ELUCIDATING THE POTENTIAL MECHANISM OF VISFATIN, AN ADIPOCYTOKINE WITH INSULIN-LIKE PROPERTIES

by

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A thesis submitted to the Graduate Council of
Texas State University in partial fulfillment
of the requirements for the degree of
Master of Science
with a Major in Human Nutrition
May 2019

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ACKNOWLEDGEMENTS

I would like to sincerely thank Dr. Ramona Price for being a wonderful thesis advisor, you are an inspiration. Thank you for your patience, kindness, and encouragement throughout this journey. I would also like to thank my thesis committee Dr. Krystal Zuniga and Dr. Christopher Jenney for taking the time to be a part of this journey with me. Also, thank you Dr. Zuniga for always taking time out of your day to give me advice on my future endeavors. I would also like to thank my mom for always pushing me to my fullest potential and my fiancé for always being there for me and offering words of encouragement through rough times.

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LIST OF ABBREVIATIONS

Abbreviation **Description**

ATCC – American Type Culture Collection

COX-2 – Cyclooxygenase-2

DMEM – Dulbecco's Modified Eagles Media

DMSO – Dimethyl sulfoxide

EMEM – Eagles Minimum Essential Medium

EMT – Epithelial to Mesenchymal Transition

eNAMPT – extracellular NAMPT

FBS – fetal bovine serum

HCC – Hepatocellular Carcinoma

HNMPA – Hydroxy-2-naphthalenylmethylphosphonic acid

IGF-1 – Insulin – like Growth Factor -1

IGF-1 R – Insulin – like Growth Factor -1 Receptor

iNAMPT – intracellular NAMPT

IR – Insulin Receptor

IRS -1 – Insulin Receptor Substrate 1

IRS -2 – Insulin Receptor Substrate 2

JRCB – Japanese Cancer Research Resources Bank

LDH – Lactate Dehydrogenase

NAFLD – Non-Alcoholic Fatty Liver Disease

NAM – Nicotinamide

NAMPT – Nicotinamide Phosphoribosyltransferase

NHANES – National Health and Nutrition Examination Survey

NMN – Nicotinamide Mononucleotide

PI3K – Phosphoinositide 3-Kinase

PPP – Picropodophyllin

TBST – Tris Buffered Saline with Tween

VEGF – Vascular Endothelial Growth Factor

I. REVIEW OF THE LITERATURE

Obesity and Liver Cancer

The prevalence of obesity has been rising over the past few decades, not only in the United States but worldwide.[1] In the U.S, according to the 2013-2014 national health and nutrition examination survey (NHANES) data, 1 in 3 people are obese, and 2 in 3 people are either overweight or obese.[2] Worldwide, according to the World Health Organization, an overwhelming 1.9 billion adults are overweight, and of these, 650 million of them are obese.[3] Obesity is a major health concern. Obesity is a risk factor for a plethora of health problems including diabetes, non-alcoholic fatty liver disease (NAFLD), cardiovascular disease.[4] Researchers are also recently becoming aware of its association to multiple types of cancers, including liver cancer.[5]

Liver cancer is the fifth most common cancer worldwide and is the 3rd leading cause of cancer death.[6] The American Cancer Society estimates that in the U.S., in the year 2018, there will be about 42,220 new cases of liver cancer diagnoses, and about 30,200 liver cancer related deaths.[7] The primary subtype for liver cancer is hepatocellular carcinoma (HCC), accounting for 70-85% of liver cancer diagnoses.[5] Liver cancer mainly stems from a history of hepatitis B virus, hepatitis C virus, or alcohol abuse; however, anywhere from 15-50% of liver cancer cases cannot be attributed to these causes.[5] Recently, researchers have found an increasing amount of HCC cases associated with NAFLD as an underlying cause.[5] NAFLD is a spectrum of liver disease that manifests in the presence of obesity and metabolic syndrome.[6]

Increasing amount of evidence has linked obesity and HCC.[8] In a study including more than 300,000 Swedish men, individuals with a BMI greater than 30 kg/m^2 had a relative risk of HCC, 3.1 times higher than normal weight individuals.[6] In another study

including more than 900,000 individuals, Calle et al. found that obese individuals were 4.5 times more likely to die from HCC compared to normal weight subjects.[6] Recent evidence found an increased association between HCC and NAFLD. In a study analyzing 623 individuals from 2000 to 2010, researchers found an unprecedented 10-fold increase in NAFLD associated HCC in this time period.[5] With obesity steadily increasing over the past decades, and considering obesity is directly linked to NAFLD, NAFLD associated HCC may also be increasing in prevalence, making it a substantial health concern. The mechanisms associated with obesity related cancer can be found in Figure 1.

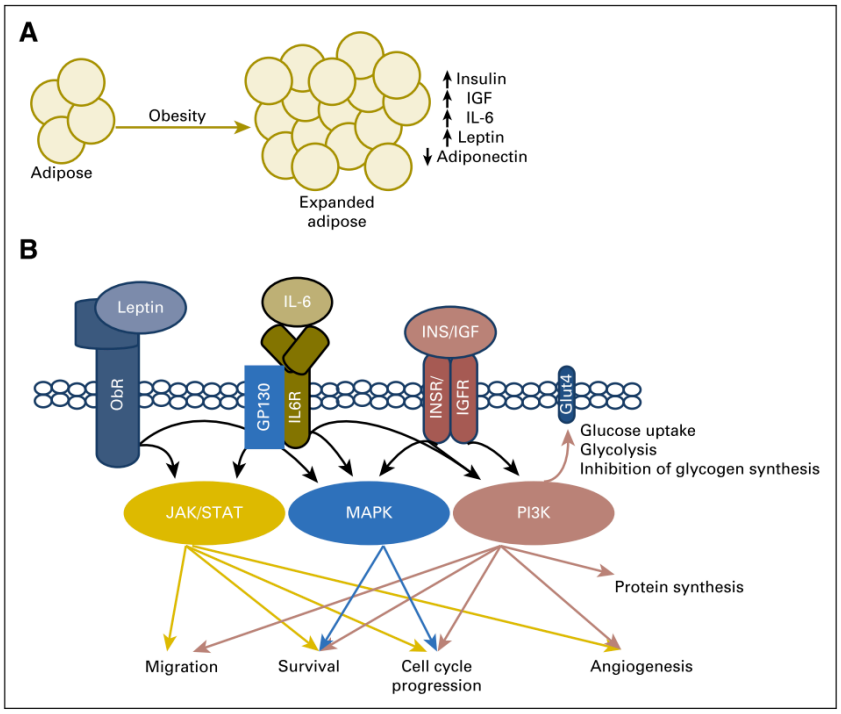

Figure 1. Mechanisms linking obesity and cancer [9]

Sorafenib

Another important issue concerning HCC is the high death rate among patients with HCC.[10] Unfortunately, HCC is commonly diagnosed at late stages and the available treatment, sorafenib, has a high incidence of resistance, with many patients showing signs

of improvement during the first months only to develop a resistance to the drug shortly after.[10] Sorafenib works as a multikinase inhibitor, with some of its main targets being several isoforms of the Raf serine/threonine kinase, three of the vascular endothelial growth factor (VEGF)-receptors, platelet derived growth factor receptor, among others.[11] By inhibiting these proteins, sorafenib acts as an anti-proliferative and anti-angiogenic chemotherapeutic drug. However, studies demonstrate that by inhibiting the known proliferative RAF/MEK/ERK pathway, an upregulation in another known proliferative pathway, the phosphoinositide 3-kinase (PI3K)/Akt pathway is occurring due to crosstalk between the two.[10] This upregulation in the Akt pathway, along with other mechanisms shown in Figure 2, may have a significant contribution to the increased resistance seen in many patients treated with sorafenib.[10] In a recent study of colon cancer patients, high insulin levels which are typically found in obese individuals, were more prevalent in patients with chemo-resistance.[1] However, by inhibiting the PI3K/Akt pathway a sensitization to the drug occurred, reversing resistance.[1] Individuals who are obese and have HCC, may be at risk of chemo-resistance, considering the Akt signaling pathway may not only be upregulated due to the crosstalk between ERK and Akt but also from high levels of insulin. Therefore, Akt is a vital target for prevention of chemoresistance.

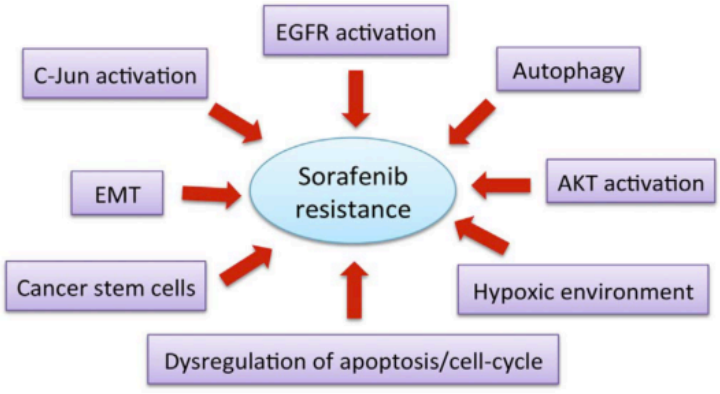

Figure 2. Mechanisms Involved in the Development of Chemo Resistance[11]

Visfatin

Overweight and obese individuals have also been found to have higher plasma levels of an adipocytokine, visfatin, also known as extracellular nicotinamide phosphoribosyltransferase (eNAMPT).[12,13] This adipocytokine may also be upregulating pathways that are very similar to those that insulin activates, making it another significant target in cancer treatment. Visfatin's intracellular counterpart, intracellular NAMPT (iNAMPT), on the other hand, is well known for its enzymatic activities such as its role in regenerating nicotinamide mononucleotide (NMN) from nicotinamide (NAM). Extensive research has been done on NAMPT's intracellular functions; however, research is still being conducted to identify eNAMPT/visfatin's role and mechanism in disease. It is hypothesized that visfatin acts as an insulin mimetic, and numerous data support this.[14] Data has shown visfatin to have insulin like effects in osteoblasts as well as in pancreatic beta-cells.[15,16] Brown et al, when assessing the effects of visfatin in pancreatic beta-cells, found that visfatin significantly increased insulin receptor phosphorylation.[16] In a similar study, Xie et al. found that visfatin stimulated the phosphorylation of insulin receptor substrate 1 and insulin receptor substrate 2 in osteoblasts.[15] However, what receptor or whether visfatin is binding to a receptor is still unknown. A previously published study, which showed visfatin bound the insulin receptor was retracted, and no new data has been published to prove or disprove that visfatin does not in fact bind the insulin receptor. Therefore, in order to move forward in finding visfatin's receptor, knowing it does or does not bind to the IR or IGF1-R can then lead to new research on identifying other possible tyrosine kinase receptor candidates.

In multiple cancer cell lines visfatin has been shown to upregulate signaling pathways involved in tumorigenesis, including the PI3K/Akt pathway, and the ERK

signaling pathway (Figure 3).[1713] NFκB, which is a known transcription factor for many tumorigenic proteins, has also been found to be upregulated by visfatin.[18] Interestingly, recent research involving sorafenib resistance has led to data revealing that both the activation of the PI3K/Akt signaling pathway and the upregulation of NFκB promoted sorafenib resistance in sorafenib resistant hepatocellular carcinoma cells.[19,20] By inhibiting PI3K/Akt and NFκB, resistance was reversed, and the resistant cells became sensitized to sorafenib. Visfatin upregulates both NFκB and the PI3K/Akt pathway, a phenotype that is very similar to the sorafenib resistant phenotype and signifies that visfatin may be an important target for obese individuals receiving sorafenib in order to potentially prevent or delay sorafenib resistance. However, understanding the mechanism by which visfatin works will be key in developing a drug that can target visfatin.

