THE DEVELOPMENT OF CHEMORESISTANT LIVER CANCER CELLS TO EXPLORE THE ROLE OF VISFATIN AND THE IGF-1R/IR/AKT AXIS

by

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Obesity

The World Health Organization reports that as of 2016, 39% of persons age 18 and older were found to be overweight and 13% obese.[1] The 2017-2018 National Health and Nutrition Examination Survey (NHANES) revealed that in the United States 42.4% of the population were obese with no significant differences by age.[2] These statistics are measured via body mass index (BMI) which is weight in kilograms divided by height in meters (kg/m^2), with 25-29 indicating someone is overweight and 30 or greater classified as obese.[1] Obesity is a worldwide public health concern due in part to the significant health detriments associated with obesity-related comorbidities.[1,3] The most severe comorbidities include cardiovascular disease, metabolic syndrome, type two diabetes, nonalcoholic fatty liver disease (NAFLD) and promotion of several cancers (e.g. liver, pancreas, colon).[1]

Hepatocellular Carcinoma (HCC)

The two main types of liver cancer are of the lobes or duct.[4] Liver cancer of the lobes is termed hepatocellular carcinoma (HCC) and is the most prevalent of the two types, in the United States.[4] HCC has been steadily rising in the United States, with incidence tripling in approximately 40 years.[4] The death rate of liver cancer has also risen by 2% within the last decade.[5] The American Cancer Society estimates that for 2019 in the United States over 40,000 new cases of HCC will be diagnosed with the potential for 75% of those cases resulting in death.[5] Liver cancer also heavily impacts developing countries, ranking as the sixth most common cancer type in the world.[5] The high death rate of liver cancer is related to symptoms and diagnosis.[5] Symptoms of liver cancer may include jaundice, fatigue, abdominal swelling, or loss of appetite, however, most often individuals with liver

cancer do not have any symptoms.[4] HCC is typically in an advanced stage by the time it is diagnosed.[5] This late stage diagnosis means it is too late to resect the cancerous portion of the liver.[5,6] Therefore, chemotherapy is the only remaining treatment for the disease.[6] The causes and contributors of HCC include prolonged use of certain oral contraceptives, cirrhosis, excessive alcohol consumption, NAFLD, hepatitis C, hepatitis B, and other unknown sources.[7,8] Herein lies a connection of HCC to obesity. NAFLD is a comorbidity of obesity, and also one of the contributors to HCC. Therefore, a rise in obesity could equate to an even greater rise in HCC. Epidemiological studies of large cohorts totaling over 300,000 individuals demonstrate a three to four-fold increase risk of HCC development in those who are obese.[9,10] Thus, there is a need to understand the mechanisms by which obesity increases the risk in the development and progression of liver cancer.

Mechanisms Linking HCC and Obesity

Potential mechanisms linking obesity and HCC pertain to adipocyte accumulation. Increases in adiposity results in elevated levels of circulating insulin, insulin growth factor (IGF-1), leptin, and interleukin 6 (IL-6), with a decrease in adiponectin expression.[11] Leptin, IL-6, IGF-1, and insulin have integral membrane receptor binding abilities that activate kinase pathways.[11] Activation of these pathways promote several cell processes that are needed for HCC survival and growth including angiogenesis, proliferation, pro-tumorigenic protein expression, and migration.[11] A summary of these substrates and their binding downstream effects can be viewed in Table 1.

Table 1. HCC Pro-tumorigenic Pathway Activation [11]

Substrate	Binding Site	Pathways Activated	Downstream Affects
Leptin	ObR	JAK/STAT, MAPK	Migration, Survival, Cell Cycle Progression
IL-6	GP130, IL6R	JAK/STAT, MAPK, PI3K	Migration, Survival, Cell Cycle Progression, Angiogenesis, Protein Synthesis
Insulin	INSR, IGF-1R	MAPK, PI3K	Migration, Survival, Cell Cycle Progression, Angiogenesis, Protein Synthesis
IGF-1	INSR, IGF-1R	MAPK, PI3K	Migration, Survival, Cell Cycle Progression, Angiogenesis, Protein Synthesis

Genetics and epigenetics for obesity-induced HCC is a growing, novel body of research. Understanding obesity-related genetics, epigenetics and its relationship with cancer are pertinent and could provide deeper insight and direction for better treating persons with HCC and obesity.[12] Obesity causes a multitude of health complications. In addition to the contribution of disease states obesity seems to contribute to genetic

instability and epigenetic changes by way of single nucleotide polymorphisms (SNPs).[13] SNPs have many ill effects including a role in cancer development and progression.[13]

For example, a single nucleotide polymorphism (SNP) in gene PNPLA3 is correlated with up to three-times higher incidence of NASH and twelve-times higher chance of developing HCC.[12,14] The relating mechanism is due to PNPLA3s role in hepatocyte lipolysis regulation.[12] The protein that SNP PNPLA3 codes for, Ile148Met, is resistant to degradation and therefore accumulates in the hepatocyte, specifically on the lipid droplets. The buildup of Ile148Met on the lipid droplets prevents their degradation thus leading to lipid droplet accumulation.[12] In a 2010 study looking at NAFLD and PNPLA3 found that in addition increased to increased instance of NAFLD development when SNP PNPLA3 was present, that individuals of the study, including healthy controls had elevated BMIs of 25 or higher.[15] Meaning that even in healthy population controls without SNP PNPLA3 there is still a risk of NAFLD due to the elevated BMI.[15] Another gene of interest is TM6SF2. Though the function of TM6SF2 is still unknown, mouse model knockouts of the genes and correlations linking a polymorphism to TAG synthesis. Lastly, a variant in gene HSD17B13 which codes for a hepatocyte lipid droplet enzyme may indicate a reduction in risk via prevention of hepatocyte injury.[12]

A review published in 2020, combined available date for all three genes HSD17B, RM6sF2, and PNPLA3. The reviewers found that when all three genetic variances were present an individual could be twelve times as likely to develop cirrhosis of the liver and would be 29 times higher to develop HCC. Genetics may play a large role of predicting liver disease in an obese society and its progression to HCC.[12,15]

Sorafenib Resistance

As previously mentioned, due to late-stage diagnosis, chemotherapy is the primary treatment for HCC. The National Cancer Institute reports 14 different FDA approved drugs for the treatment of various liver cancers.[16] Of these drugs, Sorafenib Tosylate (also known as Nexavar or Sorafenib) is the most commonly used to treat HCC.[16,17] Unfortunately, only about 30% of HCC patients respond to Sorafenib and the other 70% typically become resistant to the drug within three to six months of use.[17] Initial resistance is also termed primary resistance.[17] Resistance to Sorafenib signifies a vital need for understanding the mechanisms pertaining to development of resistance and if synergistic effects exists with increased adiposity. Sorafenib's mechanism of action (MOA) is via multiple kinase and receptor kinase inhibition.[17] Specific inhibition targets are as follows in Table 2.

