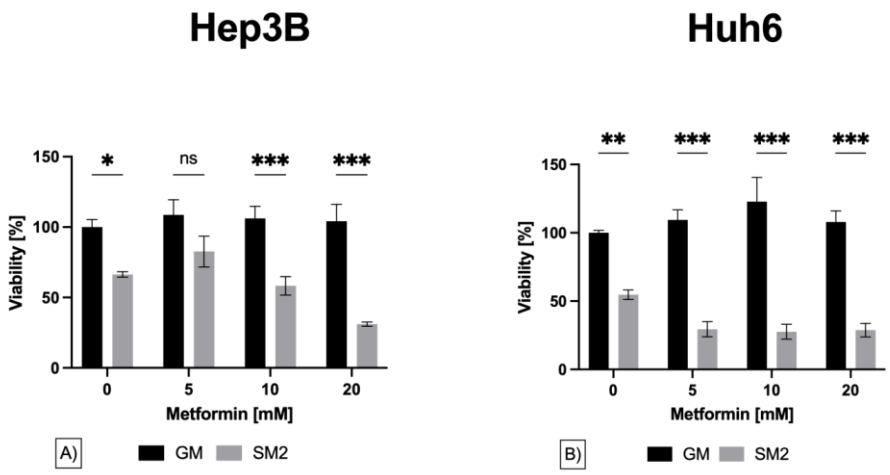

Figure 2: Viability of Hep3B (A) and Huh6 (B) cells measured after 24h treatment with indicated concentrations of metformin in growth medium (GM) and starvation medium (SM2). Average values and SEM are shown. 2-way-ANOVA was performed. Asterisks (*) indicate significance between growth medium and starvation medium for each inhibitor concentrations. *** $p<0.0001$, ** $p<0.001$, * $p<0.1$, ns no significance.

In Hep3B cells we observed a 41.73% reduction of the cell viability with 10mM metformin and 68.97% with 20mM of the inhibitor. The reduction in viability for Huh6 started at much lower inhibitor concentrations and with a stronger effect. With 5mM, 10mM and 20mM of Metformin the viability was reduced for 70.59%, 72.40% and 71.23%, respectively. Of note, SM2 alone significantly reduced cell viability in all tested cell lines.

Starvation potentiates the effect of Phenformin on cell viability

Although we observed some variability in the viability of HepG2 cells after the inhibitor treatment in GM (*Figure 3 A and B*, black bars), the viability remained above 50%.

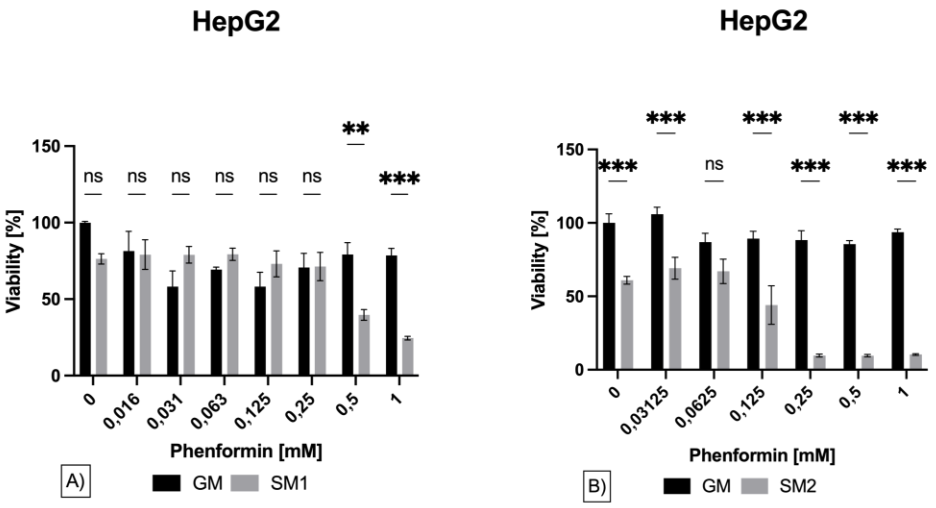

Figure 3: Viability of HepG2 cells measured after 24h treatment with indicated concentrations phenformin in growth medium (GM), HBSS + Hepes (SM1) (A) or DMEM -/-/-/- (SM2) (B). Average values and SEM are shown. 2-way-ANOVA was performed. Asterisks (*) indicate significance between growth medium and starvation medium for each inhibitor concentration. *** $p<0.0001$, ** $p<0.001$, * $p<0.1$, ns no significance.

Similar to with metformin, SM1 *(Figure 1A)* required an increased inhibitor concentration to reduce cell viability. The addition of 0.5 mM or 1 mM phenformin in SM1 reduces the cell viability by 60.22% or 75,48% compared to the control condition (GM, no inhibitor). In SM2 (*Figure 3B*), lower phenformin concentrations were sufficient to reduce cell viability: for 0.125 mM about - 55.91% and for the concentrations 0.25 mM, 0.5mM, 1 mM about -90% compared to the control medium (GM, no inhibitor).