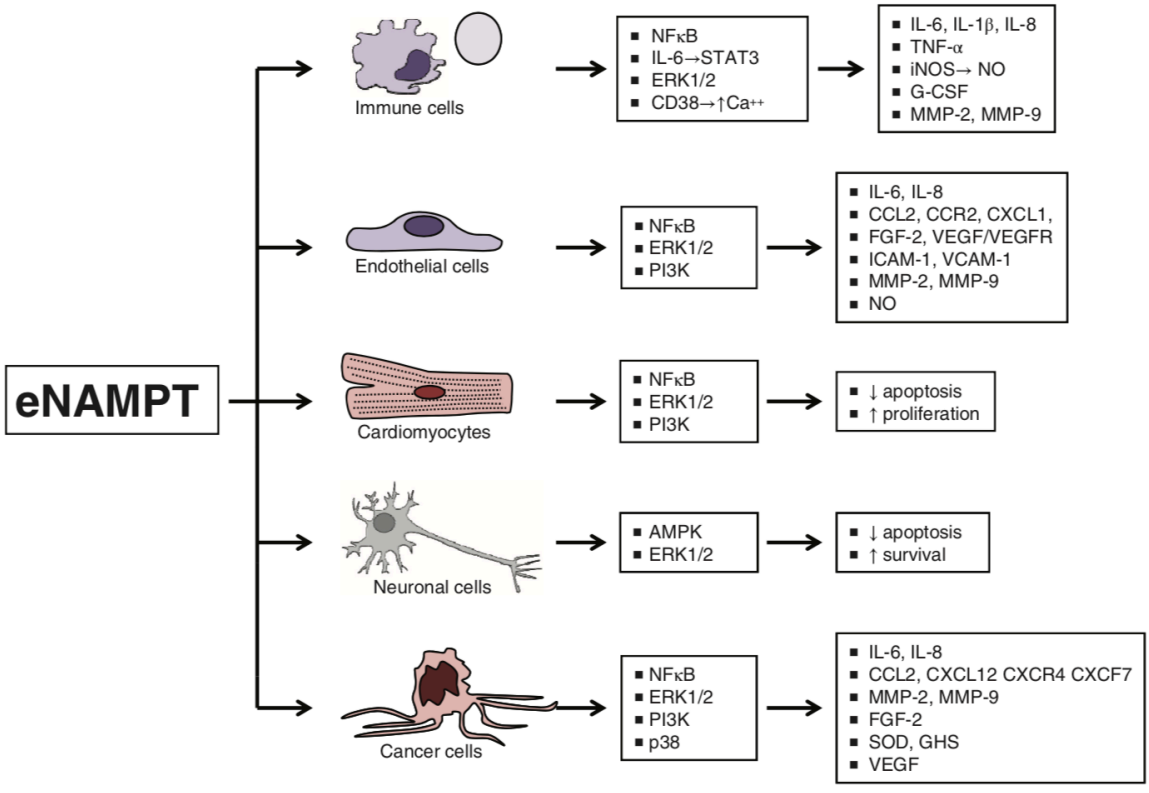

Figure 3. Effects of Visfatin in Different Types of Cells[13]

Visfatin and NFκB

A possible mechanism by which visfatin is mediating PI3K/Akt and NFκB activation, may be through the insulin or IGF signaling pathway. Research in aging and disease has shown that PI3K/Akt activation by either the insulin receptor or IGF-1R can lead to NFκB activation.[21] Because visfatin has been shown to act as an insulin mimetic, visfatin may be able to induce insulin receptor or IGF-1R activation, leading to the activation of the PI3K/Akt pathway, which may result in the activation of NFκB.

NFκB has the potential to activate the transcription of many tumorigenic genes following its translocation into the nucleus where it acts as a transcription factor.[22,23] Some of the genes activated by NFκB include: pro-angiogenic VEGF, pro-inflammatory and metastatic IL-6, anti-apoptotic Bcl-2 and Bcl-xL, and inflammatory and tumor promoting cyclooxygenase-2 (COX-2).[22,23] NFκB has not only been shown to be upregulated in sorafenib resistance but also in many other chemo-resistant phenotypes,[24] which may be related to NFκB's ability to transcriptionally activate several pro-tumorigenic genes.

Many of the genes activated by NFκB have been shown to be upregulated in chemo-resistance including IL-6, Bcl-2, Bcl-xl, and COX2.[24,23] Researchers have found that IL-6 mediates tamoxifen resistance in breast cancer by inducing epithelial to mesenchymal transition (EMT) and activating the JAK/STAT and PI3K/Akt pathways.[25] EMT plays a vital role in a tumors ability to become metastatic and infect nearby and distant tissues.[25] Bcl-2 and Bcl-xl have both been shown to be upregulated in sorafenib resistance and possibly mediate resistance through their anti-apoptotic capabilities.[11]

Sorafenib in combination with small molecular inhibitors to inhibit Bcl-xl led to a significant reduction in tumor growth.[11] In breast cancer cells, COX-2 was also upregulated in association to chemoresistance.[26]

Interestingly, studies have shown that visfatin has the potential to induce the expression or concentration of many of the pro-tumorigenic genes activated by NFκB, including IL-6, VEGF, Bcl-2, and COX-2. In pancreatic beta cells, visfatin was able to inhibit apoptosis by increasing the expression and concentration of anti-apoptotic Bcl-2 through the MAPK and PI3K/Akt pathways.[27] *In vitro*, visfatin was also able to increase the expression of COX-2 in granulosa luteal cells.[28] In human endothelial cells, visfatin upregulated the expression and protein concentrations of VEGF.[29] Finally, in a mouse model visfatin induced the expression of IL-6.[18]

Previous data suggests, visfatin acts as an insulin mimetic and can induce the PI3K/Akt and MAPK signaling pathways. Visfatin is found to be overexpressed in some cancers and is significantly elevated in obese individuals. The aim of this study is to identify if visfatin is binding the insulin and IGF-1 receptor, and if so, then block both receptors to examine if visfatin can still carry out its pro-tumorigenic effect. Additionally, sorafenib resistant cell lines will be developed to facilitate further studies on visfatin's effect in a sorafenib resistant environment.

II. ELUCIDATING THE POTENTIAL MECHANISM BY WHICH VISFATIN PROMOTES A TUMORIGENIC PHENOTYPE

Abstract

The data supporting the link between obesity and liver cancer is ever-growing. Visfatin is an adipocytokine, found at significantly higher levels in obese individuals, and is also overexpressed in certain cancers. Numerous amounts of data demonstrate that visfatin is an insulin mimetic and can promote various proliferative pathways. Our objective is to determine whether visfatin can bind either insulin or IGF-1 receptor. Following the co-immunoprecipitation assay, the resulting data suggested that visfatin was able to bind both IGF-1 receptor and insulin receptor. Both the IGF-1 and insulin receptor were inhibited to examine whether visfatin's effects could be attenuated. HepG2 and Huh7 cell lines were utilized as a model for HCC. The following experiments were conducted: coimmunoprecipitation, MTT assay, LDH (lactate dehydrogenase) Cytotoxicity Assay, Colony Formation Assay, and Western Blot Analysis. The following proteins were analyzed as downstream markers using western blot analysis: p-Akt, NFκB, COX-2, VEGF, and Bcl-xl. Inhibition of both receptors led to significant decreases in cellular proliferation and significant increases in cytotoxicity in the presence of visfatin. Inhibition of both receptors also led to decreases in protein concentrations of NFκB, COX-2, VEGF, and p-Akt in comparison to visfatin alone. Overall, visfatin bound the insulin and IGF-1 receptor and may be exerting its proliferative and pro-tumorigenic effects via the insulin and IGF-1 signaling pathways.

Introduction

Liver cancer is the fifth most common cancer worldwide, and the third leading cause of cancer deaths. Liver cancer stems from a range of causes including hepatitis B and C virus, or alcohol abuse. An increased correlation between non-alcoholic fatty disease (NAFLD) and liver cancer has been identified. NAFLD stems from the presence of obesity and metabolic syndrome. Obesity is becoming increasing prevalent with 1 in 3 people in the United States being obese and an increasing amount of evidence has linked obesity to an increased risk liver cancer. Visfatin, an adipocytokine found at significantly higher levels in obese individuals compared to normal weight and overweight individuals, has been shown to increase cellular proliferation in many cancer cell lines, by increasing, PI3K/Akt and MAPK pathways. Preliminary data has also suggested that visfatin can act as an insulin mimetic by inducing the phosphorylation of both the insulin receptor, insulin receptor substrate 1 (IRS-1) and insulin receptor substrate 2 (IRS-2). In addition, researchers have also found that visfatin has the capability to induce NFκB translocation into the nucleus. NFκB is a pro-tumorigenic transcription factor that promotes the transcription of many tumorigenic genes. Interestingly, visfatin can increase protein expression of many of the genes transcribed by NFκB. However, no data to date has explored whether visfatin is binding to a specific receptor and how exactly it is inducing the various tumorigenic responses seen in cancerous cells.

Therefore, recognizing that visfatin has the potential to induce the expression and protein concentrations of many of the genes activated by NFκB and also activate the PI3K/Akt pathway and induce the translocation of NFκB, and is an insulin mimetic; visfatin may be exerting its effects through the insulin or insulin-like growth factor – 1 (IGF-1) signaling pathway. In doing so, it may be activating the PI3K/Akt signaling

pathway, by binding the IGF-1 or insulin receptor, which may then induce the translocation of NFκB. NFκB can then act as a transcription factor, activating the transcription of tumorigenic genes.

Methods

Cell Culture

HepG2 and Huh7 cells were used as a model for hepatocellular carcinoma. HepG2 cells were purchased from the American Type Culture Collection (ATCC) and Huh7 cells were purchased from the Japanese Cancer Research Resources Bank (JCRB). HepG2 cells were cultured in Eagles Minimum Essential Medium (EMEM) which was supplemented with 10% fetal bovine serum (FBS), streptomycin, and penicillin. Huh7 were cultured in Dulbecco's Modified Eagles Media (DMEM) with low glucose which was supplemented with streptomycin, penicillin and 10% FBS. The cells were kept at 37° C in a 5% (v/v) CO_2 humidifier.

Co- Immunoprecipitation

To analyze the interaction between visfatin and the insulin receptor and IGF-1 receptor, the Pierce™ Classic Magnetic IP/Co-IP Kit (#88804) was utilized. Cells were seeded in 6-well plates at 1-2 million cells/well in triplicate per treatment. The cells were then serum starved for 3 hours and then treated for 30 minutes with either 40 ng/mL of visfatin or the cells specific media containing 20% FBS. FBS (complete media) was used as a control because it contains insulin and IGF-1, which can also bind the receptors. Following treatment, cells were lysed with ice cold lysis buffer from the kit for 5 minutes. Cells were then collected, and a protein determination was conducted using the

Pierce BCA protein assay kit (#23335). Equal amounts of protein (500-1000 µg) were transferred to a new microtube, where the final volume was brought up to 500 µL with lysis buffer. Ten µg of either anti-IGF-1 receptor antibody (Novus Biologicals, #NB110-87052) or anti-insulin receptor (Novus Biologicals, #NBP2-67726) were added to the cell lysates and incubated overnight at 4°C while rotating. 25µL of magnetic beads that had been previously washed were added to the cell lysates which contained the anti-body mixture. The magnetic beads and anti-body mixture were incubated at room temperature while rotating for one hour. The mixture was then placed on a magnetic stand where the beads were collected. The remaining mixture not bound to the magnets were collected and labeled as the preclear, which should have contained anything that was not bound to either IGF-1 or insulin receptor. The magnetic beads were then washed before the addition of 100 µL of Elution Buffer which removed the target antigen (Insulin or IGF-1 receptor) from the magnetic beads. A protein determination was then conducted to determine protein concentrations for the pre-clear solution and antigen mixture. Following the protein determination, a Western blot analysis was conducted to determine whether visfatin was binding the IR, IGF1-R or both. A NAMPT/Visfatin antibody (AdipoGen Life Sciences, #AG-40A-0056Y-CO10) was used to measure visfatin on the nitrocellulose membrane. As a secondary antibody, the Thermo Scientific Clean Blot IP Detection Reagent (#21230) was used to remove any interference from the heavy and light chain IgG bands.

Treatments

Following the co-immunoprecipitation, HepG2 and Huh7 cells were treated in serum free media (SFM) with either dimethyl sulfoxide (DMSO) as a vehicle control,

80ng/mL of visfatin[30], 80ng/mL visfatin + 10 μM LY290004[31] (Akt inhibitor), 80ng/mL visfatin + 100 μM Hydroxy-2-naphthalenylmethylphosphonic acid (HNMPA)[32] (IR-specific inhibitor), 80ng/mL visfatin + 500 nM of Picropodophyllin (PPP)[33] (IGF1-R specific inhibitor) and 80ng/mL visfatin + 100 μM HNMPA + 500 nM of PPP for the remainder of the experiments. Inhibitors were added 1 hour prior to the addition of visfatin in all experiments. Concentrations of visfatin and inhibitors used in previous studies were utilized for this study.

Western Blot Analysis

Western blot analysis was used to assess whether visfatin was able to alter the protein concentrations of certain proteins through either the insulin or IGF-1 receptor, or Akt pathway. Downstream markers of the insulin and IGF-1 signaling pathway as well as proteins transcriptionally activated by NFκB were assessed.

HepG2 and Huh7 cells were seeded in 6-well plates at 400,000 cells/well for 24 hours. Following seeding, cells were serum starved for 24 hours. Cells were then treated with either DMSO as a vehicle control, 80ng/mL of visfatin, 80ng/mL visfatin + 10 μM LY290004 (PI3K inhibitor), 80ng/mL visfatin + 100 μM HNMPA (IR-specific inhibitor), 80ng/mL visfatin + 500 nM of PPP (IGF1-R specific inhibitor) and 80ng/mL visfatin + 100 μM HNMPA + 500 nM of PPP for 30 minutes to analyze phosphorylated proteins and 24 hours to analyze all other proteins. Cell lysates were collected using lysis buffer (5 ml glycerol, 3.14 ml TRIS 1M pH 6.8, 5 ml 10% SDS, 36.86 ml ddH_2O). Samples were quantified using the Pierce BCA protein assay kit (#23335) to ensure equal protein loading. The samples were then electrophoresed through an 8% gel at 110 volts. The gel was then transferred on to a nitrocellulose membrane for 45 minutes at 100 volts.