Table 2. Sorafenib Inhibition Targets[17]

Sorafenib Inhibition Target	Forms
Raf serine/threonine kinases (intracellular)	Raf-1 (C-Raf), wild-type B-Raf, mutant B-Raf
Receptor Tyrosine Kinases	VEGRF1, PDGFR, FLT-3, RET

When Sorafenib can effectively inhibit these targets the downstream effects are anti-proliferation through Raf related pathway interference, the slowing/blocking of angiogenesis through vascular endothelial growth factor receptors (VEGFRs), and other

receptor kinases listed in table 2.[17] When certain metabolic pathways are inhibited pharmacologically, upregulation of compensatory pathways may occur.[17] The PI3k/Akt signaling cascade is one of the upregulated pathways associated with Sorafenib chemoresistance .[17] The over activation of this pathway, in concert with other factors, including presence of cancer stem cells, cell cycle errors, upregulation of epidermal growth factor receptor (EGFR), hypoxia, and epithelial mesenchymal transition (EMT) contribute to HCC resistance to Sorafenib.[18]

Visfatin and Sorafenib Resistance

Considering the potential for obesity related promotion of HCC, there is also a potential link between obesity and HCC Sorafenib resistance.[19] As discussed, individuals who are obese have higher levels of insulin and IGF-1.[9] Insulin and IGF-1 binding to their receptors cause several downstream effects including the activation of the PI3K/Akt pathway. Therefore, due to this activation, it is logical to deduce that obese HCC patients could have primary Sorafenib resistance or more rapid secondary resistance.[19] In addition to higher levels of insulin and IGF-1, obese individuals also have higher circulating amounts of the adipocytokines such as visfatin.[20] Adipocytokines are a class of proteins that are expressed from adipose tissue and have metabolic crosstalk properties in adipose tissue and other organs.[21] Visfatin, which is also referred to as nicotinamide phosphoribosyltransferase (NAMPT), has different metabolic roles dependent on its location intracellularly (aka iNAMPT) vs. extracellularly (aka eNAMPT).[20] iNAMPT works as an enzyme to convert nicotinamide (NAM) back to nicotinamide mononucleotide (NMN).[20] This regeneration of NMN is the rate limiting step to produce NAD+ which is a key component of cellular energy metabolism.[20] eNAMPT is the form found in elevated

amounts in the serum of obese individuals.[20] eNAMPT is less studied than iNAMPT but some data has indicated that eNAMPT could act similarly to insulin.[20,22,23]

A 2007 study performed using human osteoblast cell cultures, eNAMPT exhibited insulin mimetic effects via activation of the insulin receptor, and the intracellular insulin receptor substrates 1 and 2.[23] The exact mechanism of eNAMPT's influence was not determined.[23] In 2010, a mouse model study of pancreatic beta cells, the presence of eNAMPT equated to increased activation of the insulin receptor, as well as a 46% increase in insulin secretion.[24] Again, authors were unable to demonstrate the mechanism by which eNAMPT exerted its insulin-like effects.[24] A recent study published in 2020 found that in a small cohort of HCC patients, two adipocytokines, one of which was visfatin, were found to be in elevated levels compared to healthy controls. More specifically, as this pertains to obesity and HCC with visfatin as a linkage, visfatin was found to not only be elevated in HCC patients but also in those with higher levels of insulin resistance. Due to the insulin mimetic properties of visfatin this finding strengthens the body of evidence indicating the former.[24] Even without a clear understanding of eNAMPT's insulin like properties it is important to consider how these findings could translate to HCC progression and Sorafenib resistance in obese individuals. Activation of the PI3K/Akt pathway resulting from eNAMPT's insulin-like properties have been studied.

In a study performed using non-small-cell-lung cancer (NSCLC) cells and tissue NAMPT was found to be expressed more when compared to their parental cell counterparts.[25] Furthermore, the results demonstrated that visfatin also contributed to chemoresistance via upregulation of the Akt pathway.[25] Visfatin included phosphorylation and translocation of Akt to the nucleus and it's affinity to bind to ABCC1.[25] ABCC1 codes

for multidrug resistance protein-1 (MRP1).[25] Higher levels of MRP1 resulted in NSCLC cell to be more resistance to chemotherapy.[25] Use of LY294002 to suppress the Akt pathway led to lower levels of MRP1, thus increasing the chemotherapies efficacy.[25] Overall, NAMPT contributed to chemo resistance in NSCLC cells and tissue via activation on the Akt/MRP1 pathway.[25] Another study published in 2019, had some similar findings but in a different cancer cell line and with different pathway considerations.[26] In this study chemo resistant osteosarcoma cells had more expression of NAMPT than parental cells.[26] More promising though is that knockdown of visfatin resulted in recovered sensitivity to chemotherapy.[26] Researchers for this study looked at overexpression of visfatin and its effect on upregulation of Snail and increased stability of Zeb-1, which are both epithelial-mesenchymal transition-related transcription factors (EMT-TFs).[26] This is important because EMT-TFs are key in how cancer cells break away and metastasize.[25]

A 2017 study demonstrated eNAMPT's role in breast cancer progression through activation of the PI3K/Akt and the ERK/MAPK pathways.[27] Breast cancer cells were treated with eNAMPT and the results demonstrated increased cellular proliferation.[27] A further finding linking visfatin to cancer progression involves its ability to influence the upregulation and nuclear translocation of NF-κB.[28,29] This is significant because when NF-κB translocates to the nucleus it acts as a transcription factor for several proteins involved in tumor progression.[28-30] Table 3 discusses some proteins induced by NF-κB's transcriptional activity, and describes their respective roles in tumorigenesis. eNAMPT's influence on NF-κB could be due to its insulin-like properties by activating the PI3K/Akt pathway thus causing NF-κB's translocation to the nucleus.[23,31] NFκB's translocation to

the nucleus not only has implications for cancer progression but, more specifically, has also been found to contribute to HCC Sorafenib resistance.