The viability of Hep3B and Huh6 cells remained high under GM with and without phenformin (*Figure 4 A and B*, black bars).

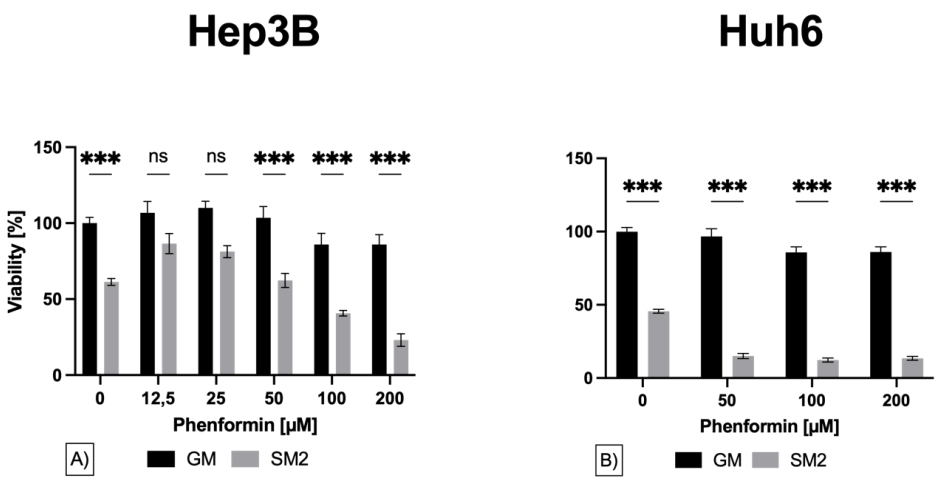

Figure 4: Viability of Hep3B (A) and Huh6 (B) cells measured after 24h treatment with indicated concentrations of phenformin in growth medium (GM) and starvation medium (SM2). Average values and SEM are shown. 2-way-ANOVA was performed. Asterisks (*) indicate significance between growth medium and starvation medium for each inhibitor concentrations. *** $p<0.0001$, ** $p<0.001$, * $p<0.1$, ns no significance.

In Hep3B cells we observed a 59.28% reduction of the cell viability with 100µM phenformin and 76.89% with 200µM of the inhibitor. The inhibitor sensitization for Huh6 seems to be more potent, because of the stronger viability suppression. With 50µM, 100µM and 200 µM of Metformin the viability was reduced for more than 85%.

Starvation does not influence the effect of mIBG on cell viability

The viability of HepG2 cells remained similar after inhibitor treatment in GM (*Figure 5 A and B*, black bars), indicating that the cells were resistant to mIBG.

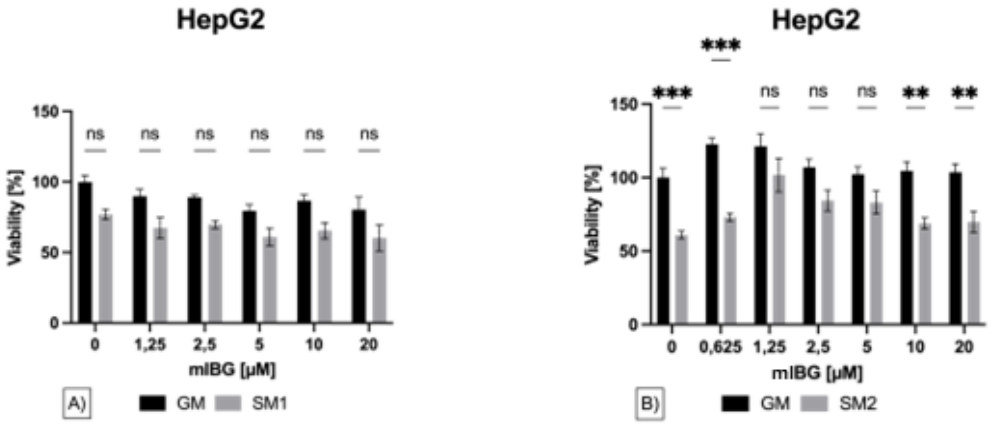

Figure 5: Viability of HepG2 cells measured after 24h treatment with indicated concentrations Meta-iodobenzylguanidine (mIBG) in growth medium (GM), HBSS + Hepes (SM1) (A) or DMEM -/-/-/- (SM2) (B). Average values and SEM are shown. 2-way-ANOVA was performed. Asterisks (*) indicate significance between growth medium and starvation medium for each inhibitor concentration. *** $p<0.0001$, ** $p<0.001$, * $p<0.1$, ns no significance.

Compared to the control condition (GM, no inhibitor), the addition of mIBG in SM1 (*Figure 5A*) had no significant impact on the cell viability. As shown in *Figure 5B*, only high mIBG concentrations reduced cell viability significantly: for 10µM and 20µM, however not more than 30% compared to the control medium (GM, no inhibitor).

The viability of HepG2, Hep3B and Huh6 cells remained high under GM with and without mIBG. (*Figure 6 A and B*, black bars).