Thereafter, the membrane was blocked in 5% nonfat dry milk in Tris buffered saline with Tween (TBST) for one hour at room temperature. Proteins were then probed utilizing corresponding primary and secondary antibodies. Primary antibodies used for probing include: p-Akt (Cell Signaling Technology, #4060) , p-Erk (Cell Signaling Technology, #4370) total-Akt (Cell Signaling Technology, #4685), total-Erk (Cell Signaling Technology, #4695) NFκB (Santa Cruz Biotechnology, #sc-8008) Cox-2 (Invitrogen, #PA5-17614), VEGF (Novus, # NB100-2381SS) and Bcl-XL (Cell Signaling Technology, #2764).

Cytotoxicity

HepG2 and Huh7 cells were seeded at 10,000 cells/well for 24 hours. Thereafter, the following treatments were added: DMSO as a vehicle control, 80ng/mL of visfatin, 80ng/mL visfatin + 10 μM LY290004 (PI3K inhibitor), 80ng/mL visfatin + 100 μM HNMPA (IR-specific inhibitor), 80ng/mL visfatin + 500 nM of PPP (IGF1-R specific inhibitor) and 80ng/mL visfatin + 100 μM HNMPA + 500 nM of PPP. Cells remained in treatments for 24 hours, thereafter the Pierce LDH Cytotoxicity Kit (#88953) was used to assess LDH secretion in cells following addition of treatments. The presence of LDH in the cells inferred increased cytotoxicity/apoptosis.

Proliferation

To analyze cellular proliferation in HepG2 and Huh7 cells an MTT assay was utilized. Cells were seeded in a 96 well plate at 10,000 cells/well for 24 hours. The cells were then serum starved for 24 hours and were then treated with the following treatments for 48 hours: DMSO as a vehicle control, 80ng/mL of visfatin, 80ng/mL visfatin + 10 μM

LY290004 (PI3K inhibitor), 80ng/mL visfatin + 100 μM HNMPA (IR-specific inhibitor), 80ng/mL visfatin + 500 nM of PPP (IGF-1 receptor inhibitor). Following the treatment, 20 μL of MTT solution was added directly to media. The cells were then incubated for 2 hours and media was aspirated before addition of 100 μL DMSO to each well. Once the solution was homogenous or after about 10 minutes, the absorbance was measured at 540 nm in the Cytation5 reader.

Colony Formation Assay

Cell survival was assessed by colony formation assay. Cells were seeded at a density of 1,000 cells per well in a 6-well plate. After 24 hours, cells were treated in 5% FBS with DMSO as a vehicle control, 80ng/mL of visfatin, 80ng/mL visfatin + 10 μM LY290004 (PI3K inhibitor), 80ng/mL visfatin + 100 μM HNMPA (IR-specific inhibitor), 80ng/mL visfatin + 500 nM of PPP (IGF-1 receptor inhibitor) for 72 hours, and then recovered in 10% FBS for 4 days. After staining with crystal violet, colonies with more than 50 cells were counted and compared to control using Gene5 software and Cytation 5 Cell Imaging Multi-Mode Reader Biotek (Winooski, VT).

Statistical Analysis

Values are presented as mean ± s.e. of the mean. Experiments were repeated at least three times. Statistical analyses were performed between treatment groups except as noted. For all tests, GraphPad Prism 7.0 software was used (GraphPad Software Inc., La Jolla, CA, USA), and $P<0.05$ is considered statistically significant. Results were compared using Student's t-test.

Results

Ability of Visfatin to Bind the Insulin Receptor and Insulin-like Growth Factor-1 Receptor

The first step in identifying visfatin's mechanism of action was to determine whether visfatin binds to with the insulin of insulin-like growth factor -1 receptor. Because visfatin has been shown to act as an insulin mimetic, we hypothesized that visfatin may be binding to either the insulin or IGF-1 receptor.

Interestingly, visfatin bound to both the insulin and IGF-1 receptor in both cell lines. In HepG2 cells treated with visfatin, 78.8 ±5.5% of the visfatin detected by western blot analysis bound to the insulin receptor and 82.8 ±1.3% of visfatin detected bound to the IGF-1 receptor (Figures 4 and 5). In HepG2 cells treated with FBS, 80.7 ±6.3% of visfatin detected was bound to the insulin receptor and 83.4 ±4.8% of visfatin detected was bound to the IGF-1 receptor (Figures 4 and 5). In Huh7 cells treated with visfatin, 80.8 ±3.9% of the total visfatin detected in the cells bound to the insulin receptor and 64.1 ±7.2% of the total visfatin detected in the cells was bound to the IGF-1 receptor (Figures 4 and 5). In Huh7 cells treated with FBS, 77.1 ± 7.6% of visfatin detected in the cells bound to the insulin receptor and 64 ± 9.8% of visfatin detected bound the IGF-1 receptor (Figures 4 and 5).

The majority of visfatin was found bound to either the IGF-1 or insulin receptors on Huh7 and HepG2 cells compared to only a small amount of visfatin being unbound. Interestingly, there weren't major differences in binding between the cells treated with visfatin and the cells treated with fetal bovine serum which contains visfatin as well as other hormones and growth factors such as insulin and IGF-1. Therefore, even in the cells treated with FBS where there may have been competitive binding if all three molecules

were present, visfatin was still able to bind at that same capacity. Future studies are necessary to determine if visfatin's binding potential would be impacted if physiological levels of insulin and IGF-1 are present during the exposure to visfatin. A study of this design would most likely be more indicative of what would happen *in vivo*.

Inhibition of Insulin and IGF-1 Receptors, and Akt Activation Block Visfatin's Proliferative Potential

Visfatin has been shown to promote growth and proliferation in many different cancer cells; however, how it stimulated pro-tumorigenic processes is unknown. Since we have determined that visfatin is binding to both the insulin and IGF-1 receptors, the next step was to identify whether inhibiting the IGF-1 and insulin receptors would block visfatin's proliferative potential. In addition, visfatin has also been shown to induce the phosphorylation of PI3K/Akt in different cancer cells; therefore, because the phosphorylation of Akt is a potential next step in the activation of both the IGF-1 and insulin receptors. Identifying whether inhibiting the activation of PI3K/Akt could reduce proliferation caused by visfatin was also explored as a potential mechanism of action.

Visfatin stimulated cellular proliferation in both HepG2 and Huh7 cells compared to the vehicle control by 51.8% and 25.3% respectively (Figures 6 and 7). Inhibition of the insulin receptor blocked proliferation caused by visfatin by 24.1% and 35.4% in HepG2 and Huh7 cells (Figures 6 and 7). By inhibiting the IGF-1 receptor cellular proliferation was decreased in HepG2 and Huh7 cells by 37.5% and 54.3% compared to visfatin (Figures 6 and 7). There was also a significant decrease in cellular proliferation in both the HepG2 and Huh7 cells following the treatment of the PI3K inhibitor (42.1% and 54.3%). Inhibition of both the IGF-1 receptor and insulin receptor inhibitors resulted

in the largest decrease in cellular proliferation by 56.9% and 67% in both cell lines in comparison to visfatin alone (Figures 6 and 7).

Inhibition of IGF-1 Receptor, Insulin Receptor and Akt Induce LDH Secretion in Liver Cancer Cells Following Visfatin Treatment

Before undergoing apoptosis, a cell will begin to leak lactose dehydrogenase through its cellular membrane, therefore, increased LDH secretion can infer increased apoptotic activity within a cell. Thus, inhibiting the receptors and possible pathway visfatin may be working through to induce proliferation, should result in an increase in LDH secretion from those specific treatments when visfatin is present. Inhibiting both the IGF-1 receptor and insulin receptor at the same time in the presence of visfatin, resulted in the most LDH secretion in both cell lines, followed by the inhibition of the IGF-1 receptor alone and Akt. Inhibiting both receptors led to a significant increase of 74 ±7.2% and 168 ±2.3% in LDH secretion in the HepG2 and Huh7 cell lines compared to visfatin alone (Figures 8 and 9). Interestingly, cellular proliferation induced by visfatin was reduced the most in the presence of both receptor inhibitors which corresponds to the ability of both receptor inhibitors to induce the highest amount of LDH secretion in the presence of visfatin. In HepG2 cells, the PI3K inhibitor and IGF-1 receptor inhibitor increased LDH secretion levels by 41.3 ± 0.53% and 47.4 ±0.46% respectively (Figures 8 and 9). In Huh7 cells, all inhibitors significantly increased LDH secretion compared to visfatin.

Effects of Inhibiting the IGF-1 Receptor, Insulin Receptor, and Akt on Protein Expression in the Presence of Visfatin

In Huh7 cells, the combination of the IGF-1 receptor inhibitor and insulin receptor inhibitor was the most effective in reducing protein concentrations of nuclear NFκB, total NFκB, COX-2, VEGF, and phosphorylated Akt in the presence of visfatin compared to visfatin alone (Figure 10). In HepG2 cells, the combination of the IGF-1 receptor inhibitor and insulin receptor inhibitor in the presence of visfatin reduced protein concentrations of nuclear NFκB, total NFκB, COX-2, and VEGF compared to visfatin alone (Figure 11). In both cell lines, the protein concentration of bcl-xL was reduced in the presence of the PI3K inhibitor and visfatin compared to visfatin alone.

Effects of Inhibiting the Insulin Receptor, IGF-1 Receptor and Akt on Survival in the Presence of Visfatin

In both liver cancer cell lines, inhibiting the insulin receptor, IGF-1 receptor, a combination of both receptor inhibitors and Akt in the presence of visfatin reduced colony formation counts in comparison to visfatin alone (Figures 12 and 13). However, only significant reductions in colony formations were observed in the HepG2 cell line when treated with the insulin receptor inhibitor, IGF-1 receptor inhibitor, and a combination of both receptor inhibitors in combination with visfatin.

Insulin Receptor

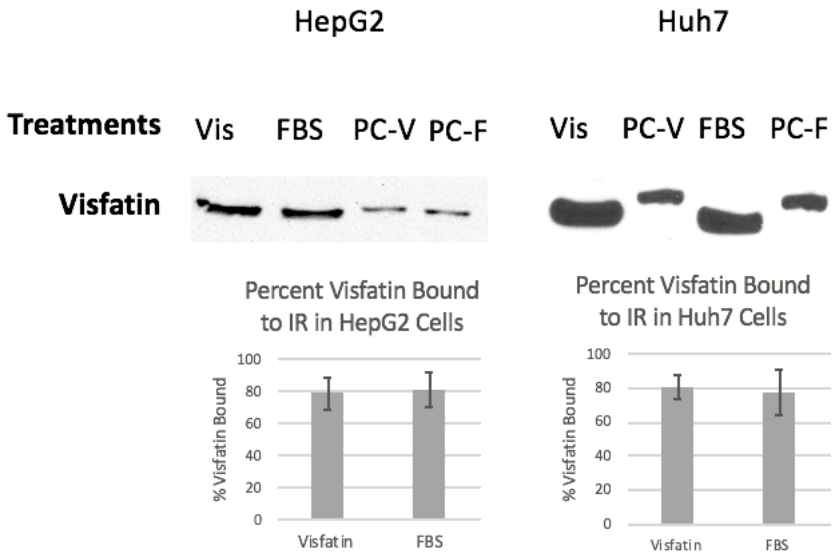

Figure 4. Visfatin's Ability to Bind to the Insulin Receptor (IR) in HepG2 and Huh7 Cells. Cells were seeded at 1-2 million cells/well, where 3 triplicate wells were used for each treatment (visfatin and FBS) in order to obtain 500-1000 µg of protein. Following cell lysis and protein determination to ensure enough protein was obtained, 10 µg of anti-insulin receptor was added to lysed cells. The mixture was allowed to incubate overnight at 4ºC in order for the insulin receptor antibody to bind to the insulin receptor in sample. Following overnight incubation, antibody-antigen mixture was added to magnetic beads, the magnetic beads and antibody-antigen mixture were incubated at room temperature for 1 hour. The magnetic beads were then collected using a magnetic stand. The leftover solution not bound to the magnetic beads was collected and labeled as the pre-clear (PC) treatment. The magnetic beads were then washed and eluted with elution buffer to remover antigen bound them. The pre-clear solution and elution mixture was then analyzed for each treatment by western blot analysis to examine whether visfatin had bound to the insulin receptor, by using an anti-NAMPT antibody.