Table 3. Pro-Tumorigenic Effects of NF-κB [29,30]

NF-κB Induced Protein Transcription	Pro-Tumorigenic Effects
VEGF	Pro-inflammatory
Bcl-xL	Anti-apoptotic
COX-2	Pro-inflammatory, Tumor promoting
IL-6	Pro-inflammatory, Metastasis

NF-κB and Sorafenib Resistance

The translocation of NF-κB may also play a role in the development of HCC Sorafenib resistance due to the unintended activation of the PI3K/Akt pathway.[17,32] Proteins transcribed as a result of NF-κB translocating to the nucleus are involved in Sorafenib resistance development and cancer progresison.[30,33] These proteins include Bcl-xL, COX-2, and IL-6.[30,33] COX-2 and IL-6 have both demonstrated to have roles in breast cancer chemoresistance.[14,34] Bcl-xL has specifically demonstrated to promote Sorafenib resistance by preventing apoptosis.[18] A study in 2017 found that by blocking Bcl-xL during Sorafenib treatment in HCC cells a slowing of tumor growth ensued.[18] Altogether, a

potential connection ensues in which high levels of eNAMPT in obese individuals could be increasing the rate and prevalence of HCC and HCC Sorafenib resistance through the over activation of PI3K/Akt and further translocation of NF-κB to the cell's nucleus.[18,19,22,28,30,31,33]

Sorafenib Resistance and Genetics

Genetics may play a role in Sorafenib resistance, though it is a largely unexplored area of research. A recent study published 2020 began to look at a noncoding RNA, IncRNA NEAT1, and how it may play a role in Sorafenib resistance.[35] Researchers took tissue samples from HCC patients and also used HCC cell lines and Sorafenib-resistant HCC cell lines to look at the expression of NEAT1 and how it relates to Sorafenib resistance.[35] It was determined that NEAT1 was found at higher levels in the HCC patient tissue samples.[35] The Sorafenib resistant HCC cell lines demonstrated that higher NEAT1 expression decreased the effectiveness of Sorafenib via miR-149-5p/AKT1 axis.[35]

Understanding genetics, molecular pathways, and cross talk as they pertain to obesity related HCC, and Sorafenib resistance will lead to a comprehensive approach in treating the disease. There are many directions for research foci for obesity related HCC and Sorafenib resistance including epidemiology, genetics, molecular signaling pathways. All of which are necessary in understanding the growing incidence of obesity and HCC. Appropriate cell models are needed to investigate the molecular signaling pathways involved in chemoresistance. Thus, the aim of this study is to develop Sorafenib resistant liver cancer cell lines and use these cells to explore visfatin's role in chemoresistance.

Materials and Methods

Cell Culture

The human HepG2, HUH7, and SNU-449 cell lines were obtained American Type Culture Collection (Manassas, VA). SNU-449 were cultured in RPMI media supplemented with 10% fetal bovine serum, and streptomycin. HepG2 cells were cultured in EMEM media supplemented with 10% fetal bovine serum. HUH7 cells were cultured in DMEM media supplemented with 10% fetal bovine serum.

Treatments to Test for Chemoresistance

Cell lines HepG2, HUH7, and SNU-449 were treated with Sorafenib at the concentration beginning at 5 μM to 10 μM gradually, over approximately four months. Specifically, cells were exposed to 7.5 μM of Sorafenib for approximately one month which allowed the cells to adapt to the new concentration. After one month, the Sorafenib dose was increased to the final concentration of 10 μM. Sorafenib treatments occurred biweekly, one day following passage of the cell lines.

Visfatin Treatments

Cell line HepG2 was exposed to the following treatments in serum-free medium (SFM): (1) 80 ng/mL visfatin + vehicle, (2) 80 ng/mL visfatin + 500 ng/mL LY294002 (PI3K inhibitor) + 10 nM Fk866, (3) 80 ng/mL visfatin + 100 μM α_2-HSG^{bac} (HNMPA) (insulin receptor inhibitor) + 10 nM Fk866, (4) 80 ng/mL visfatin + 500nM PPP (IGF-1 receptor inhibitor) + 10 nM Fk866, (5) 80 ng/mL visfatin + 10 nM Fk866, (6) 80 ng/mL visfatin + 500 ng/mL LY294002, (7) 80 ng/mL visfatin + 100 μM α_2-HSG^{bac}, (8) 80 ng/mL visfatin + 500nM PPP, (9) 500 ng/mL LY294002, (10) 100 μM α_2-HSG^{bac}, (11) 500nM PPP, and (12) vehicle. Concentration amounts are based on previous data.[10,12] Treatment times as described in below assay sections.

Table 4. Summary of Experimental Treatments

Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6
visfatin + vehicle	visfatin + Fk866 + LY294002	visfatin + Fk866 + α_2-HSG^{bac}	visfatin + Fk866 + PPP	visfatin + Fk866	visfatin + LY294002
Treatment 7	Treatment 8	Treatment 9	Treatment 10	Treatment 11	Treatment 12
visfatin + α_2-HSG^{bac}	visfatin + PPP	LY294002	α_2-HSG^{bac}	PPP	vehicle

Cellular Proliferation Assays

Cellular proliferation was determined using a MTT assay in resistant and non-resistant cell lines HepG2, HUH7, and SNU-449. These six cell lines were seeded in a 96-well plate at a concentration of 10,000 cells per each well. The seeded cells were incubated at 37°C and 5% CO_2 for 24 hours prior to treatment with either Sorafenib or DMSO as the control. After 48 hours cellular proliferation was assessed using MTT assay as per Thermo Fisher MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) M6494 instructions.

