TARGETING iNAMPT TO BREAK THE OBESITY-ASSOCIATED LIVER CANCER LINK: AN IN VITRO AND IN VIVO APPROACH

by

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DEDICATION

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ABSTRACT

Background: Studies have shown that obesity is linked to liver cancer through metabolic mechanisms. Obesity can promote tumor growth through metabolic impairment, decreased lipid metabolism, and the interference of energy balance in the liver. NAMPT is an enzyme expressed in the liver and is involved in the progression of tumors in obesogenic environments. iNAMPT is known to be the rate-limiting enzyme in the synthesis of NAD, an essential coenzyme involved in ATP synthesis which promotes a pro-growth environment in the context of obesity. Because iNAMPT and cellular energetics, a hallmark of cancer, plays an important role in liver cancer progression, it has become a target for therapies focused on inhibiting its behavior in cancer cells. The *objective* of this study was to determine the contribution of NAD biosynthesis in obesity-induced liver cancer progression.

Methods: Cell studies were conducted with serum from mice randomized to a diet-induced obesity (OB) or control chow (CR) + FK866 (iNAMPT inhibitor) in SNU, HepG2 human liver cancer cells and Hepa 1-6 murine cells. Analysis of proteins pAkt and pERK was performed by immunoblot. Proliferation, ROS, cytotoxicity, and invasion were also measured in liver cancer cells. For the mouse model, C57/BL mice were randomized to OB chow or CR chow. At 21 weeks of age, mice were injected subcutaneously with Hepa 1-6 liver cancer cells. At 23 weeks, mice received an I.P. injection of FK8666 (30 mg/kg) for 2 weeks. Tumor and mouse weights were measured.

Results: Cells exposed to OB sera increased proliferation, LDH secretion, ROS, invasion. FK866 decreased proliferation, LDH secretion, ROS, and invasion in all liver cancer cells. Cells exposed to NW sera and OB + FK866 resulted in more LDH suggesting increased apoptosis compared to OB sera. OB sera increased phosphorylation of Akt which was suppressed by

FK866 compared to OB. In liver cancer cells, physiological and cellular signaling were differentially affected when inhibiting NAD biosynthesis in an in vitro model of obesity and liver cancer. In vivo, OB mice weighed significantly more than mice fed a control diet. In addition, 66% of OB mice developed tumors compared to 16% in CR mice.

Impact: Identifying pre-clinical strategies to reverse the impact of obesity on liver cancer progression is important due to the strongly increased risk of liver cancer and its poor prognosis. Future translation research studies will build from this pre-clinical foundational research.

I. Introduction

Hepatocellular carcinoma (HCC) is a common primary liver cancer, constituting about 75% of all liver cancer types and being one of the leading causes of deaths from cancer internationally.[1] While treatment options and mortality rates have improved for HCC, the current 2-year survivability rate is 50% which decreases to 10% after 5 years.[1] Males are 2-4x more likely to develop HCC compared to females due to behavioral risk factors such as smoking and alcohol consumption, while Asian Americans, African Americans, and Hispanics have the highest rates based on ethnicity.[2] Early detection of HCC can result in effective treatments such as liver transplantation or surgery, leading to an improved prognosis over time.[1] Even so, most patients are diagnosed when HCC is in an advanced stage.[1] In late stages, chemotherapy is a common cancer treatment used to inhibit cancer cell viability and proliferation, with the most common agent being sorafenib.[1] While sorafenib's mechanism of action is to block specific kinase pathways, it is often only able to increase survival by 7-10 months, indicating the need to research and develop other effective liver cancer treatment options.[1]

Non-alcoholic steatohepatitis (NASH), alcoholic cirrhosis, hepatitis B and C, and non-alcoholic fatty liver disease (NAFLD) are major contributors to the development of HCC in the United States population.[1] Obesity, diabetes, metabolic syndrome, and a variety of other disease states increase the risk in the development and progression of HCC through exacerbation of NAFLD.[3] McGlynn et al. report that the risk of developing HCC was 2.6-fold higher in obese patients with NAFLD compared to non-obese patients with NAFLD.[3] Obesity has been shown to be a strong underlying factor to the development of NASH and NAFLD, specifically through the upregulation of certain adipokines, subsequently leading to tumorigenesis.[3] Clinical data shows a correlation between NAFLD and obesity, with steatohepatitis prevalence in those with class I-II

obesity being 65% and 85% in those with class III obesity.[4] According to data from the US Cancer Statistics database, 4.7% of cancer cases in men and 9.6% of cancer cases in women were due to excess body weight, with 51% of those cases being liver or gallbladder cancer.[5] Data has also shown that obesity is associated with a 13% risk of recurrence and poor outcomes in survivors.[5] Obesity is indicative of a high level of adipocytic tissue which plays a role in the impairment of metabolic and hormonal control, thus leading to carcinogenesis.[6] While the obesity-cancer connection is multifactorial, research has shown that there are three primary mechanisms responsible for the increased risk of cancer in obese individuals.[6] For one, adipose tissue has the ability to increase the production of hormones and adipokines that may be anti-apoptotic and pro-proliferative when excreted in excess concentrations by adipocytes.[6] The overproduction of these hormones, such as leptin, lead to proinflammatory environments with excess and sustained levels of obesity-associated hormones.[6] Leptin plays a vital role in appetite regulation.[6] However, when overexpressed through obesity, it can contribute to tumorigenesis through the activation of pro-proliferative and anti-apoptotic mechanisms by binding to its receptor and stimulating pro-tumorigenic cellular changes.[6] Next, obesity is often associated with hyperinsulinemia, with high levels of insulin and insulin-like growth factor 1 (IGF-1) aiding in the development of cancer and in certain cases can lead to progression.[6] IGF-1 is a hormone similar to insulin, which can regulate growth hormone, and is involved in bone and tissue growth.[7] Overactive production of IGF-1 can lead to tumorigenesis due to its pro-proliferative and anti-apoptotic characteristics.[7] While the hormone itself is not responsible for the appearance of cancer cells, it contributes to the uncontrolled growth and survival of tumor cells.[7] These properties that are exacerbated by obesity, such as resistance to cell death, proliferative signaling,

and cell immortality, have been deemed to be just a few of the Hallmarks of Cancer that are adversely affected by obesity.