IGF-1 Receptor

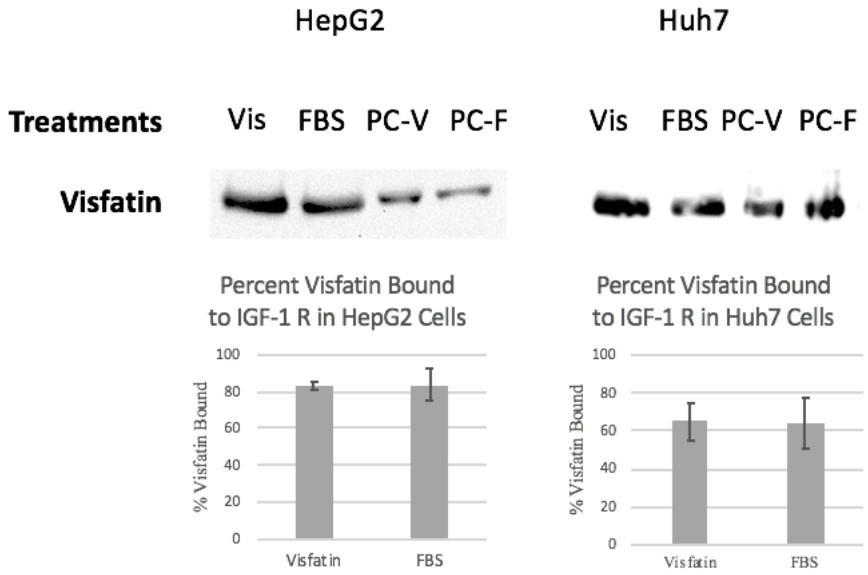

Figure 5. Visfatin's Ability to Bind to the IGF-1 receptor in HepG2 and Huh7 Cells. Cells were seeded at 1-2 million cells/well, where 3 triplicate wells were used for each treatment (visfatin and FBS) in order to obtain 500-1000 µg of protein. Following cell lysis and protein determination to ensure enough protein was obtained, 10 µg of anti-IGF-1 receptor was added to lysed cells. The mixture was allowed to incubate overnight at 4ºC in order for the IGF-1 receptor antibody to bind to the IGF-1 receptor in the sample. Following overnight incubation, the antibody-antigen mixture was added to magnetic beads, the magnetic beads and antibody-antigen mixture were incubated at room temperature for 1 hour. The magnetic beads were then collected using a magnetic stand. The leftover solution not bound to the magnetic beads was collected and labeled as the pre-clear (PC) treatment. The magnetic beads were then washed and eluted with elution buffer to remove the antigen bound to them. The pre-clear solution and elution mixture was then analyzed for each treatment by western blot analysis to examine whether visfatin had bound to the IGF-1 receptor, by using an anti-NAMPT antibody.

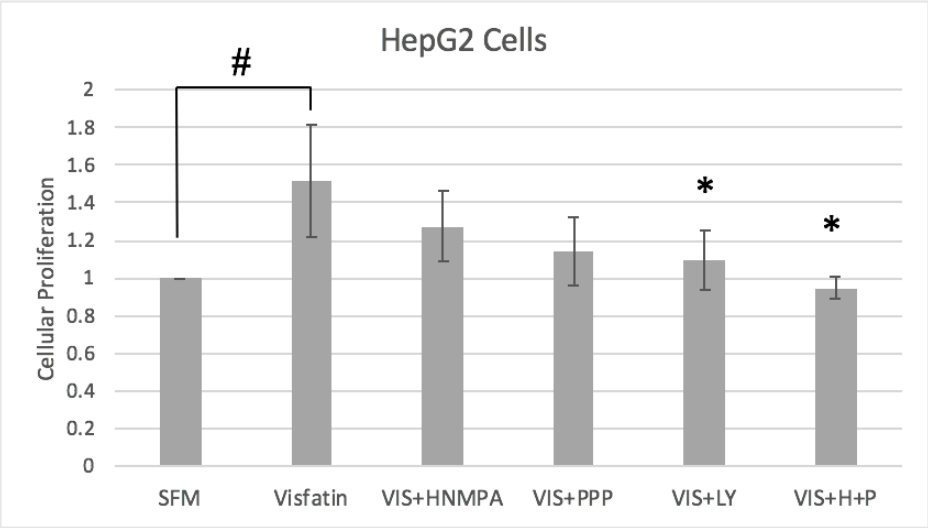

Figure 6. Effects of Inhibitors on Cellular Proliferation in HepG2 Cells. HepG2 cells were seeded at 10,000 cells/well in a 96 well plate for 24 hours. The cells were then serum starved for 24 hours. The cells were then treated in serum free media (SFM) with either DMSO as a vehicle control, 80ng/mL of visfatin, 80ng/mL visfatin + 10 µM LY290004 (PI3K inhibitor), 80ng/mL visfatin + 100 µM HNMPA (IR-specific inhibitor), 80ng/mL visfatin + 500 nM of PPP (IGF1-R specific inhibitor) and 80ng/mL visfatin + 100 µM HNMPA + 500 nM of PPP. 48 hours following treatments 20 µL of MTT reagent was added directly to media and allowed to incubate for 1.5-2 hours. Thereafter, media was aspirated and 100 µL of DMSO was added to cells. Following homogenization, the absorbance was measured at 540 nm in the Cytation5 reader. (*= $P<0.05$ vs. visfatin treated control) (#=$P<0.05$)

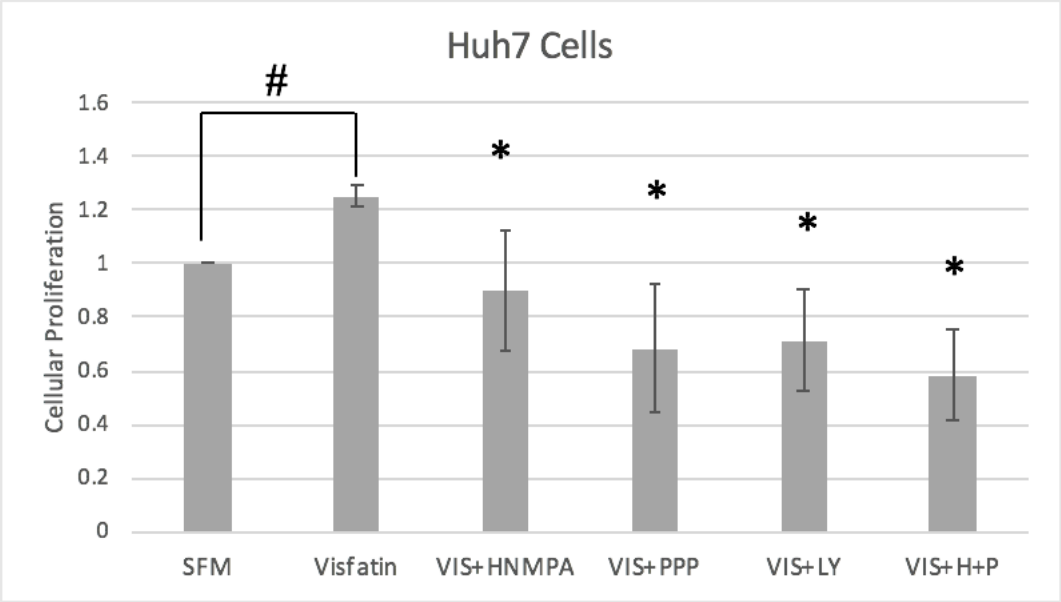

Figure 7. Effects of Inhibitors on Cellular Proliferation in Huh7 Cells. Huh7 cells were seeded at 10,000 cells/well in a 96 well plate for 24 hours. The cells were then serum starved for 24 hours. The cells were then treated in serum free media (SFM) with either DMSO as a vehicle control, 80ng/mL of visfatin, 80ng/mL visfatin + 10 µM LY290004 (PI3K inhibitor), 80ng/mL visfatin + 100 µM HNMPA (IR-specific inhibitor), 80ng/mL visfatin + 500 nM of PPP (IGF1-R specific inhibitor) and 80ng/mL visfatin + 100 µM HNMPA + 500 nM of PPP. 48 hours following treatments 20 µL of MTT reagent was added directly to media and allowed to incubate for 1.5-2 hours. Thereafter, media was aspirated and 100 µL of DMSO was added to cells. Following homogenization, the absorbance was measured at 540 nm in the Cytation5 reader. (*= $P<0.05$ vs. visfatin treated control) (#=$P<0.05$)

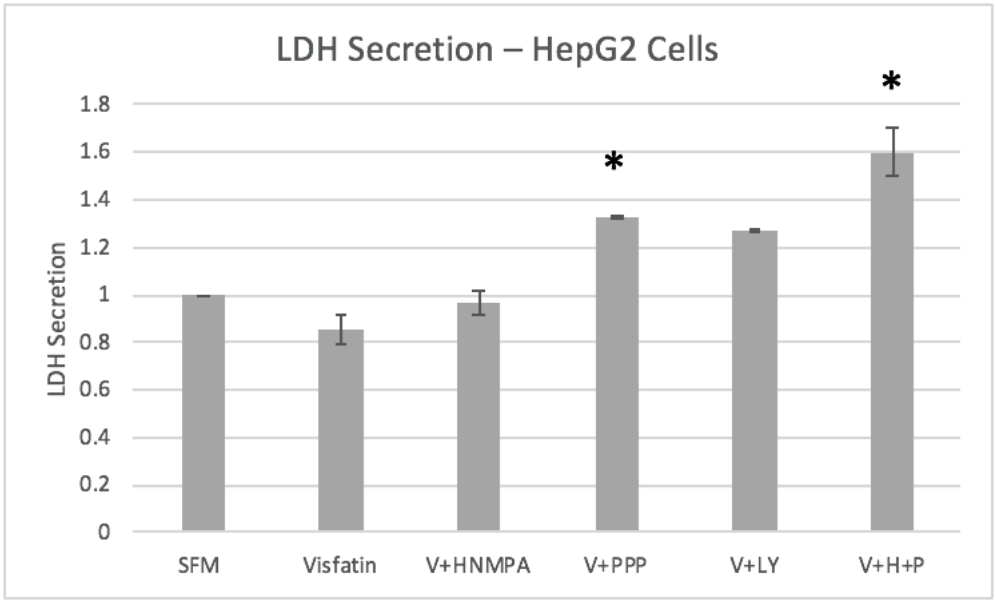

Figure 8. The effects of inhibitors on LDH Secretion I HepG2 Cells. HepG2 cells were seeded at 10,000 cells/well for 24 hours. Thereafter, the following treatments were added: DMSO as a vehicle control, 80ng/mL of visfatin, 80ng/mL visfatin + 10 µM LY290004 (PI3K inhibitor), 80ng/mL visfatin + 100 µM HNMPA (IR-specific inhibitor), 80ng/mL visfatin + 500 nM of PPP (IGF1-R specific inhibitor) and 80ng/mL visfatin + 100 µM HNMPA + 500 nM of PPP. All cells were treated in serum free media. Cells remained in treatments for 24 hours, thereafter the Pierce LDH Cytotoxicity Kit was used to assess LDH secretion in cells following addition of treatments. (*= $P<0.05$ vs. visfatin treated control)

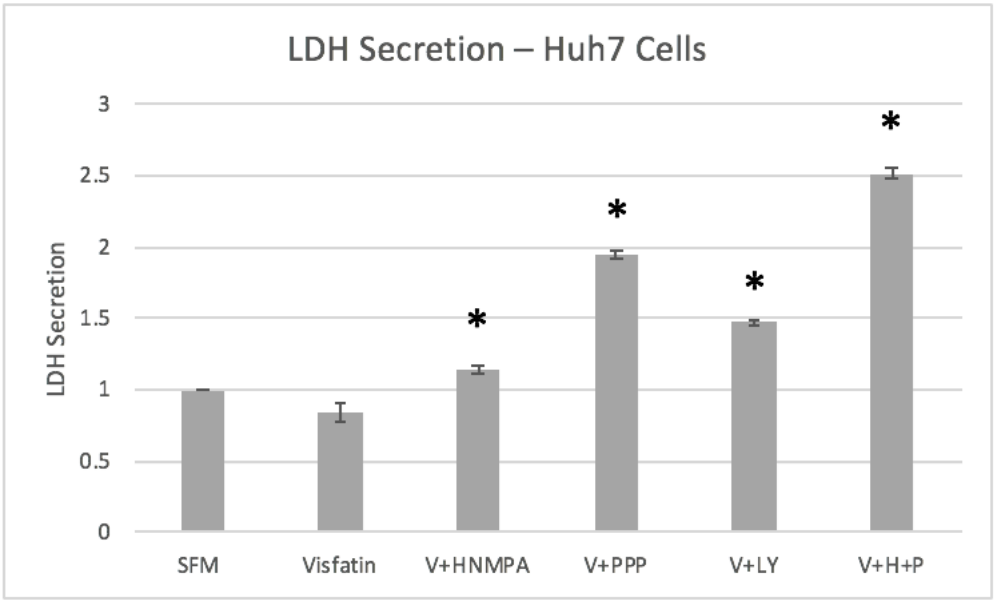

Figure 9. The Effects of Inhibitors on LDH Secretion in Huh7 Cells. Huh7 cells were seeded at 10,000 cells/well for 24 hours. Thereafter, the following treatments were added: DMSO as a vehicle control, 80ng/mL of visfatin, 80ng/mL visfatin + 10 µM LY290004 (PI3K inhibitor), 80ng/mL visfatin + 100 µM HNMPA (IR-specific inhibitor), 80ng/mL visfatin + 500 nM of PPP (IGF1-R specific inhibitor) and 80ng/mL visfatin + 100 µM HNMPA + 500 nM of PPP. All cells were treated in serum free media. Cells remained in treatments for 24 hours, thereafter the Pierce LDH Cytotoxicity Kit was used to assess LDH secretion in cells following addition of treatments. (*= $P<0.05$ vs. visfatin treated control)