Colony Formation Assays

Colony formation assay was used to determine cell survival and ability to colonize in resistant and non-resistant HepG2, HUH7, and SNU-449. The six cell lines were seeded in

24 well plates at a density of 2,000 cells per well. Sorafenib treatment was administered, with DMSO treated controls. with non-treated controls, 24 hours after seeding. Media was replaced again 24 hours after treatment. Five days later, cells were washed with PBS, and fixed in 10% paraformaldehyde. After 10 minutes, cells were washed and stained with crystal violet for 15 minutes followed by a washing with water. The number of colonies containing 50 or more cells per colony were compared between resistant and non-resistant cells to determine if Sorafenib decreased colony formation.

Western Blot Analysis

Western blot analysis was performed on resistant and non-resistant HepG2, Huh7, and SNU-449 cells to measure Bcl-xL. Five hundred thousand cells were seeded into each well of 6 well plates. Twenty-four hours after plating, cells were treated with Sorafenib or vehicle for 1 hour. Cell lysates were collected using a lysis buffer (5 ml glycerol, 3.14 ml TRIS 1M pH 6.8, 5ml 10% SDS, 36.86 ml ddH2O). Proteins were quantified using Pierce BCA assay kit (catalog #23335). The samples were then electrophoresed through a 10% gel at 110 volts and transferred to a nitrocellulose membrane for an additional 45 min, also at 110 volts. The membrane was then transferred to Tris buffered saline with TBST for blocking with 5% nonfat dry milk at room temperature for one hour. Anti-Bcl-xL antibody (#2764, Cell Signaling).

Statistical Analysis

Values are presented as mean ± SEM. All experiments were repeated at least three times. Statistical analyses were performed between treatment groups except as noted. Statistical analyses were performed to compare Sorafenib treatments and visfatin treatments to their controls. For all tests, GraphPad Prism 8.0 software was used (GraphPad Software Inc., La

Jolla, CA, USA), and $p < 0.05$ was considered statistically significant. Results for MTT, western, and colony formation assays were compared using Kruskal Wallis test followed by a Mann-Whitney U test when Kruskal Wallis test results are found statistically significant.

Results

Sorafenib Decreases Cellular Proliferation of HCC Non-resistant and Resistant Cell Lines

To demonstrate that resistant HCC cell lines, SNU-449R, HepG2R, and HUH7R can proliferate at a faster rate than their non-resistant counter parts when exposed to the HCC chemotherapy Sorafenib, MTT experiments were performed. The non-resistant and developing resistant HCC cell lines were subject to MTT experiments at four different time points throughout the process of increasing Sorafenib concentrations to reach the IC_{50}. Each increase of Sorafenib exposure to the resistant HCC cell lines took place for approximately one month. Each approximate month of exposure is termed time point and its number of months, abbreviated TP and the month number numerically. MTT assays in addition to other assays were performed after each month, prior to increasing the Sorafenib exposure to the resistant HCC cell lines. The first two time points in which cell proliferation was measured resulted in Sorafenib treatment significantly decreasing all cell line proliferation regardless of developing resistant or non-resistant vs. treatment control in all three cell lines.[36] No significant differences were observed between the developing resistant cells vs. the non-resistant cells in all three cell lines. SNU-449 and HUH7 TP4 results were uncharacteristic of TP3 (Figure 1). When treated with Sorafenib SNU-449R were 54% less proliferative then the SNU-449 non-resistant ($P< 0.05$) and similarly SNU-

449R were 56% less proliferative then SNU-449 non-resistant when both were treated with the control (*P< 0.05*) (Figure 2). HUH7R were 37% less proliferative than SNU-449N when treated with Sorafenib and HUH7R were 35% less proliferative than HUH7 non-resistant when treated with the control (*P<0.05*) (Figure 2). However, HepG2 non-resistant and resistance cell lines were consistent with time points one and two, demonstrating the resistant cells lines were more proliferative when treated with Sorafenib and the control. HepG2R were 41% more proliferative than HepG2 non-resistant when treated with Sorafenib (*P<0.05*) (Figure 2). When treated with the control, HepG2R were 24% more proliferative than HepG2 non-resistant (*P<0.05*) (Figure 2). At time point three HepG2 resistant and non-resistant cell lines were the only cell line that indicated resistant cells were more proliferative than their non-resistant counterpart (Figure 1).

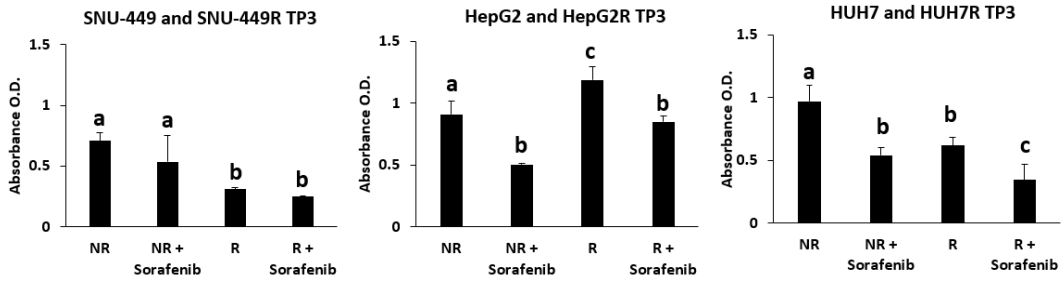

Figure 1. Liver cancer cell viability when exposed to Sorafenib at TP3. MTT assay was used to assess cell viability. Cells were cultured with treatments or control for 48 hours. Viability was assessed by MTT dye conversion. The abbreviations NR and R represent non-resistant and resistant cell lines, respectively. The data shown represents an average of a minimum of three independent experiments. The different letters indicate statistically significant differences between experimental conditions, *P<0.05*.