Obesity is the primary cause of NAFLD development with around 50% of HCC patients presenting with both.[8] NAFLD is the precursor to NASH and can be characterized by lipid accumulation in the liver without any effect on organ function.[8] In the context of obesity, this triglyceride accumulation can surpass the threshold that the liver is able to utilize, thus leading lipid retention in the liver.[8] Prolonged lipid accumulation will subsequently lead to the progression of liver disease evidenced by the presence of steatohepatitis and ballooning of the hepatocytes. NASH is the result of the enlarged hepatocytes and an inflamed liver due to steatohepatitis, eventually leading to fibrosis, cirrhosis, and the development of liver cancer if untreated.[8] Rajesh et al. suggests that insulin resistance and high-caloric intake often associated with obesity can lead to a buildup of adipocytes, thus producing a pro-inflammatory environment where hepatic cells are able to survive.[9] Chronic injury to the liver, such as cirrhosis exacerbated by NAFLD, can also result in an overexpression of nuclear factor-kB (NF-kB), a pro-tumorigenic transcription factor and inflammatory mediator associated with liver cancer.[10] NF-kB transcription can also be activated by other inflammatory stimuli, such as excess concentrations of leptin and IGF-1 as a result of obesity and disease.[10] Post-transcription and translation, NF-kB can stimulate many inflammatory responses, including the activation of the cytokines and the induction of monocyte function.[11] The release of these pro-inflammatory cytokines, such as interleukin (IL)-6, interleukin-1β (IL-1β), and tumor necrosis factor alpha (TNF-α), leads to the perpetuation of an inflammatory response, leading to increased activation of signaling pathways that are associated with the Hallmarks of Cancer, including survivability, proliferation, reactive oxygen species (ROS) production, and invasion into other tissues.[7,12]

There is a plethora of protein pathways involved in the relationship between obesity and HCC. Nicotinamide phosphoribosyl transferase (NAMPT) is an enzyme that is highly expressed in the liver and is involved in the progression of tumors in obesogenic environments, having been shown to have increased concentrations in the context of obesity.[13] NAMPT has two derivatives with varying functions, extracellular NAMPT (eNAMPT) and intracellular NAMPT (iNAMPT). eNAMPT, also known as visfatin, is an adipokine with an important and poorly understood role in carcinogenesis.[13] Recent research has shown that eNAMPT overexpression has been associated with apoptotic resistance, cancer cell proliferation, and activation of the pro-inflammatory pathways which are associated with NAD biosynthesis.[13] iNAMPT is known to be the rate-limiting enzyme in the synthesis of NAD, an essential coenzyme involved in numerous redox reactions that progress into the production of the cell's means of energy, ATP, which promotes a pro-growth environment in the context obesity.[13] While the role of iNAMPT in HCC is a novel concept, overexpressed iNAMPT levels have been detected in many other cancer types, including prostate, colorectal, and gastric tumors.[14] iNAMPT has been shown to have anti-apoptotic and proliferative properties on tumor cells and has also been correlated with increased metastasis and tumor growth.[13,14] Because iNAMPT and cellular energetics, a hallmark of cancer, play an important role in liver cancer progression, it has become a target for therapies focused on inhibiting its behavior in cancer cells.[15,16] FK866 is an FDA-approved iNAMPT inhibitor that has been shown to have antitumor properties through repression of cancer cell growth, dephosphorylation of Erk and Akt pathways, and apoptotic induction.

The purpose of this study is to determine the contribution of NAD biosynthesis in obesity-induced liver cancer progression. We hypothesized that in using cell cultures infused with obese serum, cells will show suppressed viability and increased apoptotic ability when

treated with an iNAMPT inhibitor, FK866. We also hypothesized that in using a syngeneic mouse model of liver cancer, obese mice injected with an iNAMPT inhibitor, FK866, will show a decrease in tumor incidence and growth, kinase signaling, and pro-tumorigenic transcription factors leading to apoptosis. In both, we expect to see the inhibition of iNAMPT result in suppression of tumor growth in the context of obesity. Identifying pre-clinical strategies to reverse the impact of obesity on liver cancer progression is important due to the strong increased risk of liver cancer and its poor prognosis. Future translational research studies will build from this pre-clinical foundational research.