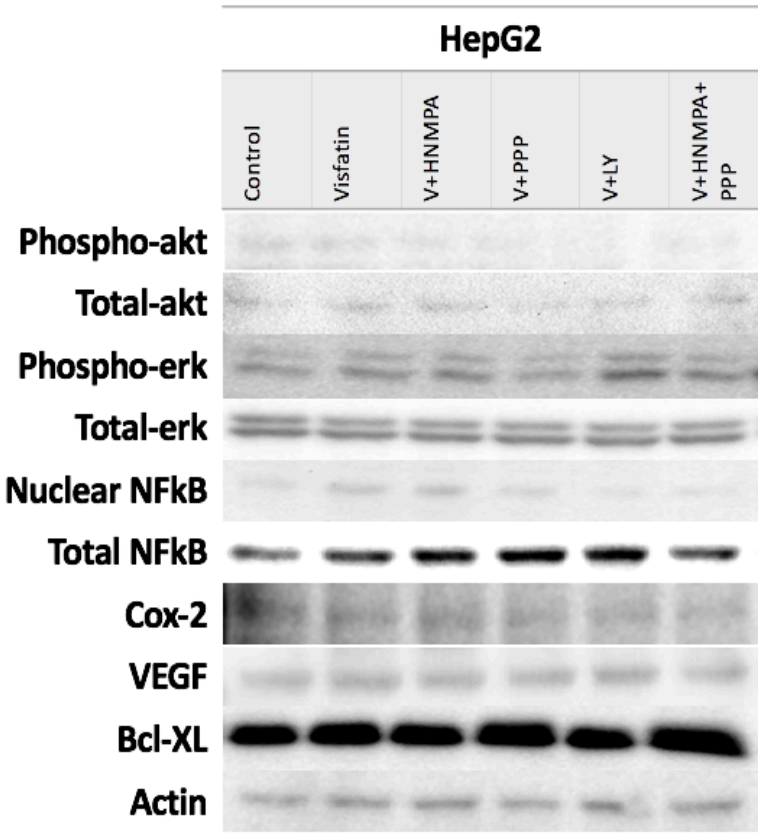

Figure 10. The Effects of Inhibitors on Protein Expression in HepG2 Cells. HepG2 cells were seeded in 6-well plates at 400,000 cells/well for 24 hours. Cells were serum starved for 24 hours and then treated in serum free media with either DMSO as a vehicle control, 80ng/mL of visfatin, 80ng/mL visfatin + 10 μM LY290004 (PI3K inhibitor), 80ng/mL visfatin + 100 μM HNMPA (IR-specific inhibitor), 80ng/mL visfatin + 500 nM of PPP (IGF1-R specific inhibitor) and 80ng/mL visfatin + 100 μM HNMPA + 500 nM of PPP.

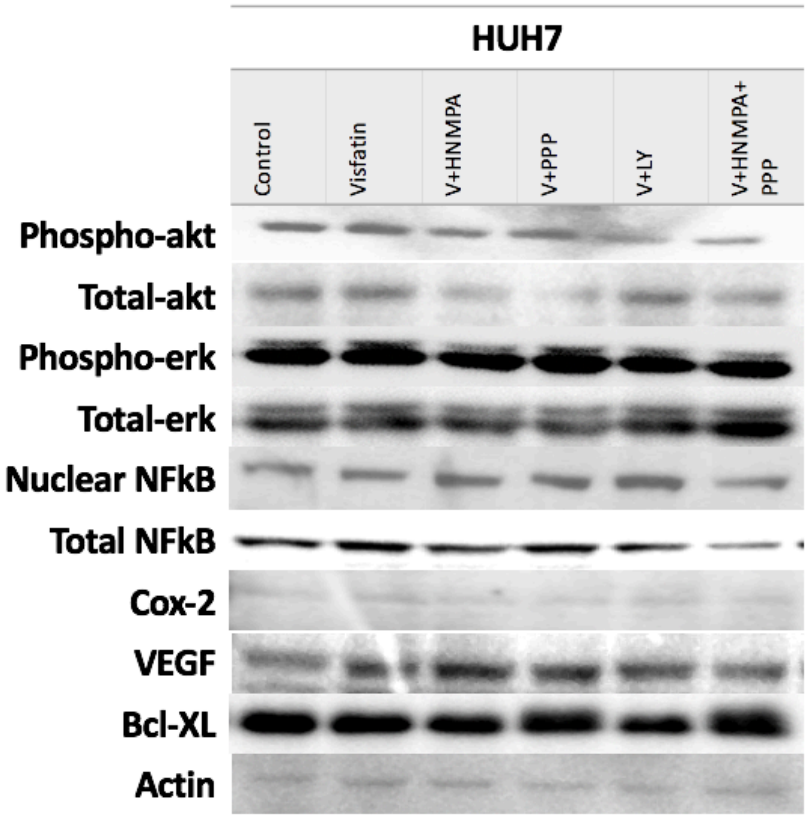

Figure 11. The Effects of Inhibitors on Protein Expression in Huh7 Cells. Huh7 cells were seeded in 6-well plates at 400,000 cells/well for 24 hours. Cells were serum starved for 24 hours and then treated in serum free media with either DMSO as a vehicle control, 80ng/mL of visfatin, 80ng/mL visfatin + 10 μM LY290004 (PI3K inhibitor), 80ng/mL visfatin + 100 μM HNMPA (IR-specific inhibitor), 80ng/mL visfatin + 500 nM of PPP (IGF1-R specific inhibitor) and 80ng/mL visfatin + 100 μM HNMPA + 500 nM of PPP.

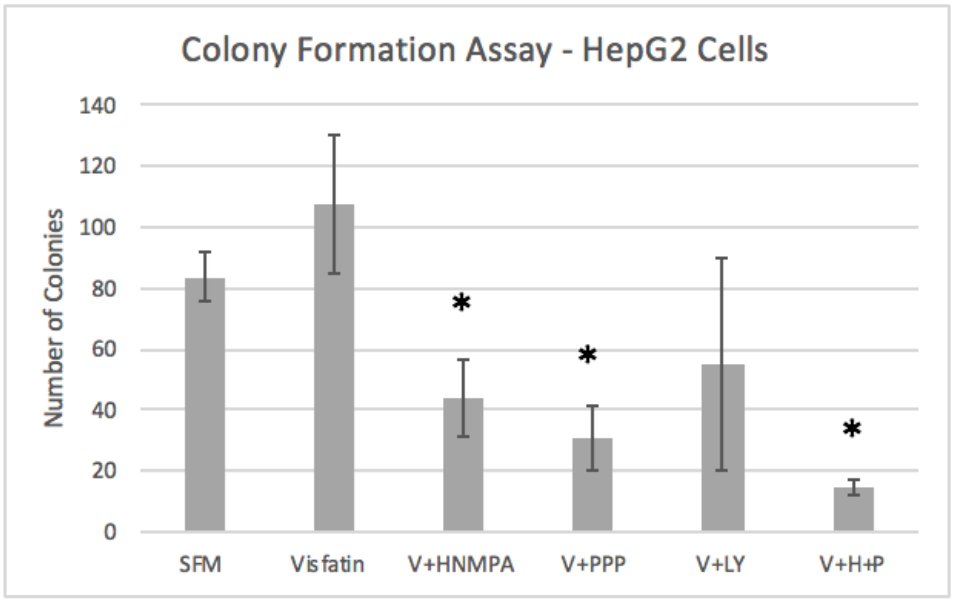

Figure 12. The Effects of Inhibitors on Cell Survival in HepG2 Cells. HepG2 cells were seeded at 1,000 cells/well in a 6 well plate for 24 hours. Cells were then treated with respective treatments in 5% FBS for 72 hours. The cells were then recovered in 10% FBS for 4 days. (*= $P<0.05$ vs. visfatin treated control)

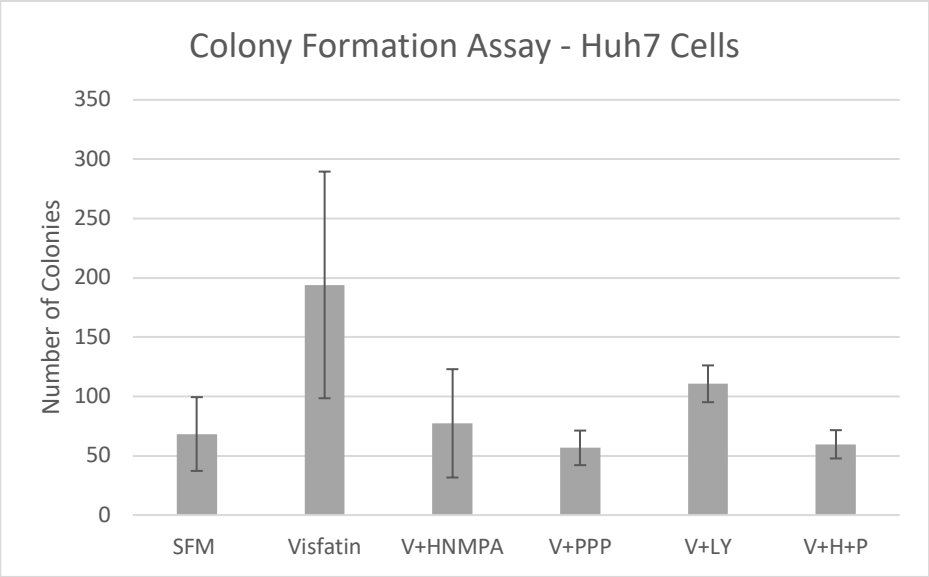

Figure 13. The Effects of Inhibitors on Cell Survival in Huh7 Cells. Huh7 cells were seeded at 1,000 cells/well in a 6 well plate for 24 hours. Cells were then treated with respective treatments in 5% FBS for 72 hours. The cells were then recovered in 10% FBS for 4 days.

Table 1. Expected and Actual Outcomes from Chapter 2.

Assay	What does the assay measure?	Expected Outcomes in Presence of Visfatin	Expected Outcomes in Presence of Inhibitors and Visfatin	Actual Outcomes in Presence of Both Receptor Inhibitors and Visfatin
Co-immunoprecipitation	Protein Binding	Visfatin is binding either IR or IGF-1 R	N/A	N/A
MTT Assay	Cellular Proliferation	↑ proliferation	↓ proliferation	↓ proliferation
LDH Cytotoxicity Assay	LDH Secretion/ Cytotoxicity	↓ LDH Secretion	↑ LDH secretion	↑ LDH secretion
Colony Formation Assay	Cell Survival	↑ Cell survival	↓ Cell survival	↓ Cell survival
Western Blot Analysis	Protein Concentrations	↑ phosphorylated and pro-tumorigenic proteins	↓ phosphorylated and pro-tumorigenic proteins	↓ phosphorylated and pro-tumorigenic proteins

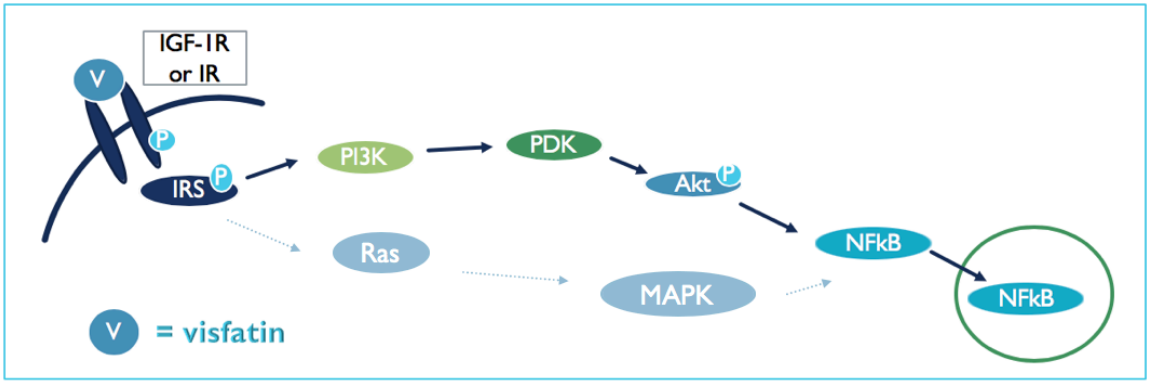

Figure 14. Potential Mechanism of Visfatin. Visfatin was able to bind both the insulin and IGF-1 receptor. Inhibition of both receptors in the presence of visfatin led to significant decreases in cellular proliferation in HepG2 and Huh7 cells as well as significant increases in LDH secretion. The combination of inhibiting both receptors also led to decreases in NFkB, VEGF, and COX-2 suggesting that visfatin may be carrying out its pro-tumorigenic effects by initiating the activation of NFkB through the IGF-1 and insulin signaling pathways. Inhibition of the PI3K pathway did not consistently decrease concentrations of NFkB suggesting visfatin may also be working through the MAPK signaling pathway.