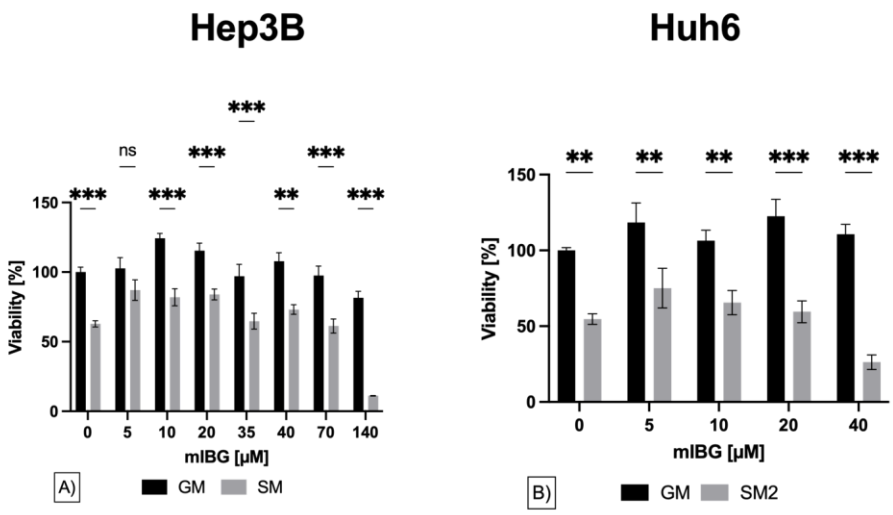

Figure 6: Viability of Hep3B (A) and Huh6 (B) cells measured after 24h treatment with indicated concentrations of Meta-iodobenzylguanidine (mIBG) in growth medium (GM) and starvation medium (SM2). Average values and SEM are shown. 2-way-ANOVA was performed. Asterisks (*) indicate significance between growth medium and starvation medium for each inhibitor concentrations. *** $p<0.0001$, ** $p<0.001$, * $p<0.1$, ns no significance.

In Hep3B we observed a 38.70% reduction of the cell viability with an inhibitor concentration of 70mM and 88.68% with 140mM mIBG. The inhibitor sensitization for Huh6 started at much lower inhibitor concentrations and with a stronger effect. With 20mM and 40mM of mIBG the viability was reduced for 40.50% and 73.75%.

Starvation potentiates the effects of IM156 on cell viability

After the inhibitor treatment in GM (*Figure 7 A and B*, black bars), the viability of HepG2 cells remained similar to control except for 70µM and 100µM,.

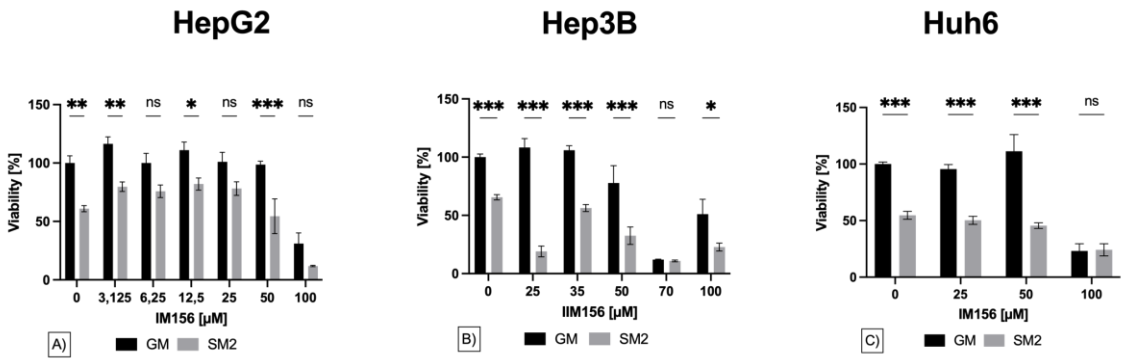

Figure 7: Viability of HepG2 (A), Hep3B (B) and Huh6 (C) cells measured after 24h treatment with indicated concentrations of IM156 in growth medium (GM) and starvation medium (SM2). Average values and SEM are shown. 2-way-ANOVA was performed. Asterisks (*) indicate significance between growth medium and starvation medium for each inhibitor concentrations. *** p<0.0001, ** p<0.001, * p<0.1, ns no significance.

In glucose depleted medium (SM2), IM156 concentration of 50µM was sufficient to reduce HepG2 cell viability for 45.61% compared to the control medium (GM, no inhibitor) (*Figure 7A*). Higher concentrations like 100µM had an impact on HepG2 cells treated with IM156 both in GM and SM2, indicating that this concentration has a possible cytotoxic effect. For Hep3B (*Figure 7B*) we observed an 80.91% reduction of the cell viability with 25µM, 43.72% with 35µM and 67.40% with 50µM IM156. The higher concentrations of 70µM and 100µM IM156 showed a possible cytotoxic effect on Hep3B cells independent of the cell culture media. Sensitization of the Huh6 cells seems to be similar to Hep3B (*Figure 7C*). With 25µM and 50µM of IM156 the viability was reduced for 50% or more. The impact of higher inhibitor concentrations on cells treated with normal glucose was also seen in Huh6.