II. METHODS

Cell Culture

Both human and mouse liver carcinoma cell lines, SNU-449 and Hepa1-6 respectively, were purchased from the American Type Tissue Culture Collection (ATCC). SNU-449 cells were cultured in RPMI plus 10% fetal bovine serum (FBS) and both HepG2 and Hepa 1-6 cells were cultured in EMEM plus 10% FBS. All cells were maintained at 37 °C in a 5% (v/v) CO2 humidified incubator. Cells were exposed to one of five experimental conditions: 5% mouse obese (OB) serum, 5% OB serum + FK866 (10 nM), 5% mouse control (CR) serum, and 5% CR + FK866 (10 nM). The times of exposure will be explained further in each assay described below. The concentrations of obese serum and FK866 were selected based on previous studies.[17,18]

Mouse Serum

Mouse serum was purchased from Jackson Laboratories. Post weaning, mice were randomized to a high fat diet (60% kcal from fat) or control diet and continued their respective diets for the remainder of the study. For the OB group, 12 mice sera were pooled at 14 weeks of age. For the CR group, 12 mice sera were pooled at 14 weeks of age. OB and CR sera were used for in vitro experiments using a concentration of 5%.

MTT Assay

To measure cell viability, MTT assay was performed. HepG2, SNU-449, and Hepa 1-6 cells were seeded in a 96-well plate with 10,000 cells per well. Cells were incubated overnight in FBS. After 24 hr, cells were treated with the treatments stated above for 72 hr. After 72 hr, 20 µL of MTT reagent (5 mg/mL) was added to the well plate for 1.5 hr. Then, the media along with the reagent was aspirated, and 100 µM of dimethyl sulfoxide (DMSO) was added to the well

plate and shaken for 10 minutes at room temperature. Absorbance was measured at 540 nm using Cytation 5.

ROS Assay

Intracellular levels of reactive oxygen species (ROS) were assessed using a cell-permeable dichlorofluorescin diacetate (DCFH-DA) probe (ab113851) from Abcam according to the manufacturer's procedure. SNU-449, HepG2, and Hepa 1-6 cells were seeded in a dark, clear bottom 96-well microplate. On day 2, cells were exposed to the same experimental conditions described above. On day 3, the media was removed, and cells were washed and incubated for 45 min with 25 μM DCFDA at 37 °C. Fluorescent intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 529 nm with a Cytation 5 from BioTek Instruments, Inc.

Cytotoxicity

To assess cytotoxicity and early indicators of cell death, a lactate dehydrogenase (LDH) Assay was used in SNU-449, HepG2, and Hepa 1-6 cell lines according to the manufacturer's instructions from Sigma Lactate Dehydrogenase Activity Assay Kit MAK066-1KT.

Invasion Assay

The Corning BioCoat Matrigel Invasion Chamber (#354480) assay was utilized to assess the addition of FK866 and its ability to decrease the invasive capacity of HepG2, SNU-449, and Hepa 1-6 cell lines when exposed to treatments described above. The Matrigel chamber was hydrated in serum free media for 2 h prior to seeding. After the chambers were rehydrated, cells were seeded on the upper side of the chamber at a concentration of 80,000 cells per well in a 24-well plate and were treated with the conditions described above. The chemoattractant, 10% FBS, was added to the well below the chamber. After 48 h cells were stained with crystal violet, and

images were captured using a Cytation 5 from BioTek Instruments, Inc. After imaging, the crystal violet stain was dissolved using a distain solution (methanol, acetic acid, and water), and absorbance was measured with the Cytation5 microplate reader.

Immunoblot

To measure protein expression differences, SNU-449, HepG2 and Hepa 1-6 cells were plated at a density of 4 × 105 cells per well in a 6-well plate. After 24 h, the cells were serum starved for 6 h. The cells will then be exposed to their respective experimental conditions for 15 min. After the treatment, cells were harvested using lysis buffer (5 mL glycerol, 3.14 mL TRIS 1 M pH 6.8, 5 mL 10% SDS, 36.86 mL ddH2O) and quantified using Pierce BCA protein assay kit Thermo Fisher. Fifty μg of protein lysate was electrophoresed through a 10% SDS PAGE gel. Proteins were then transferred to a nitrocellulose membrane. The membrane was blocked with 5% bovine serum albumin for 45 min. Protein lysates were subjected to immunodetection with rabbit anti-phospho-NF-kB, rabbit anti-phospho- ERK, rabbit anti- total ERK, rabbit anti-phospho-Akt, and rabbit anti- total Akt. Images were acquired using a Fotodyne gel documentation system. After measuring phosphorylated proteins, blots were stripped with Restore Plus western blot stripping buffer before total levels of protein were measured. Protein levels were normalized to total kinase or β-actin and quantified by densitometry using ImageJ.. Pierce BCA kit was used to determine protein concentration.

Animal Model/Study Design

Thirty-two 8-week-old were ordered from Jackson Laboratories. Post weaning, half of the 32 mice were randomized to either (ad libitum) a high fat diet chow that induces obesity, 60% kcal from fat chow D12492 or control diet (10% kcal from fat) D12450B and remained on this diet for the duration of the study. Mice received new food 2 times per week. The food given

and uneaten were weighed at every feeding (this is to calculate average kcal intake). Mice were weighed weekly to monitor weight gain. At 17 weeks of age, all mice were injected with 500,000 Hepa 1-6 liver cancer cells suspended in PBS for a total volume of 100 μl. At 23 weeks of age, FK866 was administered at the dose of 30mg/kg dissolved in DMSO and phosphate buffered saline (PBS) for 3 days over 2 weeks. OB and CR mice were injected with a vehicle control consisting of DMSO and (PBS) in the same volume as the inhibitor. The concentration of FK866 was based on previous cancer mouse models.[19] Mice were euthanized via isoflurane overdose followed by cardiac puncture and cervical dislocation after treatments were finished.