Discussion

Visfatin has been shown in previous studies to act as an insulin mimetic, Xie et al, when evaluating the effects of visfatin in osteocytes found that visfatin had the ability to phosphorylate not only the insulin receptor but also the insulin receptor substrate 1 and insulin receptor substrate 2.[15] Brown et al, when assessing visfatin's effects in pancreatic beta cells, also found that visfatin was capable of insulin receptor phosphorylation.[16] However, since these studies have been published there has been no published research on whether visfatin is binding the insulin receptor to induce phosphorylation of the insulin receptor and IRS-1 and IRS-2 that was observed in these studies. Because the insulin receptor and IGF-1 receptor have similar homologies it was decided that a co-immunoprecipitation would be completed using both insulin and IGF-1 receptor antibodies to determine whether visfatin could bind to one of the two. To our surprise, visfatin was able to bind both receptors in both cell lines at a similar capacity ranging anywhere from 60-80% (visfatin bound to receptor/ (visfatin bound to receptor + free visfatin in pre-clear solution)) of visfatin bound. Two sources of visfatin were used to treat the liver cancer cells, recombinant visfatin and fetal bovine serum. The visfatin from both treatments were able to bind both receptors at near the same capacity. Future studies could be designed to determine if visfatin still has the same ability to bind both receptors in the presence of physiological levels of both insulin and IGF-1.

Because visfatin was able to bind both receptors it was hypothesized that visfatin may initiate the phosphorylation of the insulin receptor, IRS-1, and IRS-2 by binding to the insulin receptor and IGF-1 receptor. Visfatin has also been found to initiate cellular proliferation in many cancerous cell lines by initiating the phosphorylation of Akt, as well as Erk. Therefore, the inhibition of the IGF-1 receptor, insulin receptor, and PI3K

was analyzed in combination with visfatin to elucidate how visfatin might be carrying out its proliferative and pro-tumorigenic effects. Inhibition of PI3K led to significant decreases in cellular proliferation when treated in combination with 80ng/mL of visfatin. Wang et al., when analyzing visfatin's proliferative effects in endometrial cancer cells found that the inhibition of PI3K using 10 and 50μM of LY294002 in endometrial cancer cells also significantly reduced proliferation caused by visfatin.[31] Gholinejad et al found similar results in breast cancer cells.[17] The IGF-1 receptor and insulin receptor inhibitors alone only significantly reduced cellular proliferation in Huh7 cells; however, the combination of the two receptor inhibitors significantly reduced cellular proliferation in both cell lines(Table 1). Xie et al., also used an insulin receptor inhibitor ($HNMPA(AM)_3$) when analyzing visfatin's effects in osteocytes and found a significant reduction in cellular proliferation.[15]

LDH secretion was analyzed to infer apoptosis; therefore, it was hypothesized that by inhibiting the receptors and pathway visfatin may be working through, an increase in LDH secretion would be seen, due to the inhibitors blocking visfatin's proliferative effects. Confirming the results previously seen from the proliferation assays, treatment with the combination of the two receptor inhibitors led to a significant increase in LDH secretion compared to visfatin alone. (Table 1) Although not significant in both cell lines, the PI3K inhibitor, insulin receptor inhibitor, and IGF-1 receptor inhibitor significantly increased LDH secretion in Huh7 cells but not in HepG2 cells.

Consistent with cellular proliferation and cytotoxicity results, the combination treatment of the IGF-1 receptor inhibitor and insulin receptor inhibitor resulted in the most consistent decrease in protein concentrations of proteins down and upstream of NFκB and, importantly, NFκB itself. (Table 1) Although not consistent in both cell lines,

the receptor inhibitors together were able to decrease the phosphorylation of Akt and Erk. Interestingly, treatment of both inhibitors in both cell lines consistently led to an increase in the protein concentration of anti-apoptotic Bcl-XL. This data is the opposite of what was expected because the combination of both inhibitors decreased cellular proliferation and increased cytotoxicity. However, the data suggests that with the treatment of the combination of both inhibitors, antiapoptotic activity is going on in the cell. This may be occurring as a resistance mechanism by the cell to overcome the apoptotic activity occurring in the cell due to the inhibition of the IGF-1 and insulin receptor.

Because visfatin is able to bind both the insulin receptor and IGF-1 receptor, it was expected that each of the receptor inhibitors alone may not have been as effective as both together. This rationale stems from the idea that even though one of the inhibitors is blocked, visfatin can still bind to the other effective receptor and carry out its functions. Interestingly, data has also shown that if either the IGF-1 receptor or insulin receptor is inhibited, a compensatory increase in activation of the other receptor occurs due to crosstalk between the two receptors.[34] Therefore, inhibiting just one receptor could make visfatin's effects even stronger compared to when neither receptor is being inhibited.

When analyzing the effects of inhibiting PI3K, decreases in protein concentrations of COX-2, Bcl-XL and nuclear NFκB in HepG2 cells, and total NFκB in Huh7 cells were seen. The inconsistencies between the cell lines in PI3K inhibition leading to decreased levels of total NFκB and NFκB in the nucleus could be a result of visfatin's ability to activate the MAPK pathway which has also been shown to initiate NFκB activation. However, without protein quantification these results have yet to be confirmed which is a limitation of this study.

The data from this study suggest that visfatin may be carrying out its tumorigenic effects by binding and activating to both the IGF-1 and insulin receptors, leading to the activation of growth pathways including the PI3K/Akt pathway and the ERK1/2/MAPK pathway. (Figure 14) Future research should be done to investigate the inhibition of the MAPK pathway as well as the inhibition of the PI3K/Akt in combination with the MAPK pathway when cancer cells are exposed to visfatin, to determine the capacity visfatin has to activate these proliferative pathways and initiate downstream effects when present in cancer cells.

III. DEVELOPMENT OF SORAFENIB RESISTANT HEPATOCELLULAR CARCINOMA CELL LINES

Introduction

Unfortunately, high death rates among patients with HCC are commonly seen, due to diagnoses at late cancer stages. The chemotherapeutic drug, sorafenib, has a high incidence of resistance, with many patients showing signs of improvement during the first months only to develop a resistance to the drug shortly after.[10] Sorafenib works as a multikinase inhibitor, with some of its main targets being several isoforms of the Raf serine/threonine kinase, three of the vascular endothelial growth factor (VEGF)-receptors, platelet derived growth factor receptor, among others.[11] However, in the process of inhibiting these proteins another known proliferative pathway, the PI3K/Akt pathway increases due to crosstalk.[10] Scientists believe this upregulation in the Akt pathway, along with other mechanisms, have a significant contribution to the increased resistance seen in many patients treated with sorafenib.[10] NFkB has also been shown to be upregulated in sorafenib resistance.[10] Visfatin an adipocytokine found in obese individuals can upregulate many of the same proteins and signaling pathways upregulated in sorafenib resistance; therefore, understanding how this molecule effects sorafenib resistant cells is of importance.[12] In order to carry out these experiments sorafenib resistant hepatocellular cell lines are needed. The objective of this study is to develop sorafenib resistant hepatocellular carcinoma cell lines that can be used for future research on visfatin's role in sorafenib resistance.

Methods

Cell Culture

HepG2, Huh7, and SNU-449 cells were used as a model for hepatocellular carcinoma. HepG2 and Snu-449 cells were purchased from the American Type Culture Collection (ATCC). Huh7 cells were purchased from the Japanese Cancer Research Resources Bank (JCRB). HepG2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) which was supplemented with 10% fetal bovine serum (FBS), streptomycin, and penicillin. Huh7 cells were cultured in DMEM with low glucose supplemented with streptomycin, penicillin and 10% FBS. SNU-449 cells were cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with streptomycin, penicillin and 10% FBS. The cells were kept at 37° C in a 5% (v/v) CO_2 humidifier.

Sorafenib Treatment

Cells were initially exposed to 3.75 μM of sorafenib during the first month (timepoint 1(TP1)), and 5 μM during the second month of developing their resistance (timepoint 2 (TP2)). Developing resistant cells were labeled as Huh-7 R, Snu-449 R, and HepG2-R. Cells treated with sorafenib were labeled as the cell line (+) and cells without sorafenib were labeled as the cell line (-).

Developing Sorafenib Resistant Cell Lines

Resistant HepG2, Huh7, and Snu-449 cells were split every 3-4 days or once the cells were 70-80% confluent. Every 7 days the cells were treated with sorafenib. Each month, cellular proliferation assays were conducted to assess if the cells were becoming

resistant by comparing them to the normal cells and treating them with and without sorafenib.

Proliferation

An MTT assay was used to assess proliferation of HepG2, Huh7, and SNU-449 cells. Normal cells and resistant cells from all three cells lines were seeded in a 96-well plate at 10,000 cells/well for 24 hours. The normal and resistant cells were then treated with the corresponding concentration of sorafenib the cells were currently being exposed to (3.75 μM or 5 μM) or DMSO as a control for 48 hours. After 72 hours, 20 μL of MTT solution was added directly to media. The cells were then incubated for 1 and half to 2 hours. The media was aspirated and 100 μL of DMSO was added to each well. Once the solution was homogenous or after about 10 minutes, absorbance was measured at 540 nm using the Cytation 5 Cell Imaging Multi-Mode Reader Biotek (Winooski, VT).

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Colony Formation Assay

Cell survival was assessed by colony formation assay. Cells were seeded at a density of 1,000 cells per well in a 6-well plate for 24 hours. The normal and resistant cells were then treated with the corresponding concentration of sorafenib the cells were currently being exposed to (3.75 μM or 5 μM) or DMSO as a control in 5% FBS medium. Treatments were removed after 72 hours and the cells were recovered in 10% FBS for 4 days and then stained with crystal violet. Colonies with more than 50 cells were counted and compared to control using Gene5 software and Cytation 5 Cell Imaging Multi-Mode Reader Biotek (Winooski, VT).

Western Blot Analysis

In order to assess apoptosis, western blot analysis was used to assess the PARP cleavage and Bcl-XL protein concentrations. HepG2, Huh7, and Snu-449 normal and resistant cells were seeded in 6-well plates at 400,000 cells/well for 24 hours. Cells were then treated for 24 hours with either 5 μM of sorafenib or DMSO as a control. Cell lysates were collected using lysis buffer (5 ml glycerol, 3.14 ml TRIS 1M pH 6.8, 5 ml 10% SDS, 36.86 ml ddH_2O). Thereafter, the samples were quantified using the Pierce BCA protein assay kit to ensure equal protein loading. The samples were then electrophoresed through an 8% gel at 110 Volts. The gel with attached proteins was then transferred on to a nitrocellulose membrane at 100 volts for 45 minutes. Bcl-xl antibody (Cell Signaling Technology, #2764), and cleaved PARP antibody (Cell Signaling Technology, #5625) were used to measure protein concentrations of bcl-xL and cleaved PARP.

Statistical Analysis

Values are presented as mean ± s.e. of the mean. Experiments were repeated at least three times. Statistical analyses were performed between treatment groups except as noted. For all tests, GraphPad Prism 7.0 software was used (GraphPad Software Inc., La Jolla, CA, USA), and $P<0.05$ is considered statistically significant. Results were compared using Student's t-test.

Results

Differences in Cellular Proliferation, Cell Survival, and Protein Expression Between Sorafenib Resistant Cells and Normal Cells

Following the first month and second month of exposure to sorafenib, statistically significant differences in cellular proliferation in both the normal and resistant cells were observed between the cells exposed to sorafenib and the cells not exposed to sorafenib (Figures 15-17). However, following the second month of exposure to sorafenib more of the resistant cells were viable compared to normal cells when treated with sorafenib (Figure 18-20). Following the second month of exposure to sorafenib, no statistically significant differences in cell survival were observed in the normal and resistant snu-449 cells when comparing the cells treated with and without sorafenib (Figures 21-23). Significant differences in cell survival were observed in normal and resistant Huh7 cells when comparing the cells treated with and without sorafenib. In all cell lines, resistant cells treated with and without sorafenib had increased protein expression of Bcl-XL in comparison to their normal cell counterparts (Figures 24-26). Unfortunately, PARP cleavage could not be detected (Figures 25 and 26).