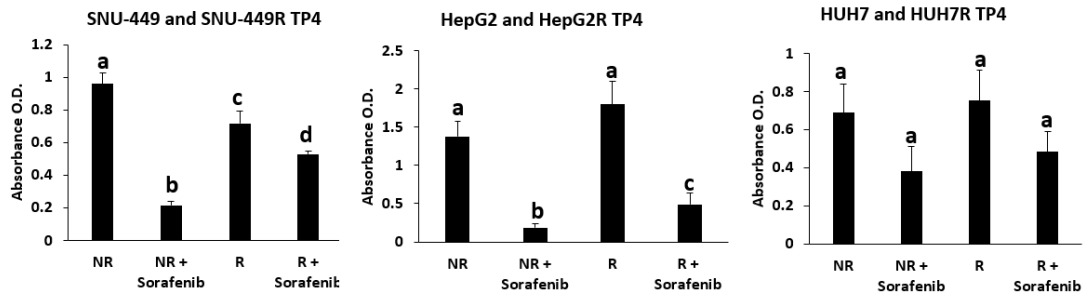

Figure 2. Liver cancer cell viability when exposed to Sorafenib at TP4. MTT assay was used to assess cell viability. Cells were cultured with treatments or control for 48 hours. Viability was assessed by MTT dye conversion. The abbreviations NR and R represent non-resistant and resistant cell lines, respectively. The data shown represents an average of a minimum of three independent experiments. The different letters indicate statistically significant differences between experimental conditions, $P<0.05$.

SNU-449R and HUH7R cells were not more proliferative when compared to non-resistant HCC cells. HepG2R cells were 62% more proliferative than non-resistant cells when treated with Sorafenib ($P< 0.05$). HepG2R cells were also 23% more proliferative than the non-resistant cells when treated with the control ($P< 0.05$). These results indicate that of the three cell lines HepG2R cells are more proliferative in the presence of Sorafenib than HepG2 non-resistant cells suggesting these cells are becoming resistant to the chemotherapy.

Cell Survival of HCC Non-resistant and Resistant Cell Lines

Colony formation assay was used to assess cell survival of SNU-449, HepG2, and HUH7 non-resistant and resistant cells. The ability of a single cell to form a colony is indicative of cell survival and proliferation. For the first two time points none of the cell lines demonstrated developing resistance.[36] At time point three all cell lines yielded varying results. HUH7 non-resistant and HUH7R cells had no significant differences when treated with Sorafenib or the control (Figure 3). SNU-449 began to demonstrate resistance at TP3.

Specifically, SNU-449R vs. SNU-449 non-resistant had 55% higher survival (*P< 0.05*) (Figure 3). The SNU-449R vs. the SNU-449 non-resistant when treated with Sorafenib had an even higher survival difference of 66% (*P< 0.05*) (Figure 3). HepG2 non-resistant cells showed 40% less ability to survive when treated with Sorafenib vs. the control (*P< 0.05*), while HepG2 resistant showed 23% less ability to survive when treated with Sorafenib vs. the control (*P< 0.05*) (Figure 3). HepG2 non-resistant cells demonstrated 26% higher survival when compared to HepG2R with control treatment (Figure 3). No difference was observed when comparing HepG2R when exposed to Sorafenib treatment (Figure 3).

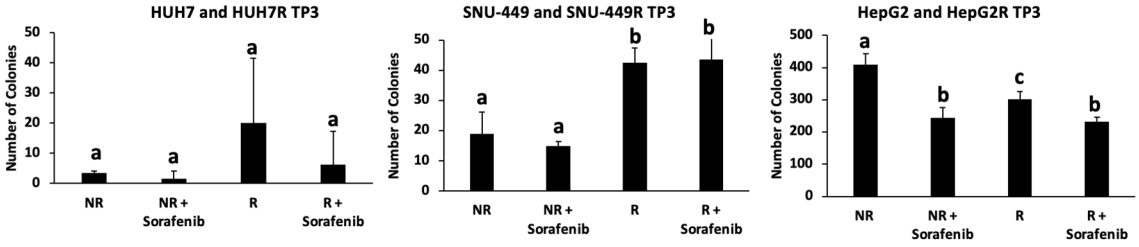

Figure 3. Colony formation in liver cells exposed to Sorafenib at TP3. Colony formation assay was used to assess cell survival and ability to colonize. Cells were cultured with treatments or control for 24 hours. Media was replaced was replaced again 24 hours after treatment and cells were incubated for 5 days. The abbreviations NR and R represent non-resistant and resistant cell lines, respectively. The data shown represents an average of a minimum of three independent experiments. The different letters indicate statistically significant differences between experimental conditions, *P< 0.05*.

At TP4, SNU-449 cells continued to demonstrate the development of resistance. SNU-449R cells had 84% higher survival then SNU-449 non-resistant cells when both were treated with Sorafenib (*P< 0.05*) (Figure 4). In addition, SNU-449 non-resistant cells had a 99% higher survival rate than SNU-449 non-resistant cells treated with Sorafenib (*P< 0.05*) (Figure 4). Thus, in the presence of Sorafenib SNU-449R cells demonstrated ability to survive over SNU-449 non-resistant cells. This shows that SNU-449R cells were developing resistance to the drug and therefore able to survive. HUH7 colony formation assay (CFA) experiment averages were highly skewed. The cell line was unavailable to

repeat experiments. TP4 HepG2 cells demonstrated increased survival. HepG2R cells had a 66% higher survival rate than the HepG2 non-resistant ($P< 0.05$) when exposed to Sorafenib (Figure 4). HepG2 non-resistant cells were 80% less likely to survive when treated with Sorafenib when compared to HepG2 non-resistant cells without Sorafenib ($P< 0.05$) (Figure 4). HepG2R cells exposed to Sorafenib had a 45% decrease in colonies when compared to HepG2R cell without Sorafenib (Figure 4). Although, there was a decrease in HepG2R colonies with Sorafenib, this cell line had more colonies than HepG2 non-resistant.