Anesthesia

Prior to subcutaneous Hepa 1-6 cancer cell injections, the mice were anesthetized via isoflurane. For induction, a concentration of 3-5% isoflurane and a maintenance concentration of 1-2% isoflurane, along with oxygen, was used during the procedure. The time of exposure to isoflurane was less than 3 minutes, as the time it takes to inject the mice with the cells was very quick. The mouse was monitored to avoid excessive depression of cardiac and respiratory functions, or insufficient anesthesia, which is characterized by poor muscle relaxation, or movement in response to surgical stimulation. To ensure the mouse was anesthetized we performed a toe pinch to ensure lack of reflex response. For respiratory rate and depth, the chest was monitored for up and down movement in a slow and regular rhythm, as well as avoidance of gasping. The mice recovered in a clean cage (without bedding), where each mouse was monitored until they were fully ambulatory. Once the mice were ambulatory, they were placed back into their assigned cage with access to their chow and water.

Tumor Injection and Growth

For each cancer cell injection, a 500,000 Hepa 1-6 tumor liver cancer cell suspension was added to PBS and 25% Matrigel. The ratio of cell suspension to PBS was 1:1 with a maximum volume of 250 uL. The cell suspension was injected subcutaneously on the flank of the C56BL/6 mouse with a 25g needle. Mice were monitored daily until a palpable tumor was detected. As a baseline, mice were weighed on the day of implantation. Once a palpable tumor was detected, mice were monitored daily.

Tumor Incidence

Twenty-four hours post cancer cell injections mice were monitored for palpable tumors. Tumor incidence was determined by counting the number of palpable tumors in each experimental group.

Statistical Analysis

All experiments were replicated at least 3 times to ensure validity. GraphPad Prism was used to analyze results via one-way ANOVA with a Tukey's post hoc test. Results containing $p<0.05$ were considered statistically significant.

III. RESULTS

NAD inhibition reduces serum-induced viability

Cell proliferation is important to assess in cancer progression as excessive cell viability can lead to dysregulation in cellular homeostasis, thus leading to an increase in tumorigenesis.[7] All three cell lines were exposed to obese (OB) and control (CR) sera with or without FK866 to assess viability through MTT assay. When comparing OB and control sera, Hepa 1-6, SNU-449, and HepG2 were shown to have an increase in viability in the OB serum by 98%, 98%, and 31% respectively ($P < 0.05$) (Figure 1A/B/C). The addition of FK866, an NAD inhibitor, to OB serum suppressed cell viability by 189% in Hepa 1-6, 291% in SNU-449, and 167% in HepG2 cell lines ($P < 0.05$). The addition of FK866 to CR in all cell lines also showed a decrease in cell viability, with significant changes observed in SNU-499 and HepG2 ($P < 0.05$) (Figure 1B/C). These data suggest that FK866 does reduce obesity-induced viability.

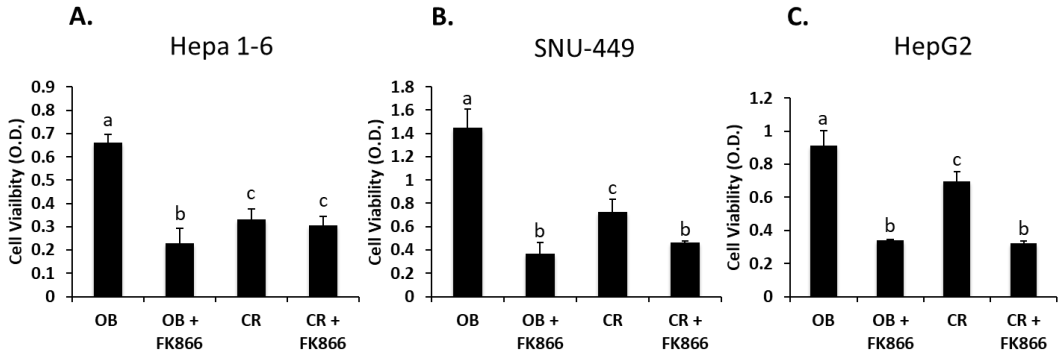

Figure 1. MTT assay was used to assess cell viability. Viability was assessed by MTT dye conversion. Hepa 1-6 (A), SNU-449 (B) or HepG2 (C) cells were exposed to the treatments for 72 h. OB= Obese sera, FK866= iNAMPT inhibitor, and CR= Control sera. Data shown represents the average of at least three independent experiments. Results were compared using ANOVA followed by Tukey's post hoc test. Different letters indicate significant differences between experimental conditions, $p<0.05$.

Addition of FK866 decreases obesity-induced ROS production

In areas of prolific and chronic inflammation, macrophages and neutrophils will produce ROS in order to destroy cancer cells.[20] Using a cell-permeable (DCFH-DA) probe, liver cancer cell lines were exposed to the previously mentioned treatments to assess ROS production through fluorescence. Hepa 1-6, SNU-449, and HepG2 were shown to have an increase in ROS production when exposed to OB serum by 27%, 41.37%, and 133.72% respectively in comparison to CR serum ($P < 0.05$) (Figure 2A/B/C). In HepG2, the addition of FK866 to OB serum decreased ROS production by 95.58% when compared to OB serum alone ($P < 0.05$) (Figure 2C). A similar decrease in ROS production can also be seen in Hepa 1-6 and SNU-499 cell lines ($P < 0.05$) (Figure 2A/B). No significant differences were observed when comparing CR serum to the addition of FK866 in CR serum in all three cell lines (Figure 2A/B/C). These data suggest that obesity-induced ROS production is inhibited by the addition of FK866.