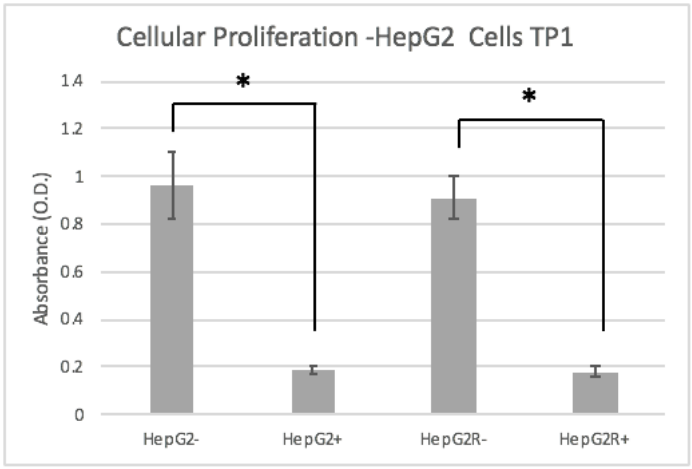

Figure 15. Effect of Sorafenib on Cellular Proliferation in Normal and Resistant HepG2 Cells Following First Month of Exposure to Sorafenib. Cells were seeded at 10,000 cells/well in a 96 well plate for 24 hours. The normal and resistant cells were then treated with 3.75 µM of sorafenib or DMSO as a control. (R= resistant) (+: with sorafenib, -: without sorafenib) (TP1 = timepoint one) (*= P<.05)

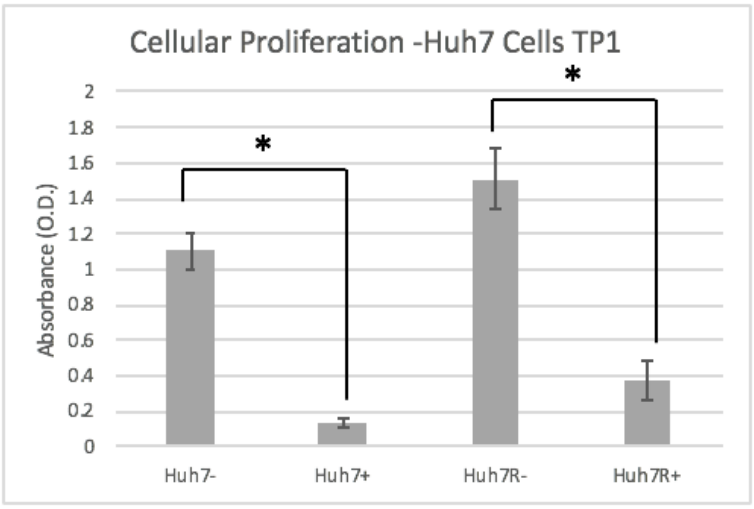

Figure 16. Effect of Sorafenib on Cellular Proliferation in Normal and Resistant Huh7 Cells Following First Month of Exposure to Sorafenib. Cells were seeded at 10,000 cells/well in a 96 well plate for 24 hours. The normal and resistant cells were then treated with 3.75 µM of sorafenib or DMSO as a control. Resistant Huh7 cells when exposed to sorafenib were statistically more proliferative when compared to the normal cells exposed to sorafenib. (R= resistant) (+: with sorafenib, -: without sorafenib) (TP1 = timepoint one) (*= P<.05)

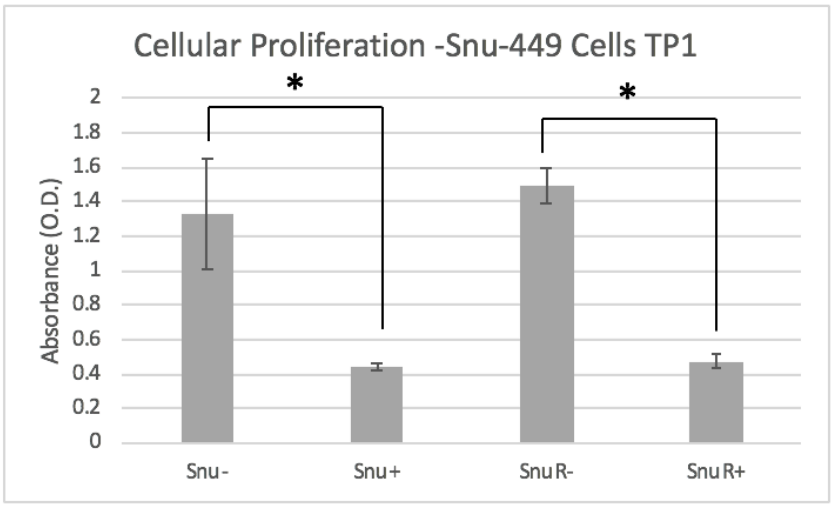

Figure 17. Effect of Sorafenib on Cellular Proliferation in Normal and Resistant Snu-449 Cells Following First Month of Exposure to Sorafenib. Cells were seeded at 10,000 cells/well in a 96 well plate for 24 hours. The normal and resistant cells were then treated with 3.75 µM of sorafenib or DMSO as a control. (R= resistant) (+: with sorafenib, -: without sorafenib) (TP1 = timepoint one) (*= P<.05)

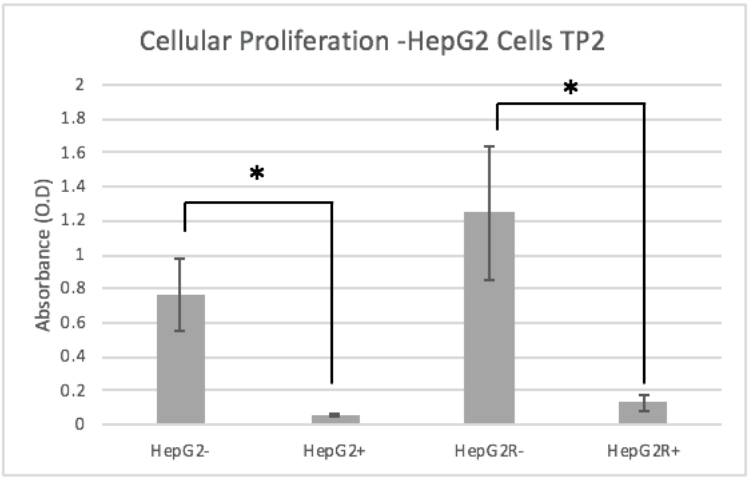

Figure 18. Effect of Sorafenib on Cellular Proliferation in Normal and Resistant HepG2 Cells Following Second Month of Exposure to Sorafenib. Cells were seeded at 10,000 cells/well in a 96 well plate for 24 hours. The normal and resistant cells were then treated with 5 µM of sorafenib or DMSO as a control. (R= resistant) (+: with sorafenib, -: without sorafenib) (TP2 = timepoint two) (*= P<.05)

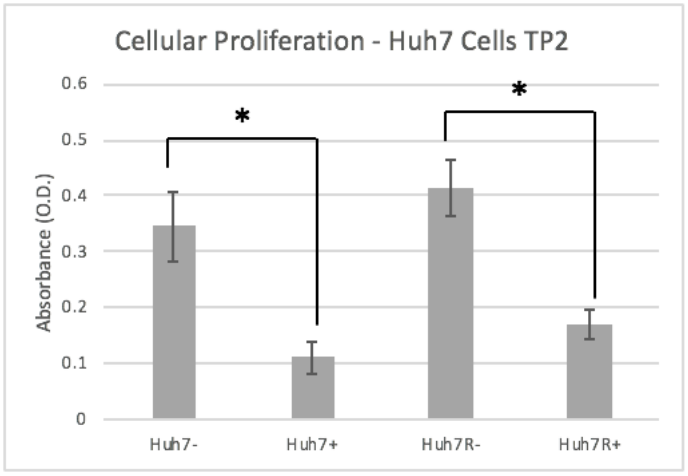

Figure 19. Effect of Sorafenib on Cellular Proliferation in Normal and Resistant Huh7 Cells Following Second Month of Exposure to Sorafenib. Cells were seeded at 10,000 cells/well in a 96 well plate for 24 hours. The normal and resistant cells were then treated with 5 μM of sorafenib or DMSO as a control. A significant increase in cellular proliferation following treatment with sorafenib in Huh7 resistant cells compared to the huh7 normal cells (R= resistant)
(+: with sorafenib, -: without sorafenib) (TP2 = timepoint two) (*= P<.05)

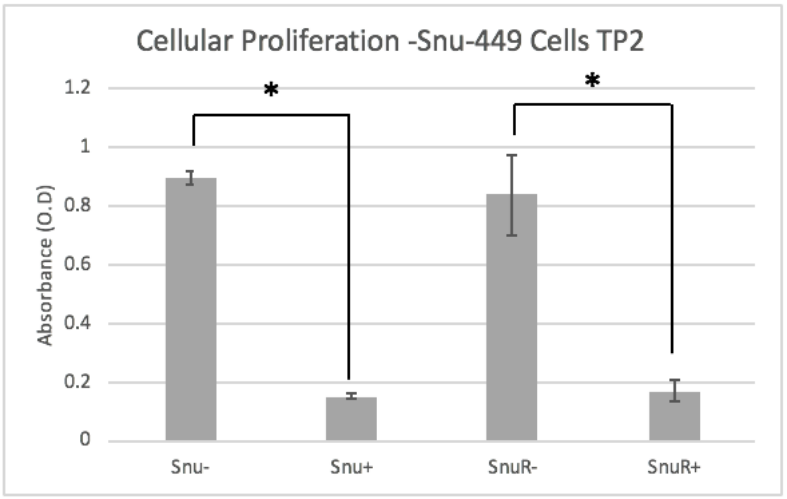

Figure 20. Effect of Sorafenib on Cellular Proliferation in Normal and Resistant Snu-449 Cells Following Second Month of Exposure to Sorafenib. Cells were seeded at 10,000 cells/well in a 96 well plate for 24 hours. The normal and resistant cells were then treated with 5 μM of sorafenib or DMSO as a control.
(R= resistant) (+: with sorafenib, -: without sorafenib) (TP1 = timepoint two) (*= P<.05)

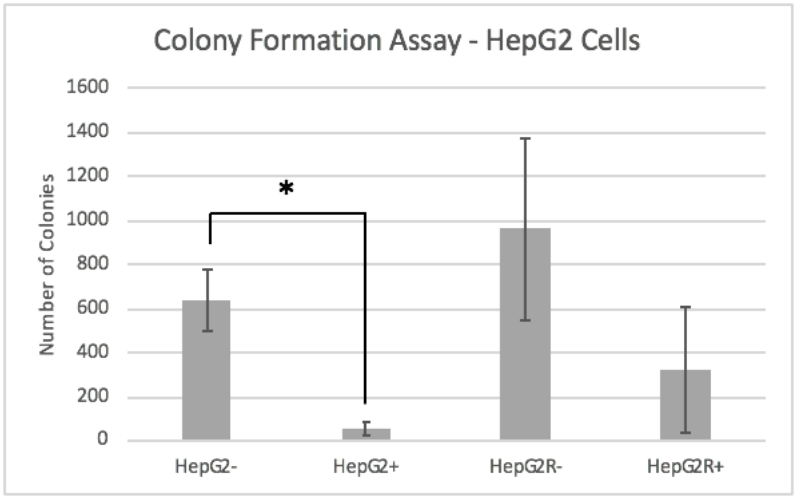

Figure 21. Effect of Sorafenib on Cell Survival in Normal and Resistant HepG2 Cells. Following Second Month of Exposure to Sorafenib. Following the second month of exposure to sorafenib, cells were seeded for a colony formation assay (CFA). Cells were seeded at 1,000 cells/well in a 6 well plate for 24 hours. Cells were then treated with 5 µM of sorafenib for 72 hours. Following treatment cells were recovered in 10% FBS for 4 days. (R= resistant) (+: with sorafenib, -: without sorafenib) (*= P<.05)