3.1.2 Determination of the limiting metabolites for cells' sensitization to OXPHOS inhibitors

To determine at which glucose concentration the cells' sensitivity to inhibitors is abolished, HepG2, Hep3B and Huh6 cells were treated with 0.5mM phenformin and 50µM IM156 in SM2 with the addition of glucose in concentrations ranging from 1.5 – 25mM. In addition, 100mM pyruvate, 200mM glutamine and 10% FCS were also each added-back to SM2 to assess their relevance for the survival of the cells. Phenformin and IM156 were chosen as inhibitors because their concentrations in the previous experiments were considered for potential clinical use.

Increasing glucose concentrations protect HepG2 cells from sensitization to Phenformin and IM156

The viability of HepG2 cells remained higher than in all conditions without treatment (*Figure 8*, black bars), indicating that the addition of glucose and other medium supplements do not significantly affect the viability of cells.

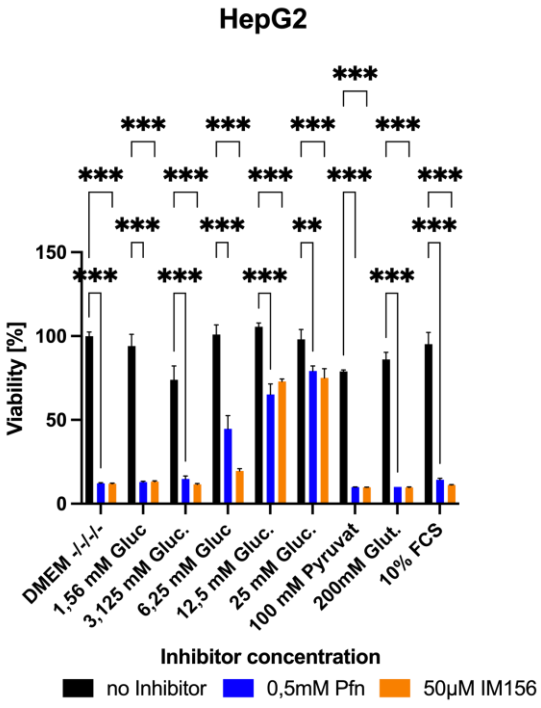

Figure 8: Viability of HepG2 cells measured after 24h treatment with concentrations of 0.5mM phenformin (Pfn), 50µM IM156 in mediums with indicated glucose (Gluc.), 100mM pyruvate, 200mM glutamate (Glut.) and 10% FCS concentrations. Average values and SEM are shown. 2-way-ANOVA was performed. Asterisks (*) indicate significance between growth medium and starvation medium for each inhibitor concentrations. *** $p<0.0001$, ** $p<0.001$, * $p<0.1$, ns – not shown.

As expected, HepG2 cells were sensitized to the tested inhibitors at low glucose concentrations (1.25mM and 3.125mM). This was reflected in cell viability less than 15% for these concentrations. The cell sensitization to phenformin became weaker with a glucose concentration of 6.25mM. The reduction of the cell viability for this glucose concentration was about 55% for phenformin and 80% for IM156. By the addition of 12.5mM glucose the inhibitor sensitization for both tested inhibitors become weaker. For phenformin the cell viability reduction was less than 35% and for IM156 about 26%. This trend continued with increasing glucose concentrations such that the reduction in cell viability for both inhibitors was less than 25% after 25mM glucose addition. The addition of 100mM pyruvate, 100x glutamate and 10% FCS could not protect the cells from death (viability was higher than 85%) and thus has no negative impact on the inhibitor sensitization of HepG2 cells at these concentrations.

Increasing glucose concentrations protects Hep3B cells from sensitization to Phenformin and IM156

The viability of Hep3B cells remained higher than in all conditions without treatment (*Figure 9*, black bars), indicating that the addition of glucose and other medium supplements do not significantly affect the viability of cells.

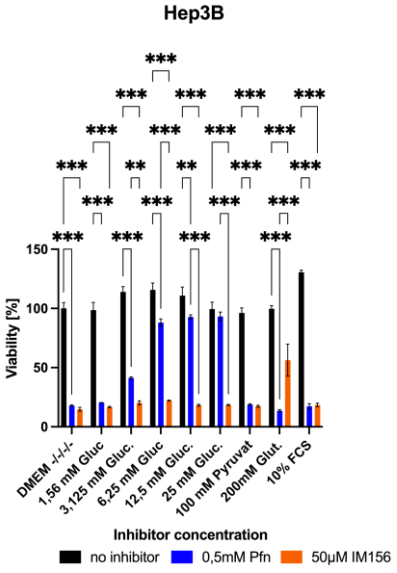

Figure 9: Viability of Hep3B cells measured after 24h treatment with concentrations of 0.5mM phenformin (Pfn), 50µM IM156 in mediums with indicated glucose (Gluc.),100mM pyruvate, 100x glutamate (Glut.) and 10% FCS concentrations. Average values and SEM are shown. 2-way-ANOVA was performed. Asterisks (*) indicate significance between growth medium and starvation medium for each inhibitor concentrations. *** p<0.0001, ** p<0.001, * p<0.1, ns – not shown.