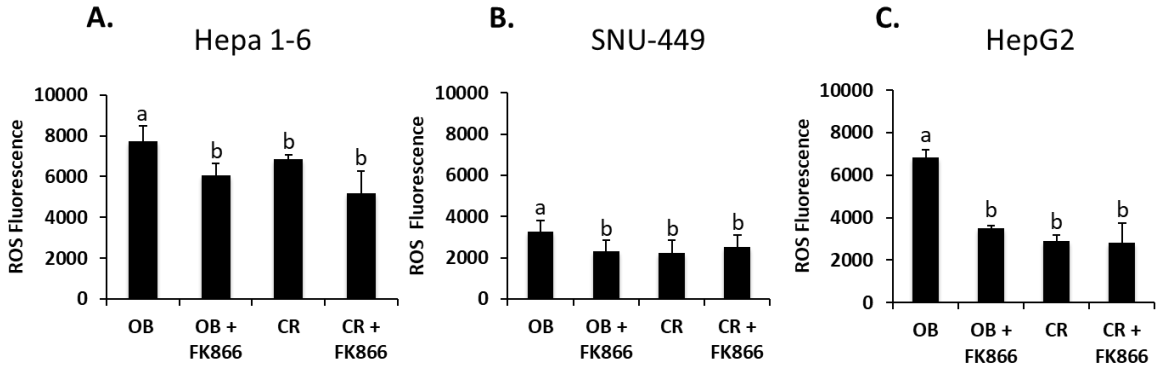

Figure 2. Hepa 1-6 (A), SNU-449 (B) or HepG2 (C) cells were exposed to the treatments for 24 hours. After 24 hours, cells were labeled with DCFDA (20 μM) and then analyzed on a fluorescent plate reader. OB= Obese sera, FK866= iNAMPT inhibitor, and CR= Control sera. Data shown represents the average of at least three independent experiments. Results were compared using ANOVA followed by Tukey's post hoc test. Different letters indicate significant differences between experimental conditions, $p<0.05$.

The effect of the addition of FK866 on LDH secretion

Cytotoxicity assessment via LDH secretion was used to assess the apoptotic ability of tumor cells when exposed to the inhibitor.[21] OB serum decreased LDH secretion by 52% in Hepa 1-6, 59.7% in SNU-449, and 51.1% in HepG2 when compared to the CR serum ($P < 0.05$) (Figure 3A/B/C). The addition of FK866 to OB serum showed an additional 18% decrease in Hepa 1-6, 27.19% decrease in SNU-449, and 57.7% decrease in HepG2 cell lines ($P < 0.05$). No significant differences in LDH secretion were observed between CR serum and the addition of FK866 to CR serum in all three cell lines. These data suggest that LDH secretion is reduced, and cell death increases with the addition of an NAD inhibitor in an obesogenic environment.

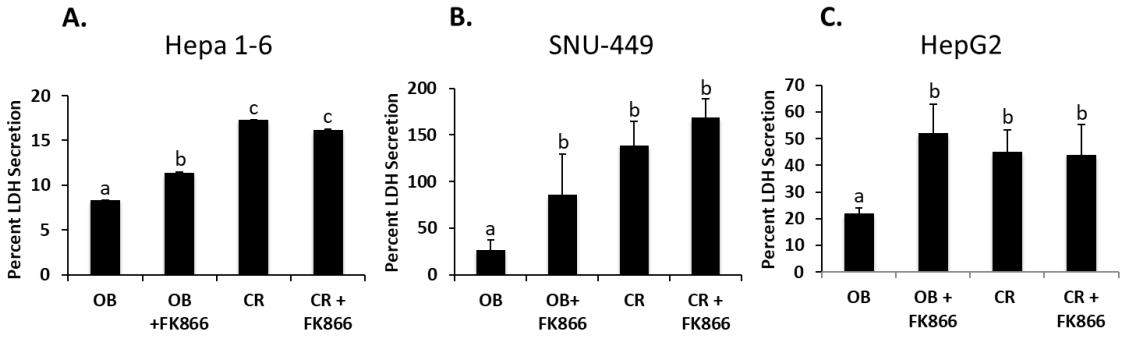

Figure 3. Hepa 1-6 (A), SNU-449 (B) or HepG2 (C) were exposed to treatments in serum free media. OB= Obese sera, FK866= iNAMPT inhibitor, and CR= Control sera. Cells remained in treatments for 24 hours, thereafter the Invitrogen LDH Cytotoxicity Kit was used to assess LDH secretion in cells following addition of treatments. Different letters indicate significant differences between experimental conditions, $p<0.05$.

Inhibition of NAD reduces invasive potential

Cellular invasion is a metastatic process where malignant tumor cells spread to surrounding tissues. The invasion assay was used to determine the efficacy of inhibiting NAD synthesis to reduce the invasive potential of cancer cells exposed to sera. Invasive capacity was

increased by 22.28% in Hepa 1-6, 32.61% in SNU-449, and 50.51% in HepG2 when cells were exposed to OB serum ($P < 0.05$) (Figure 4A/B/C). Furthermore, the addition of FK866 to OB serum reduced invasive capacity in Hepa 1-6, SNU-449, and HepG2 by 26.24%, 28.15%, and 40.57% respectively ($P < 0.05$). No significant differences were observed with the addition of FK866 to CR serum. These data suggest that FK866 decreases the invasive capacity of cancer cells by inhibiting a pro-growth enzyme.