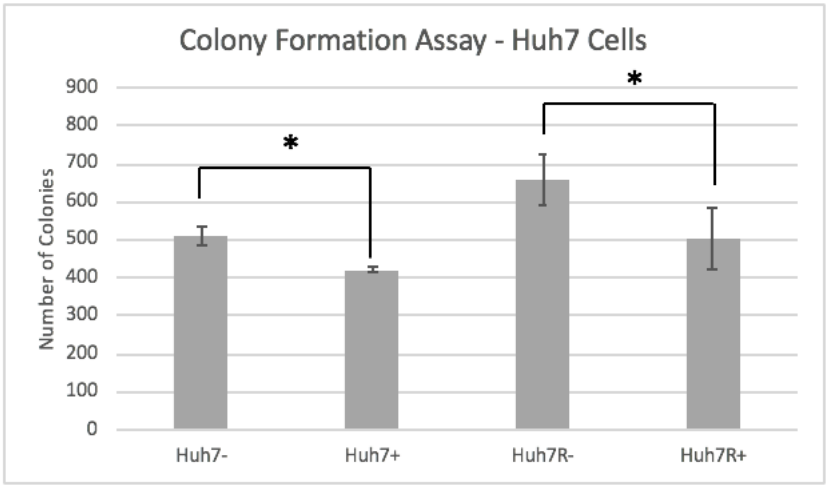

Figure 22. Effect of Sorafenib on Cell Survival in Normal and Resistant Huh7 Cells. Following Second Month of Exposure to Sorafenib. Following the second month of exposure to sorafenib, cells were seeded for a colony formation assay (CFA). Cells were seeded at 1,000 cells/well in a 6 well plate for 24 hours. Cells were then treated with 5 µM of sorafenib for 72 hours. Following treatment cells were recovered in 10% FBS for 4 days. (R= resistant) (+: with sorafenib, -: without sorafenib) (*= P<.05)

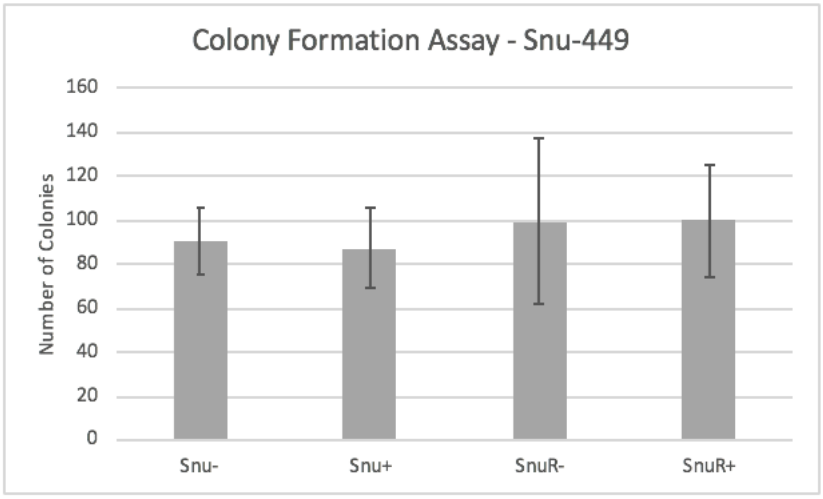

Figure 23. Effect of Sorafenib on Cell Survival in Normal and Resistant Snu-449 Cells. Following Second Month of Exposure to Sorafenib. Following the second month of exposure to sorafenib, cells were seeded for a colony formation assay (CFA). Cells were seeded at 1,000 cells/well in a 6 well plate for 24 hours. Cells were then treated with 5 μM of sorafenib for 72 hours. Following treatment cells were recovered in 10% FBS for 4 days. (R= resistant) (+: with sorafenib, -: without sorafenib)

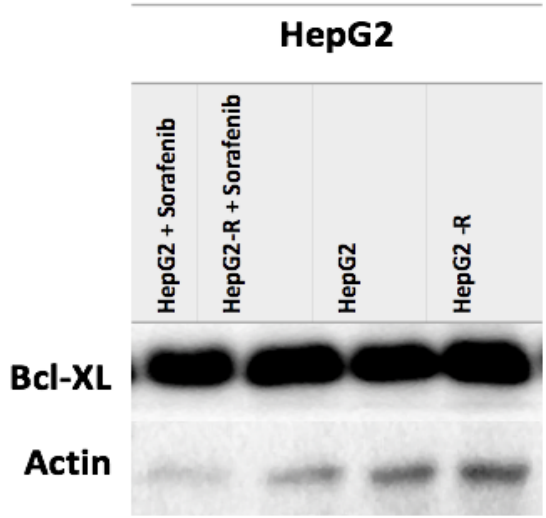

Figure 24. Protein Expression of Anti-apoptotic Bcl-XL in Normal and Resistant HepG2 Cells Following Second Month of Exposure to Sorafenib. (R= resistant)

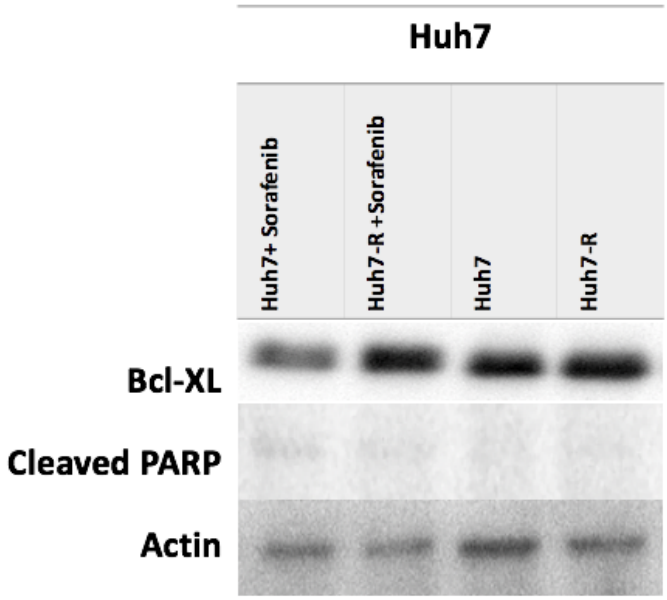

Figure 25. Protein Expression of Anti-apoptotic Bcl-XL in Normal and Resistant Huh7 Cells Following Second Month of Exposure to Sorafenib. Bcl-XL proteins are found at higher levels in resistant cells compared to normal cells, indicating they are becoming more anti-apoptotic. (R= resistant)

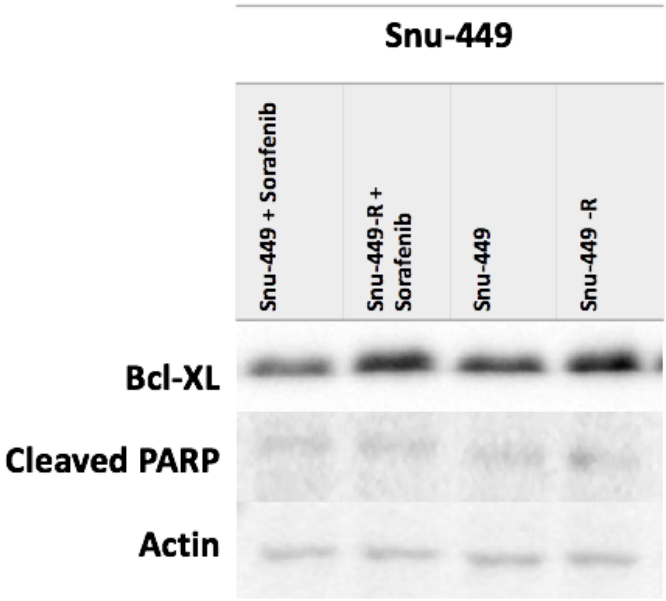

Figure 26. Protein Expression of Anti-apoptotic Bcl-XL in Normal and Resistant HepG2 Cells Following Second Month of Exposure to Sorafenib. Bcl-XL proteins are found at higher levels in resistant cells compared to normal cells, indicating they are becoming more anti-apoptotic. (R= resistant)

Discussion

Overall, cellular proliferation was higher in the resistant cells compared to the normal cells, although, statistically significant differences were observed in all cell lines, both normal and resistant when comparing cells that were treated with and without sorafenib. As the progression of sorafenib resistance continues, we hope to observe smaller differences in cellular proliferation in resistant cells when comparing sorafenib treated and untreated cells, signifying their ability to grow in the presence of sorafenib due to their constant exposure to the chemotherapeutic drug. Unfortunately, PARP cleavage was not assessed in any of the cell lines, this may be due to too short of a treatment time resulting in PARP cleavage not having occurred yet. Bcl-XL protein expression was higher in all resistant cell lines indicating they're becoming more anti-apoptotic compared to the normal cells and are resisting death. Smaller differences in cellular proliferation in resistant cells may not have been observed due to the early stages of the cells becoming resistant. In many other studies sorafenib resistance at the clinically relevant dose of 10 μM[19] did not occur until after 6 or 7 months of exposure.[35] The cells were at the time only being exposed to half of the final concentration of sorafenib that they will end up being exposed to. Therefore, the cells may still be in the early stages of developing resistance mechanisms.

Other experiments that could potentially be conducted to determine if the cells are becoming more resistant, include a assessing the phosphorylation of Erk. Because sorafenib inhibits the MAPK pathway, p-Erk shouldn't be detected following the treatment of sorafenib in non-resistant cells, however, as the cells become resistant an increase in the phosphorylation of Erk should be detected.

Following the development of the sorafenib resistant cell lines, studies can begin to be conducted to elucidate visfatin's pro-tumorigenic affect in a sorafenib resistant environment and thus be compared to its effects in normal cancerous cells.

Summary and Future Directions

Overall, the data observed from this study, supports the idea that visfatin may be an insulin mimetic, carrying out many of its pro-tumorigenic effects through the insulin and IGF-1 signaling pathways. Data from the co-immunoprecipitation assay, revealed that visfatin had the potential to bind both the insulin and insulin-like growth factor-1 receptors. Following the co-immunoprecipitation, several experiments were carried out, including an MTT assay, LDH cytotoxicity assay, a colony formation assay and western blot analysis, to examine visfatin's pro-tumorigenic effects in the presence of the IGF-1 receptor inhibitor, insulin receptor inhibitor, a combination of both receptor inhibitors and the PI3K inhibitor.

Because we hypothesized that visfatin was carrying out many of its tumorigenic effects through the insulin and IGF-1 signaling pathways, we expected the inhibitors to block visfatin's effects in liver cancer cells. Interestingly, the IGF-1 and insulin receptor inhibitors alone did not have as great of an impact in blockading visfatin's effects as did the combination of the two inhibitors. The combination of both receptor inhibitors led to a significant decrease in cellular proliferation and a significant increase in LDH secretion in both cells when compared to visfatin alone. The combination of both inhibitors also led to decreases in protein concentrations of VEGF, COX-2 and NFκB in both cell lines. Decreases, in cellular proliferation, and increases in LDH secretion were also observed following the inhibition of PI3K, however, not to the extent of the combination of the two receptor inhibitors, therefore the MAPK signaling pathway may be of importance as well

in the signaling of visfatin. The data from this study suggest that visfatin indeed may be activating both the IGF-1 and insulin signaling pathways by acting as a ligand and binding both receptors. It is well known that following the phosphorylation of the insulin and IGF-1 receptors, an induction in both the PI3K/Akt and MAPK pathways can occur, and it has been consistently observed that visfatin has the potential to induce both of these signaling pathways. Therefore, visfatin may be inducing it's pro-tumorigenic effects by binding the insulin and IGF-1 receptors, leading to the activation of the Akt/PI3K and MAPK pathway.

Future research should be done to examine the effects of inhibiting the MAPK pathway in the presence of visfatin, to elucidate the importance of MAPK signaling in visfatin's pro-tumorigenic effects. More research is also needed to confirm NFκB's role in visfatin signaling. Experiments such as immunofluorescence could be utilized to visualize nuclear localization of NFκB. Gene knockdown could also be utilized to determine visfatin's mechanism of action, knockdown of the insulin and IGF-1 receptors, as well as NFκB, could determine the contribution of NFκB's transcriptional activity in visfatin's pro-tumorigenic effects. Because visfatin was able to increase protein expressions of both VEGF and COX-2, and the protein concentrations decreased following the inhibition of the IGF-1 and insulin receptor, kinetic assays and a PGE2 ELISA should be conducted. The results from this study provide the foundation for future mechanistic studies that could be designed to reverse the impact of obesity on liver cancer progression by targeting the visfatin signaling pathway.

In chapter 3, consistent exposure to sorafenib, led to significant increases in cellular proliferation in developing Huh7 resistant cells when compared to normal Huh7 cells exposed to sorafenib. Resistant cells from Huh7 and Snu-449 cell lines expressed

increased concentrations of bcl-XL when exposed to sorafenib compared the normal cells exposed to sorafenib, inferring the resistant cells are becoming more anti-apoptotic in the presence of sorafenib. Although large differences were not observed between the developing resistant cells and the normal cells, the cells were only being exposed to half of the concentration of the clinically relevant dose of 10 μM of sorafenib. Larger differences are expected to be observed as the cells become more resistant. Because PARP cleavage was undetectable, later experiments should use a treatment timepoint longer than 1 hour. Phosphorylated Erk should also be analyzed as the cells become more resistant. As the cells are becoming resistant, experiments examining visfatin's effects in combination with sorafenib would be informative and may determine if visfatin has the ability to desensitize the cells to sorafenib. These future studies are imperative to understanding how obesity associated cytokines such as visfatin, play a role in chemo-resistance.

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