Similar to HepG2 cells, Hep3B cells become sensitized to the tested inhibitors at low glucose concentrations (1.25mM). This was reflected in cell viability lower than 20% for these concentrations. The cell sensitization to phenformin was weaker at glucose concentration of 3.125mM. The reduction of the cell viability for this glucose concentration was about 59% for phenformin and 80% for IM156. Increasing glucose concentrations increased the cell viability of Hep3B cells under phenformin. For cells treated with IM156 the cell viability was still unchanged and lower than 23% in all glucose concentrations. The addition of 100mM pyruvate and 10% FCS could not protect the cells from death (viability reduction was more than 80%) and thus has no negative impact on the inhibitor sensitization of Hep3B cells at these concentrations. For 100x glutamate we saw that it could not protect the cells from phenformin, but cells treated with IM156 had a lower reduction of cell viability around 43.66%.

Increasing glucose concentrations protects Huh6 cells from sensitization to Phenformin but not to IM156

The viability of Huh6 cells remained higher than in all conditions without treatment (*Figure 10*, black bars), indicating that the addition of glucose and other medium supplements do not significantly affect the viability of cells.

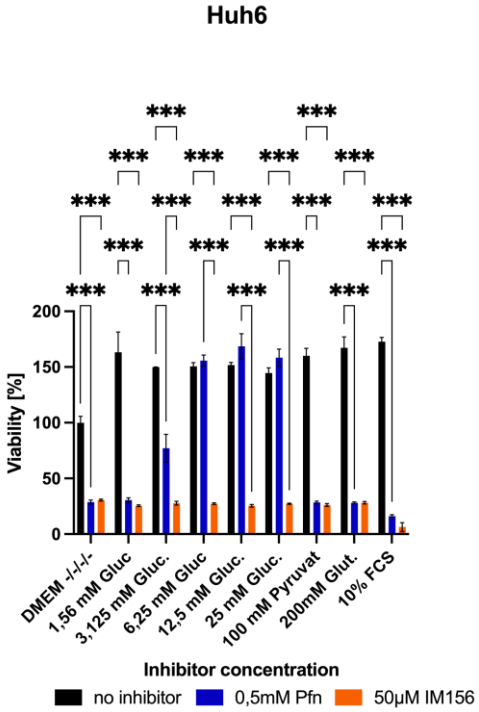

Figure 10: Viability of Huh6 cells measured after 24h treatment with concentrations of 0.5mM phenformin (Pfn), 50µM IM156 in mediums with indicated glucose (Gluc.),100mM pyruvate, 200mM glutamate (Glut.) and 10% FCS concentrations. Average values and SEM are shown. 2-way-ANOVA was performed. Asterisks (*) indicate significance between growth medium and starvation medium for each inhibitor concentrations. *** $p<0.0001$, ** $p<0.001$, * $p<0.1$, ns - not shown.

Huh6 cells become sensitized for the tested inhibitors at low glucose concentrations (1.25mM), similar to as HepG2 and Hep3B. This was reflected in cell viability less than 30% for these concentrations. The cell sensitization for phenformin became weaker at glucose concentration of 3.125mM. The reduction of the cell viability for this glucose concentration was about 22.90% for phenformin and 72.27% for IM156. Increasing glucose concentrations increased the cell viability of Hep3B cells under phenformin. For cells treated with IM156 the cell viability remained lower than 28% in all glucose concentrations. The addition of 100mM pyruvate, 200mM glutamate and 10% FCS could not protect the cells from death (viability reduction was more than 70%) and thus has no negative impact on the inhibitor sensitization of Huh6 cells at these concentrations.

4 Discussion

In this study we wanted to research which concentrations of OXPHOS inhibitors metformin, phenformin, mIBG and IM156 influence the cell viability of HepG2, Hep3B and Huh6 cell lines under growth and starvation medium.

In summary the experiments suggest that metformin reduces cell viability when combined with starvation and in higher concentrations (10mM, 20mM), possibly acting as an OXPHOS inhibitor. HepG2 and Hep3B cells showed similar sensitivity to Metformin under SM2. The Huh6 cells seem to be more sensitive to combination treatment. Studies with H460 lung tumor cells showed a similar synergistic inhibiting effect of metformin in combination with starvation. Cell viability in this studies was reduced for approximately 90% under 2.5mM metformin (28). Mice treated to 24h feeding and fasting cycles and metformin demonstrated reduced tumor progression. In vitro experiments from the same study with HCT116 colon tumor cells and HeLa cervical tumor cells showed that reduced glucose concentrations lead to a greater decrease in cell viability under metformin, in agreement with our results (29).

Phenformin seems to reduce cell viability when used under starvation and in lower concentration compared to metformin. HepG2 and Hep3B cells showed similar sensitivity to phenformin under SM2. Huh6 cells seem to be more sensitive to combination treatment. Studies with Caco-2 colon cancer cells showed a similar synergistic inhibiting effect of metformin in combination with starvation (30). Cell viability in this study was reduced for approximately 75% under 0.1mM metformin (30). Phenformin with a concentration of 100µM inhibited cell viability of SH-SY5Y neuroblastoma cells more effectively in the presence of low glucose concentrations (31).