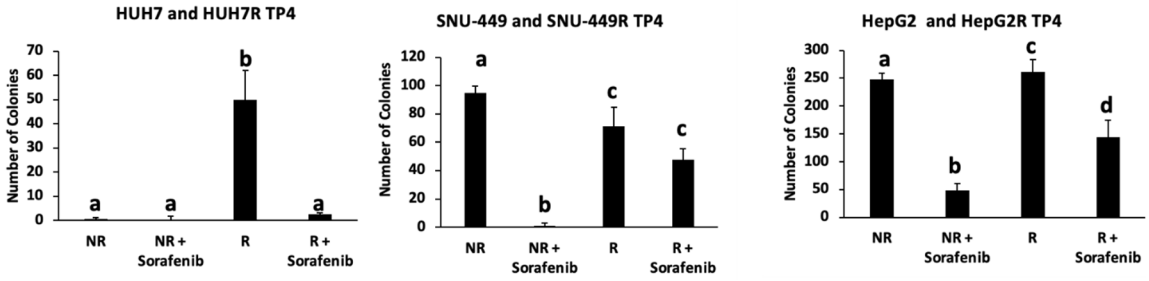

Figure 4. Colony formation in liver cells exposed to Sorafenib TP4. Colony formation assay was used to assess cell survival and ability to colonize. Cells were cultured with treatments or control for 24 hours. Media was replaced was replaced again 24 hours after treatment and cells were incubated for 5 days. The abbreviations NR and R represent non-resistant and resistant cell lines, respectively. The data shown represents an average of a minimum of three independent experiments. The different letters indicate statistically significant differences between experimental conditions, $P< 0.05$.

Bcl-xL Protein Expression in HCC Non-resistant and Resistant Cell Lines

Bcl-xL is an anti-apoptotic protein which promotes cell survival.[30,33] Therefore, the higher the amounts expressed in a cell post treatment reflects how much the cells are resisting cell death. TP3 resulted in higher amounts of Bcl-xL in all resistant cell line, thus demonstrating developing resistance in early stages (Figure 5). At TP3, HUH7 non-resistant cells had 2-fold more Bcl-xL than the HUH7R cells when treated with the control

(*P< 0.05)* (Figure 5). There were no differences between other groups (Figure 5). SNU-449 non-resistant and SNU-449R cells did not show differences between groups regardless of treatment vs. control (Figure 5).

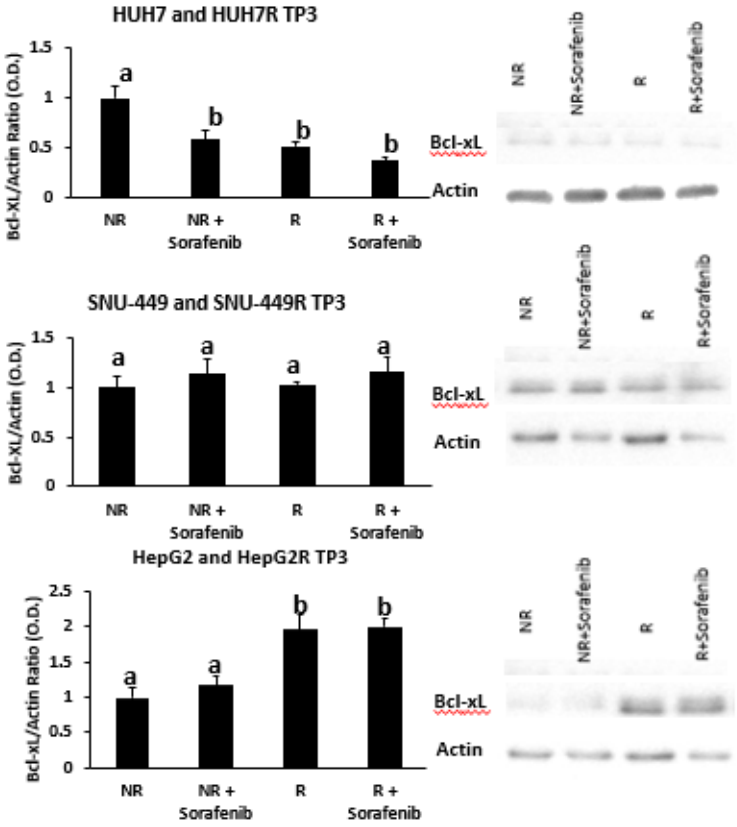

Figure 5. Bcl-xL protein expression in cells exposed to Sorafenib at TP3. Cells were cultured in experimental conditions for 15 min. Western blot analysis was used to measure amount of Bcl-xL and actin. Data shown represent the average of at least three independent experiments. The different letters indicate statistically significant differences between experimental conditions, *P< 0.05*.

At TP4, there were no differences in Bcl-xL protein expression in HUH7 non-resistant and HUH7R cells when treated with Sorafenib compared to the control (Figure 6). SNU-449R cells had 3-fold more Bcl-xL than SNU-449 non-resistant cells when treated with Sorafenib (*P< 0.05)* and SNU-449 resistant cells also had 3-fold more Bcl-xL than the SNU-449 non-resistant when treated with the control (*P<0.05)* (Figure 6). No differences were observed between other groups (Figure 6). HepG2R at TP4 resulted in

2.5-fold higher Bcl-xL in resistant vs. non-resistant SNU-449 when treated with Sorafenib and 64% more Bcl-xL protein in SNU-449R when compared to SNU-449 non-resistant when treated with the control (Figure 6). At time point four, SNU-449 and HepG2 resistant cells lines demonstrate resistance to cell death over their non-resistant counterparts while HUH7 resistant cells did not show the same results in Bcl-xL protein expression when compared to HUH7 non-resistant cells (Figure 6).