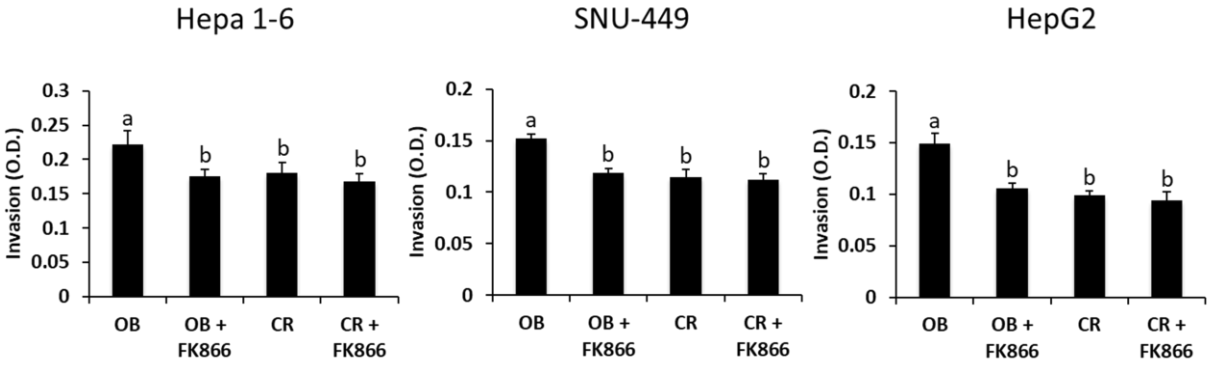

Figure 4. Hepa 1-6 (A), SNU-449 (B) or HepG2 (C) liver cancer cells were seeded in BD Biocoat Matrigel Chambers and OB, OB+FK866, CR, and CR+FK866 were added to the top of the chambers and FBS was added in the bottom chamber. After 48 hours, cells were fixed, stained, and counted. Data shown represents the average of at least three independent experiments. Different letters indicate significant differences between experimental conditions, $p<0.05$.

Differential effects of NAD inhibition on kinase signaling

Activation of kinase signaling pathways, such as ERK and Akt, have been shown to be associated with pro-tumorigenic characteristics, such as tumor growth, proliferation, and invasion into other tissues.[22] Akt and ERK kinase signaling pathways were assessed via immunoblot to investigate the effect of obesity and the impact of FK866 on protein levels. In the Hepa 1-6 cell line, pAkt was shown to be upregulated in OB serum and reduced with the addition of FK866 (Figure 5A). No differences were seen between the OB and CR group for pERK,

however, FK866 was shown to decrease ERK signaling. The SNU-449 cell line was shown to have an increase in pERK in the presence of obesity, but with differential effects with the addition of FK866 (Figure 5B). Lastly, differences were not observed between OB, CR, and the addition of FK866 to either group in the HepG2 cell line (Figure 5C).

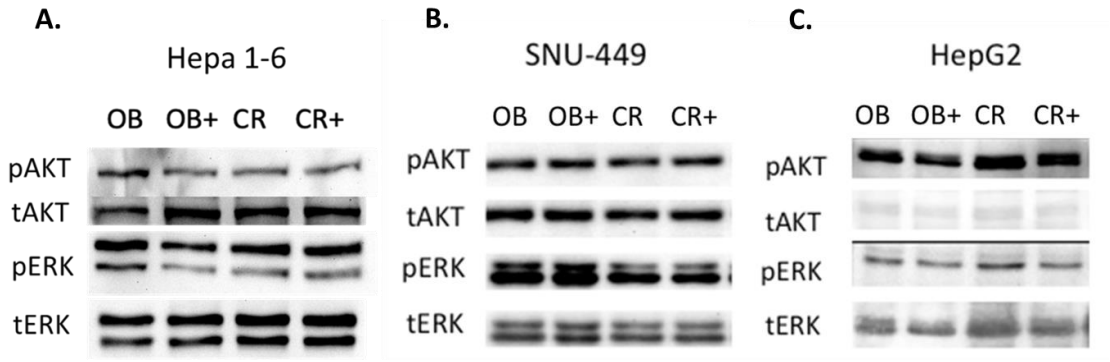

Figure 5. Hepa 1-6 (A), SNU-449 (B) or HepG2 (C) cells were seeded at 250,000 cells/well for 24 h. Cells were serum starved for 24 h and then treated in SFM with 5% OB sera, 5% OB sera + FK866 (iNAMPT inhibitor) 10 nM, 5% CR sera, 5% CR sera + FK866 10 nM for 1 hour. Western blot analysis was used to measure phospho-ERK, total ERK, phospho-Akt, and total Akt.

Body weight and tumor incidence

Due to this being an ongoing study, only mouse body weights and tumor formations were collected. Body weight was used to assess the effect of high fat diet on weight gain by increased adiposity in mice. Mice on a high fat diet had a 54% increase in body weight, which shows a significant difference in weight compared to those on a control diet ($P < 0.05$) (Figure 6A). Tumor formation was determined by daily palpation of tumor injection sites. At the time of reporting, 66% of OB mice had the presence of a tumor, while only 16% of CR mice were determined to have tumors (Figure 6B). As anticipated, mice fed a high diet demonstrated increased body weight and tumor incidence.