The experiments suggest that mIBG reduces cell viability when used under starvation only in high concentrations (40mM, 70mM, 140µM), and to a lower extent than Metformin and Phenformin. HepG2 and Hep3B cells showed similar sensitivity to mIBG under SM2. The Huh6 cells seem to be more sensitive to combination treatment. The tested inhibitor concentrations for HepG2 didn’t induce strong decrease in the cell viability, so it can be assumed that concentration over 20µM is required. The massive drop in cell viability between 70µM and 140µM suggests that the optimal inhibitor concentration for Hep3B must be in this range. Huh6 has a higher affinity for the combination of starvation and inhibitor compared to HepG2 and Hep3B, changes can be achieved even at lower

concentrations like 40µM. A previous study showed that mIBG at 10µg ml-1 inhibited the mitochondrial respiration of leukemic cells, resulting in enhanced glucose consumption and lactate production (32). In combination with anti-cancer drugs mIBG potentiated the cytotoxic effect of mitomycin C, chlorambucil melphalan, EO9 and cisplatin (32).

The results from IM156 suggest that this inhibitor reduces cell viability when used under starvation and in lower concentration. Higher concentrations of IM156 seems to act cytotoxic an reduce cell viability of cells treated with normal glucose medium. For Hep3B we observed lower cell viability of cells treated with 25µM compared to 35µM or 50µM IM156. A greater reduction at higher concentrations would be expected, but these did not occur. One explanation could be the use of cells with higher passage for the 25µM treatment. One experiment compared the impact of phenformin and IM156 on the cell viability of *Eμ-Myc+* lymphoma cells (26). Based on cell viability measurements, IM156 (12.5µM) exhibited higher potency and induced lymphoma cell death at lower concentrations than phenformin (62µM) (26).

To find out which glucose concentration is effectively causing cell sensitization we apply Phenformin and IM156 in combination with starvation medium and different glucose concentrations in the glucose add-back experiments.

Rising glucose concentration seems to provide the substrate for energy production and allows HepG2 cells to survive upon inhibitor treatment. These data suggest that glucose concentration needs to be lower than 6.25mM in order to sensitize HepG2 cells to phenformin and IM156. IM156 appears to be a more potent inhibitor since the inhibitory effect of phenformin was attenuated by a glucose concertation of 6.25mM. A concentration of 12.5mM glucose was sufficient for the cells to survive IM156 treatment. Concentrations between 6.25mM and 12.5mM give HepG2 cells enough substrate to produce energy and to survive under 0.5mM phenformin and 50µM IM156. 100mM pyruvate, 200mM glutamate and 10% FCS could not be used as energy sources, hence they did not protect HepG2 cell from phenformin and IM156.

For Hep3B, rising glucose concentration seems to give the cells more energy to survive under phenformin. These data suggest that glucose concentration lower than 6.25mM sensitize Hep3B cells for phenformin. IM156 appears to be a more potent inhibitor because of the unchanged cell viability of less than 23% in all glucose concentrations. It seems that higher glucose concentrations are necessary to protect Hep3B cells from IM156.

Concentrations higher than 6.25mM give Hep3B cells enough substrate to produce anaerobe energy and survive under 0.5mM phenformin. 100mM pyruvate and 10% FCS failed to provide energy source to protect Hep3B cell from phenformin and IM156. The concentration of 200mM glutamate provided a source of energy for Hep3B cells to survive under IM156, but not for cells treated with phenformin.

Rising glucose concentration seems to protect Huh6 cells from phenformin treatment. These data suggest that glucose concentration lower than 6.25mM sensitize Huh6 cells to phenformin. IM156 appears to be a more potent inhibitor because of the unchanged cell viability of less than 28% in all glucose concentrations. It seems that higher glucose concentrations are necessary to protect Huh6 cells from IM156. Concentrations higher than 3.125mM give Huh6 cells enough substrate for survival under 0.5mM phenformin. 100mM pyruvate, 100x glutamate and 10% FCS could not be used as energy source to protect Huh6 cell from phenformin and IM156.

Similar studies found that cell lines which are most sensitive to low glucose were defective in upregulation of OXPHOS (33). This is typically triggered by limited glucose availability, either due to alterations in complex I genes of the mtDNA or impaired glucose metabolism. These defects predicted sensitivity to biguanides, when tumor cells were grown in low glucose or as tumor xenografts. The conclusion was that mtDNA mutations and impaired glucose metabolism are potential biomarkers for identifying tumors with increased sensitivity to OXPHOS inhibitors (33).

4.1 Conclusion and Outlook

Normal liver cells primarily rely on glucose and fatty acids as energy sources, while liver cancer cells have a higher dependence on glucose and glutamine metabolism. OXPHOS inhibitors can potentially target the energy production pathways in both normal liver cells and liver cancer cells, leading to reduced ATP production and cell death. However, under fasting conditions, cancer cells have a reduced ability to adapt to this change in metabolite availability (14). This might be the reason why starvation sensitizes liver cancer cells to OXPHOS inhibitors, leading to increased cell death.