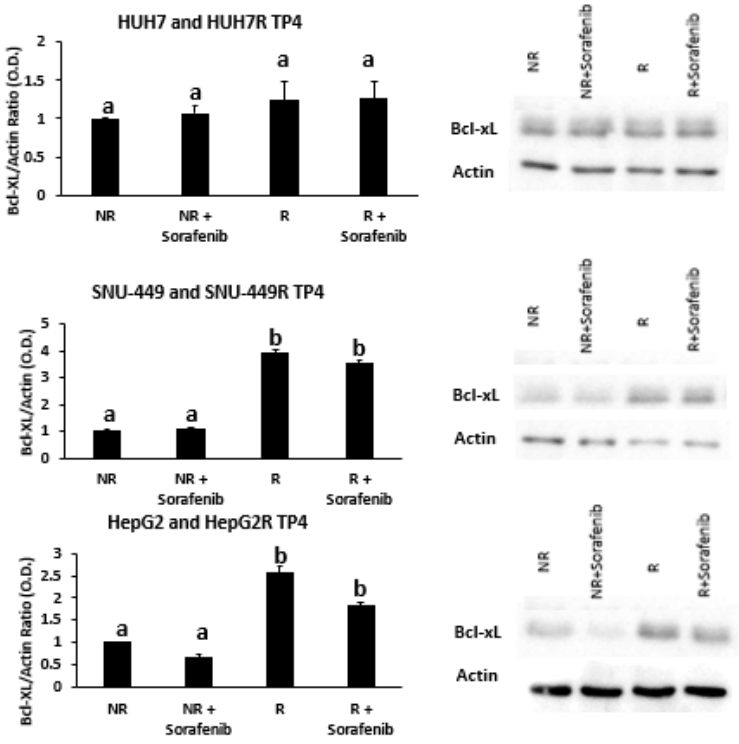

Figure 6. Bcl-xL protein expression in cells exposed to Sorafenib at TP4. Cells were cultured in experimental conditions for 15 min. Western blot analysis was used to measure amount of Bcl-xL and actin. Data shown represent the average of at least three independent experiments. All conditions were normalized to NR. The different letters indicate statistically significant differences between experimental conditions, $P< 0.05$.

Visfatin's Effects on HepG2R Cell Proliferation

HepG2 cells exposed to vistfatin were 22% more proliferative than the control (*P< 0.05)* (Figure 7). Visfatin was 30% and 29% more proliferative than visfatin + FK866 + LY and visfatin + FK866 + PPP, respectively (*P< 0.05)* (Figure 7). However, no difference was observed when comparing visfatin to the inhibitors PPP, and LY alone. Visfatin + FK866 + LY was 29% less proliferative when compared to visfatin + LY (*P< 0.05)* (Figure 7). Similarly, visfatin + FK866 + PPP, was 28% less proliferative than visfatin + PPP (*P< 0.05)* (Figure 7). HNMPA was 32% more proliferative than the control and 13% more proliferative than visfatin + control (*P< 0.05)*. No differences were observed between HNMPA and visfatin alone or LY and PPP alone or combined with visfatin (Figure 7). However, when compared to visfatin + FK866 + HNMPA, HNMPA was 39% more proliferative (*P< 0.05)* (Figure 7).

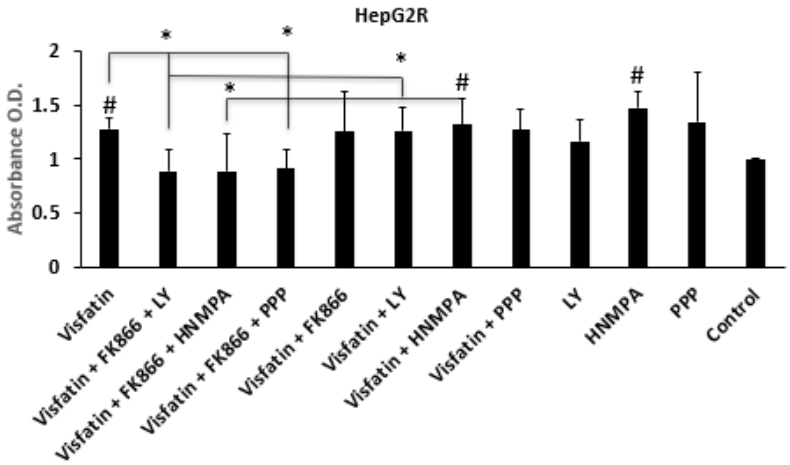

Figure 7. Effects of visfatin on HepG2R cells. MTT assay was used to assess cell viability. Cells were cultured with treatments or control for 48 hours. Viability was assessed by MTT dye conversion. The data shown represents an average of a minimum of three independent experiments. The asterisks indicate statistically significant differences between experimental conditions, $P < 0.05$ and the pound sign indicates statistical significance compared to control $P < 0.05$.

Discussion

Chemoresistance, inherent or developed, in hepatocellular carcinoma is one of the main reasons survival rates are devastating low.[5,37] Understanding the mechanisms by which hepatocytes are resistant or how they develop resistance is key to creating more affective approaches to treatment. In patients who develop resistance, chemotherapy is given in increasing doses over time and resistance is developed within three to six months.[4,18] To create and study chemoresistance in liver cancer cell lines, similar conditions must be created to recapitulate chemoresistance. Through exposing hepatocellular carcinoma cell lines HepG2, HUH7, and SNU-449 to increasing doses of Sorafenib for several months up to the IC50 resistance was demonstrated in HepG2 and SNU-449.[37]

In the first two months of continued exposure to Sorafenib, results were inconsistent.[36] This is expected due to the low Sorafenib exposure and the short length of time.[18] In the 3rd month of treatment, an array of expected and unexpected results emerged. HUH7 cells continued to show no indication of developing resistance, no significant difference between the HUH7R and HUH7 non-resistant SNU-449 cell lines begin to demonstrate resistance. In the presence of Sorafenib SNU-449R had a significantly higher survival rate and avoidance of cell death which might be through the upregulation of Bcl-xL. The reverse was true of proliferation in the SNU-449R cells. SNU-449R cell were significantly less proliferative than their non-resistant counterparts when treated with Sorafenib. Also, mixed results were observed with the HepG2 cells. Bcl-xL in HepG2R and HepG2 non-resistant were insignificant and HepG2 non-resistant cells had a higher survival rate when treated with Sorafenib than HepG2R cells. Proliferation, however, was

significantly higher in HepG2R cells then HepG2 non-resistant cells when treated with Sorafenib in the third month of treatment. This could be due to other compensatory mechanisms not yet examined during the development of Sorafenib resistance.[18]

In the fourth month of Sorafenib treatment, the final time goal, resulted in two of the three cell lines and their resistant counterparts, SNU-449R, and HepG2R, exhibiting chemo resistant characteristics. HUH7 had no significant findings across all experiments. This could be due to sensitivity and passage differences in the HUH7 cell line when compared to SNU-449 and HepG2 cell lines. HUH7 cells did not have a comparable confluency prior to biweekly passages when compared to the HepG2 and SNU-449 cells lines unless the HUH7 cells were centrifuged to fully remove trypsin before putting them into a new flask. The higher sensitivity coupled with the additional steps of passage made HUH7 cells at a higher risk for cell death. SNU-449R and HepG2R cell lines both had higher proliferation, cell death resistance, increased Bcl-xL protein expression, and survival when compared to non-resistant cells treated with Sorafenib. Thus, indicating that the increasing doses of Sorafenib administered to the SNU-449R and HepG2R cells resulted in developed chemoresistance.