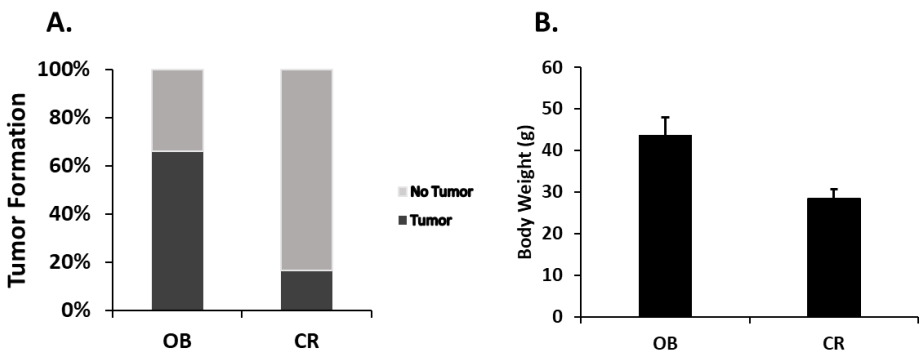

Figure 6. At 21 weeks, C57/BL6mice were injected with 500,000 Hepa 1-6 liver cancer cells and palpable tumors were detected at 23 weeks (A). These mice were randomized to a high fat diet (OB) or control (CR) diet for 23 weeks and final body weights were measured (B).

IV. DISCUSSION

Excess adiposity as a result of obesity produces pro-inflammatory adipokines and cytokines that support tumorigenesis by increasing proliferation and enhancing the biomolecules necessary for growth. One adipokine, NAMPT, and its different derivatives (eNAMPT and iNAMPT), are key targets for novel therapeutics. iNAMPT has been previously established as being overexpressed in other types of cancers and has been positively correlated to high levels of adiposity. As obesity increases, iNAMPT levels also increase as result, leading to the upregulation of harmful and pro-tumorigenic changes in the liver such as increased proliferation, ROS production, invasion, and survivability.[14] The connection between iNAMPT in the presence of obesity and its role in tumorigenesis make it an important target of inhibition in order to possibly halt cancer progression.

Studies have shown that obese serum increases cell proliferation compared to serum from a lean phenotype.[23] The data in this study follows a similar trend, where the liver cancer cell lines treated with obese serum resulted in increased proliferation compared to the control group, thereby demonstrating that obesity promotes cell growth. On the other hand, other studies involving the use of FK866 as a NAMPT inhibitor have shown that FK866 reduces cell viability of other hepatic cancer cell lines in high doses (5 nM to 40 nM).[18,24] According to the data from this study, FK866 treatment in all three cell lines resulted in a similar trend, with cell proliferation being decreased with the addition of the NAMPT inhibitor. This suggests that blocking NAD synthesis with FK866 has a significant effect on reducing liver cancer cell viability in the context of obesity.

Oxidative stress as a result of ROS production from inflammation and metabolic processes is common in many disease states, including cancer and increased adiposity.[25] The

intracellular destruction that follows the production of ROS creates an ideal environment for cancer to progress. The presence of obesity has been shown to be a pathological process that may induce oxidative stress due to the excretion of pro-inflammatory cytokines from adipocytes such as IL-6 and TNF-α. When inflammation is exacerbated by obesity due to secretion of adipocytokines, mitochondrial function can be altered, leading to the increased production of ROS.[26] One study demonstrated a reversal in ROS production with the introduction of FK866 to cells treated with H_2O_2.[27] The data from the current study shows that cells treated with obese serum had a significant increase in ROS production compared to control, whereas the addition of FK866 to the obese group decreased ROS in all three cell lines. The data from this study may suggest that increased ROS production from the presence of obesity can increase tumor growth and cause larger tumors. The high levels of adiposity that come with obesity create a pro-inflammatory environment that degrades metabolic processes, possibly leading to the accumulation of ROS and thus perpetuating inflammation. Previous research on ROS levels in tumorigenesis have shown that ROS, as well as other molecules, are responsible for the activation of signaling cascades that lead to proliferation, invasion, suppression of apoptosis and thus the formation of larger tumors.[28] Larger tumor size can have an effect on prognosis and treatment outcomes in those with HCC. The data collected from the current study also suggests that the inhibition of NAD biosynthesis via FK866 decreases ROS production, and thus tumor growth. On another note, while the murine Hepa1-6 and human HepG2 liver cancer cell lines had significant decreases in ROS production with the addition of FK866, the same pattern was not as robust in SNU-449. This difference may be due to metabolic differences in enzymatic pathways and genetic background between the liver cell lines used in this study. Because ROS is a byproduct of cellular metabolism, future directions may involve investigating the mechanisms

by which NAD inhibition slows the release of ROS in order to decrease the overall production of ROS in an obesogenic environment.

Cell survivability and the avoidance of cell death is one of the Hallmarks of Cancer and has become a popular point of interest for treatment.[7] LDH is commonly excreted by cells in the early stages of undergoing cell death, also known as apoptosis.[29] In other words, LDH secretion is a precursor event prior to initiating the steps leading to cell death. One study found that leptin, an adipocytokine known to have increased levels during cancer development, impaired natural killer cell cytotoxicity. While the methods in this study assessed cytotoxicity through the secretion of LDH, the data remains consistent as the presence of obesity decreased LDH secretion and thus cytotoxicity. In another study, it was shown that treating glioblastoma cells with FK866 increased LDH release, suggesting that more cells were undergoing apoptosis when NAD biosynthesis was inhibited.[29] The findings of this study follow a similar pattern, with OB by itself decreasing LDH secretion and the addition of FK866 increasing secretion in all three cell lines. The decrease in LDH indicates less cytotoxicity, meaning cells are not undergoing apoptosis. This may suggest that the presence of obesity protects cells from cell death. Despite this, the addition of FK866 shows that there is a key pathway that can be blocked with the inhibition of NAD biosynthesis, thus sensitizing cells to apoptosis. These data are also supported by the cell viability assay used in this study, where viability was shown to have an increase in the presence of obesity yet decreased with the addition of FK866. To extend these findings, future studies may investigate analyzing the specific mechanisms behind cytokines released by adipocytes and its protective ability against cell death. On the other hand, future studies may also investigate strategies to sensitize cells to apoptosis in order to reverse the protective ability of obesity-associated tumorigenic proteins.