However, the use of OXPHOS inhibitors for cancer therapy is still in the early stages of research. While preclinical studies have shown promising results, clinical trials have not yet demonstrated significant efficacy in treating cancer patients. One of the challenges in developing OXPHOS inhibitors as cancer therapies is the potential for off-target effects on

normal cells, which can lead to toxicity and adverse side effects. Additionally, cancer cells can develop resistance to OXPHOS inhibitors, which can limit their effectiveness over time.

In conclusion, our in vitro experiments have demonstrated that the combination of starvation with OXPHOS inhibitors is efficient in reducing the viability of HCC-derived cell lines. Glucose was identified as the limiting metabolite in most of the cell lines and combination treatments, suggesting its critical role in supporting cell survival. These findings highlight the potential of targeting OXPHOS and glucose metabolism as a promising therapeutic strategy for HCC treatment. The next step in this research would be to conduct in vivo experiments to further validate the effectiveness and safety of these inhibitors and combination treatments in more complex physiological context.

IM156 demonstrated a significant reduction in cell viability at lower concentrations compared to metformin and phenformin. The viability of HCC-derived cell lines was reduced by IM156 at concentrations as low as 25 μM.

On the other hand, metformin and phenformin required higher concentrations to achieve similar reductions in cell viability. Additionally, mIBG showed relatively weaker effects on cell viability under starvation.

Thus, IM156 could be a promising candidate for further investigation as it exhibits a potent inhibitory effect on cell viability at lower concentrations, which may be advantageous for potential therapeutic applications.

Overall, while the combination of OXPHOS inhibitors with fasting therapy holds promise as a potential treatment for liver cancer, more research is needed to fully understand its mechanisms of action and potential clinical applications.

5 References

1. 39-All-cancers-fact-sheet.pdf [Internet]. [cited 2021 Oct 11]. Available from: https://gco.iarc.fr/today/data/factsheets/cancers/39-All-cancers-fact-sheet.pdf

2. Asafo-Agyei KO, Samant H. Hepatocellular Carcinoma. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2021 [cited 2021 Oct 11]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK559177/

3. Hepatocellular carcinoma - CORE Reader [Internet]. [cited 2021 Oct 11]. Available from: https://core.ac.uk/reader/154917403?utm_source=linkout

4. Yang JD, Hainaut P, Gores GJ, Amadou A, Plymoth A, Roberts LR. A global view of hepatocellular carcinoma: trends, risk, prevention and management. Nat Rev Gastroenterol Hepatol. 2019 Oct;16(10):589–604.

5. tecentriq-epar-product-information_de.pdf [Internet]. [cited 2023 Mar 14]. Available from: https://www.ema.europa.eu/en/documents/product-information/tecentriq-epar-product-information_de.pdf

6. Garten A, Grohmann T, Kluckova K, Lavery GG, Kiess W, Penke M. Sorafenib-Induced Apoptosis in Hepatocellular Carcinoma Is Reversed by SIRT1. Int J Mol Sci. 2019 Aug 19;20(16):4048.

7. Sorafenib kills liver cancer cells by disrupting SCD1-mediated synthesis of monounsaturated fatty acids via the ATP-AMPK-mTOR-SREBP1 signaling pathway - Liu - 2019 - The FASEB Journal - Wiley Online Library [Internet]. [cited 2021 Oct 11]. Available from: https://faseb.onlinelibrary.wiley.com/doi/full/10.1096/fj.201802619RR

8. Li Y, Xia J, Shao F, Zhou Y, Yu J, Wu H, et al. Sorafenib induces mitochondrial dysfunction and exhibits synergistic effect with cysteine depletion by promoting HCC cells ferroptosis. Biochemical and Biophysical Research Communications. 2021 Jan 1;534:877–84.

9. Li Z jie, Dai H qi, Huang X wei, Feng J, Deng J huan, Wang Z xuan, et al. Artesunate synergizes with sorafenib to induce ferroptosis in hepatocellular carcinoma. Acta Pharmacol Sin. 2021 Feb;42(2):301–10.

10. Liberti MV, Locasale JW. The Warburg Effect: How Does it Benefit Cancer Cells? Trends Biochem Sci. 2016 Mar;41(3):211–8.

11. Vaupel P, Schmidberger H, Mayer A. The Warburg effect: essential part of metabolic reprogramming and central contributor to cancer progression. Int J Radiat Biol. 2019 Jul;95(7):912–9.

12. DeBerardinis RJ, Chandel NS. Fundamentals of cancer metabolism. Sci Adv. 2016 May;2(5):e1600200.

13. Park JH, Pyun WY, Park HW. Cancer Metabolism: Phenotype, Signaling and Therapeutic Targets. Cells. 2020 Oct 16;9(10):2308.

14. Buono R, Longo VD. Starvation, Stress Resistance, and Cancer. Trends Endocrinol Metab. 2018 Apr;29(4):271–80.

15. Ashton TM, McKenna WG, Kunz-Schughart LA, Higgins GS. Oxidative Phosphorylation as an Emerging Target in Cancer Therapy. Clin Cancer Res. 2018 Jun 1;24(11):2482–90.