HepG2R cells were treated with visfatin in combination with inhibition of visfatin's intracellular action (FK866), PI3K inhibitor (LY), insulin receptor inhibitor (HNMPA), and IGF-1 receptor inhibitor (PPP) in a variety of combinations. Visfatin alone when compared to the control had 22% more proliferation. Visfatin contributing to higher proliferation was expected as discussed in the background information above. Cells treated with visfatin alone were more proliferative compared to cells in which visfatin intracellular action, PI3K and IGF-1 were inhibited. However, there were no differences when

comparing cells treated with visfatin alone to to cells treated with visfatin and PI3K and IGF-1 inhibitors. This data shows that when combining receptor inhibitors and FK866, which blocks visfatin's intracellular action of regenerating NAD+ cellular proliferation is decreased. This is likely due to cellular energetics slowing the rate of growth due to less ATP production.[20] Cells treated with visfatin alone were not more proliferative compared to visfatin treatment combined with insulin receptor inhibition. Furthermore, when the insulin receptor was inhibited, cells were more proliferative than the control even in the presence of visfatin. These results appear to be counterintuitive since inhibiting the insulin receptor would seem to indicate less proliferation.[38] Due to these contradicting results a compensatory mechanism may be the reasoning. In a 2013 study involving insulin resistance it was found that growth factors and hormones could be responsible for increased cellular proliferation even when insulin resistance was present.[39] The two hormones found to be part of the compensatory mechanism for increased proliferation of pancreatic beta cells were betatrophin and hepatocyte growth factor (HGF).[39] This type of compensatory mechanism could potentially explain why cells increase cell proliferation with the insulin receptor is inhibited.[39] Another recent paper from 2019 demonstrated similar findings but through a different mechanism.[40] Pires K, et al. found a novel mechanism in which insulin receptor deficient cardiomyocytes could still resist cell death in the presence of insulin, in part through the IGF-1 signaling pathway.[40] The insulin receptor deficient cardiomyocytes when treated with insulin resisted cell death when compared to cells treatment group without insulin.[40] The authors report that this is the first observation of this mechanism.[40] Though a different cell line, this data could also indicate the possibility and explanation for increased cellular proliferation when the insulin receptor is inhibited in the HepG2R cells.

Inhibiting the IGF-1 and insulin receptors simultaneously with other treatment conditions could unveil if this theory that insulin can promote resistance to cell death through the IGF-1 receptor warrants further investigation.

II. Future Directions

Further Exploration of Visfatin's Effects on HCC Chemoresistant Cell Lines

Based on what is known about the connection between obesity and cancer, continuing to examine the problem at a molecular level is warranted.[11] HCC and obesity are both increasing in prevalence and HCC's main treatment, Sorafenib, is ultimately ineffective due to primary and developed resistance.[5,11,37] Obesity can perpetuate HCC via upregulation of cancer promoting pathways.[11] Visfatin, an adipocytokine, has demonstrated to be one specific protein that can mimic like insulin, potentially furthering the progression of HCC but also primary and/or secondary resistance.[23,28] Using Sorafenib resistant cell lines and examining how visfatin could have effects on chemoresistance could be meaningful when determining how to decrease the high death rate associated with HCC.

Visfatin alone promoted proliferation of HepG2R cells when compared to the control. Another assessment of proliferation with a treatment group including the insulin receptor inhibitor, IGF-1 receptor inhibitor and the inhibitor to block visfatin intracellular action vs. a visfatin alone treatment group would be helpful in determining if visfatin may be working extracellularly though other means or working via alternating between the insulin receptor and the IGF-1 receptor. In addition to assessing cellular proliferation, it would be valuable to access cell survival, cell death, and pro-tumor mechanisms (e.g., translocation of NF-κB to the nucleus). These assessments would provide further insight into visfatin's overall and extracellular effects on chemoresistant liver cancer cell lines. In addition to the HepG2 cells SNU-449R demonstrated resistance at 4 months of treatment, indicating these cells could also be used to explore mechanisms of chemoresistance.

Compensatory Mechanisms of Insulin and IGF-1 Receptor Inhibition

Further exploration of the compensatory mechanisms associated with inhibition of the insulin receptor could also be promising. Betatrophin and HGF were found to be involved in cell survival of pancreatic beta cells.[39] Assessing the amount of or presence of betatrophin and HGF in cells exposed to an insulin receptor inhibitor and visfatin compared to cells with inhibition of IGF-1 receptor and the insulin receptor could demonstrate these possible bypasses which may promote cell survival and proliferation. In a 2010 study involving breast cancer cells it was found that inhibiting both the IGF-1 receptor and insulin receptor decreased cell survival vs. only single inhibitor treatment groups.[41] Determining if visfatin could be mediating its effects through IGF-1 receptor in addition to its already known insulin mimetic effects would be important due to the downstream signaling effects of Akt and tumorigenesis.[14,24,28,41]

In summary, due to the increase in liver cancer incidence and chemoresistant-associated mortality, it is imperative to better understand the mechanisms of chemoresistance to improve liver cancer outcomes. Appropriate cell models are needed to investigate the development of chemoresistance. Thus, we developed two liver cancer chemoresistant cell models which can used to identify strategies to sensitize cancer cells to chemotherapy such as targeting obesity associated mechanisms.

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