Cellular invasion is a critical step in metastasis, which is the process of cancer cells spreading to other tissues and causing destruction to other body systems. One study shows that the addition of adipocytes to prostate cancer cells increased cell invasion significantly compared to cells that were not treated with adipocytes.[30] In another study, when MHCC97-H cells were treated with the NAMPT inhibitor FK866, cells were shown to be significantly less invasive than those without the inhibitor.[24] Thus, previous research aligns with the results observed in this study, which showed that all cell lines exposed to OB serum had an increase in invasion compared to control, whereas the addition of FK866 to OB groups significantly decreased invasion in all three lines. These results suggest that the presence of obesity-associated proteins, such as hormones and cytokines, may increase the invasive capacity of tumor cells but can be decreased via inhibition of NAD biosynthesis. In addition to invasion, future studies could investigate the role of obesity-associated hormones in enhancing the metastatic cascade, such as the role of visfatin in promoting the epithelial to mesenchymal transition, which is a key process in the early stages of invasion.

ERK and Akt are key signaling pathways that are known to be upregulated in both obesity and cancer. In one study, both kinases were shown to be increased in the presence of adipocytes, confirming the results found in this study where pAkt and pERK protein levels were increased in the presence of obesity-associated hormones and cytokines.[28] Studies have shown that the upregulation of these kinase pathways have been implicated in obesity-induced cancer progression through stimulating signal cascades that increase cell proliferation, growth, and metabolism.[28] Another study demonstrated that IGF-1, a hormone elevated in obesity, plays a role in activating downstream ERK and Akt signaling pathways, showing that upregulation of the pathways via IGF-1 led to the promotion of cancer cell invasion and proliferation.[31] The

study demonstrated that the inhibition of IGF-1 signaling reduced Akt and ERK levels, and thus slowed cancer progression.[31] The results of this study were shown to be consistent with those findings, where the inhibition of NAD via FK866 reduced Akt protein levels in Hepa 1-6. However, the results were not consistent amongst all three cell lines, indicating that each cell line may have altered pathways associated with tumorigenesis. Obesity associated hormones activate kinases that can promote downstream transcription factors, such as NF-kB, which lead to the production of more cytokines and proteins that affect invasion and proliferation. The mechanism by which FK866 inhibits the signal mediating these physiological outcomes needs to be explored further. Further studies may also investigate the mechanisms by which NAD inhibition mediates ERK and Akt signaling differentially in liver cancer cell lines.

A high-fat diet has been previously shown to alter the metabolic ability of liver cells, eventually leading to liver cancer progression through increased ROS production and DNA damage.[32,33] Mice fed a high-fat diet have been shown to accelerate liver cancer development.[33] This aligns with the results found in this study, where 66% of mice fed a high fat diet developed tumors while only 16% of mice on a control diet developed tumors. This data suggests that an obese environment plays a significant role in the development and sustainability of liver cancer.

The data found in this study is a big step in further understanding the impact that obesity has on tumorigenesis and possible interventions that may block obesity-induced progression. Still, further analyses need to be performed to understand the role of energy production and cancer development, such as looking at the NAD/NADH ratio to assess FK866's efficacy in blocking NAD synthesis, MMP-9 to assess inflammatory markers in tissue, and completion of serum analysis of the current mouse model. A strength of this study is the novel strategy to break the obesity-liver cancer link through the exploitation of cellular energetics, specifically through

blocking NAD biosynthesis. Currently, cancer therapeutics primarily focus on inhibition angiogenesis, signal interruption to inhibit cell growth, and promoting cancer cell death through apoptotic induction or through delivery of toxins, radiation, and other cell-killing substances. Another strength of the study was the use of pooled obese serum to look at the collective effects of combined hormones, though this may also be considered a limitation since it is difficult to assess the specificity of what hormones or enzymes in the serum are promoting pro-tumorigenic effects. In future studies, it may be useful to determine the contribution of individual hormones through the addition of a neutralizing antibody to target specific proteins upregulated in obesity. Finally, while the primary aim of the study was to look at the efficacy of FK866 in an obesity-indued liver cancer model, the mechanistic actions behind FK866 should be elucidated in order to improve understanding of its role in NAD inhibition.

V. CONCLUSION

In conclusion, the purpose of the study was to determine the role obesity plays in liver cancer progression, and how targeting cellular energetics through NAD inhibition can lessen the impact of obesity on carcinogenesis. This study found that the presence of obesity significantly increases cancer cell proliferation, invasion, ROS production, and decreases cytotoxicity, all of which are Hallmarks of Cancer. On the other hand, it was found that NAD inhibition via FK866 was able to reverse the obesity-induced hallmarks, thus signifying its efficacy as a potential intervention for liver cancer in an obesogenic environment.

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