16. Farhadi P, Yarani R, Dokaneheifard S, Mansouri K. The emerging role of targeting cancer metabolism for cancer therapy. Tumour Biol. 2020 Oct 1;42(10):1010428320965284.

17. Ganapathy-Kanniappan S, Geschwind JFH. Tumor glycolysis as a target for cancer therapy: progress and prospects. Mol Cancer. 2013 Dec 3;12:152.

18. Bonnet S, Archer SL, Allalunis-Turner J, Haromy A, Beaulieu C, Thompson R, et al. A mitochondria-K+ channel axis is suppressed in cancer and its normalization promotes apoptosis and inhibits cancer growth. Cancer Cell. 2007 Jan;11(1):37–51.

19. Deberardinis RJ, Sayed N, Ditsworth D, Thompson CB. Brick by brick: metabolism and tumor cell growth. Curr Opin Genet Dev. 2008 Feb;18(1):54–61.

20. Lv Z, Guo Y. Metformin and Its Benefits for Various Diseases. Front Endocrinol (Lausanne). 2020 Apr 16;11:191.

21. García Rubiño ME, Carrillo E, Ruiz Alcalá G, Domínguez-Martín A, A. Marchal J, Boulaiz H. Phenformin as an Anticancer Agent: Challenges and Prospects. Int J Mol Sci. 2019 Jul 5;20(13):3316.

22. Loesberg C, van Rooij H, Smets LA. Meta-iodobenzylguanidine (MIBG), a novel high-affinity substrate for cholera toxin that interferes with cellular mono(ADP-ribosylation). Biochim Biophys Acta. 1990 Jan 19;1037(1):92–9.

23. Sharp SE, Trout AT, Weiss BD, Gelfand MJ. MIBG in Neuroblastoma Diagnostic Imaging and Therapy. Radiographics. 2016 Feb;36(1):258–78.

24. Cornelissen J, Wanders RJ, Van Gennip AH, Van den Bogert C, Voûte PA, Van Kuilenburg AB. Meta-iodobenzylguanidine inhibits complex I and III of the respiratory chain in the human cell line Molt-4. Biochem Pharmacol. 1995 Feb 14;49(4):471–7.

25. Willette RN, Mangrolia P, Pondell SM, Lee CYW, Yoo S, Rudoltz MS, et al. Modulation of Oxidative Phosphorylation with IM156 Attenuates Mitochondrial Metabolic Reprogramming and Inhibits Pulmonary Fibrosis. J Pharmacol Exp Ther. 2021 Sep 30;JPET-AR-2021-000811.

26. Izreig S, Gariepy A, Kaymak I, Bridges HR, Donayo AO, Bridon G, et al. Repression of LKB1 by miR-17∼92 Sensitizes MYC-Dependent Lymphoma to Biguanide Treatment. Cell Rep Med. 2020 May 19;1(2):100014.

27. Krstic J, Reinisch I, Schindlmaier K, Galhuber M, Riahi Z, Berger N, et al. Fasting improves therapeutic response in hepatocellular carcinoma through p53-dependent metabolic synergism. Sci Adv. 2022 Jan 21;8(3):eabh2635.

28. Yakisich JS, Azad N, Kaushik V, Iyer AKV. The Biguanides Metformin and Buformin in Combination with 2-Deoxy-glucose or WZB-117 Inhibit the Viability of Highly Resistant Human Lung Cancer Cells. Stem Cells Int. 2019;2019:6254269.

29. Elgendy M, Cirò M, Hosseini A, Weiszmann J, Mazzarella L, Ferrari E, et al. Combination of Hypoglycemia and Metformin Impairs Tumor Metabolic Plasticity and Growth by Modulating the PP2A-GSK3β-MCL-1 Axis. Cancer Cell. 2019 May 13;35(5):798-815.e5.

30. Lea MA, Chacko J, Bolikal S, Hong JY, Chung R, Ortega A, et al. Addition of 2-Deoxyglucose Enhances Growth Inhibition But Reverses Acidification in Colon Cancer Cells Treated with Phenformin. Anticancer Research. 2011 Feb 1;31(2):421–6.

31. Caraci F, Chisari M, Frasca G, Chiechio S, Salomone S, Pinto A, et al. Effects of phenformin on the proliferation of human tumor cell lines. Life Sciences. 2003 Dec 19;74(5):643–50.

32. Kuin A, Aalders M, Lamfers M, van Zuidam DJ, Essers M, Beijnen JH, et al. Potentiation of anti-cancer drug activity at low intratumoral pH induced by the mitochondrial inhibitor m-iodobenzylguanidine (MIBG) and its analogue benzylguanidine (BG). Br J Cancer. 1999 Feb;79(5–6):793–801.

33. Birsoy K, Possemato R, Lorbeer FK, Bayraktar EC, Thiru P, Yucel B, et al. Metabolic determinants of cancer cell sensitivity to glucose limitation and biguanides. Nature. 2014 Apr 3;508(7494):108–12.