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The Warburg Effect and Glutamine: Targeting the Deregulation of Metabolism in Human Hepatocellular Carcinoma Cells

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I. Abstract:

Hepatocellular carcinoma (HCC) is the most common type of liver cancer in the world. Effective therapy options are limited; sorafenib is currently the only FDA-approved medication for the disease. In order improve clinical prognoses, alternative treatment options must be assessed and their mechanisms of action elucidated. An important hallmark of cancer is the deregulation of normal cellular energetics, resulting in a number of crucial metabolic adaptations to promote excessive proliferation. These changes include: the upregulation of aerobic glycolysis (the “Warburg Effect”), coordinate over-expression of the tertiary nutrient transporter system ASCT2 and LAT1, and enhanced glutamine uptake to support biosynthesis and stimulate growth signaling. It is important to discover, understand, and exploit the metabolic differences between normal and cancerous hepatocytes in order to develop successful therapies for HCC.

This project is comprised of three objectives: (1) to assess the efficacy and effective median dosage of a multitude of metabolic inhibitors on a panel of epithelial and mesenchymal-derived HCC cell lines, (2) to determine the growth impact of depriving SK-Hep-1-derived shRNA-mediated ASCT2 and LAT1 knockdown cell lines of glutamine, and finally (3) to quantify the expression of ASCT2 and LAT1 in cell lines under specific treatment conditions. These objectives were accomplished through the combined usage of cell culture techniques, proliferative assays, and western blot analysis. The most significant results include the effects of metformin, a drug currently prescribed to patients with type 2 diabetes. The efficacy of metformin in vitro was determined to be comparable to the FDA-approved medication sorafenib. Metformin was also found to upregulate ASCT2 expression in both epithelial and mesenchymal cell lines. Assessing the biochemical effects of metformin on HCC is necessary to answer a
critical question in cancer biology: how does metformin induce cell death in human hepatocellular carcinoma?

II. Introduction:

Human life expectancy is increasing, especially in modern times, due to a variety of factors, including improved nutrition, sanitation, and medical advancements. Humanity has effectively developed methods to manage and prevent severe diseases. While increasing life expectancy has been a monumental development, it has also exposed an ancient disease: cancer. Cancer is defined as a group of over 200 diseases that involve abnormal cellular growth and proliferation, with the potential to metastasize to other tissues (NIH, 2015). In 2016, an estimated 1,685,210 cases of cancer will be diagnosed in the United States; consequently, approximately 595,690 people are predicted to succumb cancer. A startling 40% of men and women will be diagnosed with cancer at some point in their lives (American Cancer Society, 2016). Cancer has failed to gain prevalence until the last century because the disease manifests in aging. Contributing to aging, the apparent incidence of cancer has increased substantially because of improvements in screening techniques, such as mammography and PET-CT. Because of the increasing frequency of cancer, the United States alone is expected to spend nearly $156 billion in 2020 (NIH, 2016). Current treatment methods for cancer include surgery, chemotherapy, and radiation. Depending on the individual, these options can be quite effective. However, there are potentially substantial side effects to these treatments. Select chemotherapies and radiation have an inherent risk of producing highly drug-resistant neoplasms in the affected tissue after treatment. Another problem with chemotherapy and radiation is that for some cancers, the therapies do little except to shortly extend the lifespan of the patient. Because of these issues, it
is crucial for research to be dedicated to understanding the mechanisms by which a cancer manifests and persists in the face of aggressive treatment regimens. This effort will eventually result in an era of personalized medicine, where a plethora of treatments are available and utilized based on an individual genetic assessment of the cancer.

Hepatocellular Carcinoma (HCC), the most common type of liver cancer, is the third leading cause of cancer mortality worldwide. It is the fourth most common cancer in the world, and in North America the incidence is approximately two people out of every 100,000, whereas in China, the incidence is higher at a rate of eighty people per 100,000 (Altekruse et al, 2009). While the United States has a much lower incidence compared to areas of Asia and sub-Saharan Africa, the prevalence has been increasing. In 2015, roughly 25,000 people in the United States died from HCC (American Cancer Society, 2016). In most cases, HCC is a secondary manifestation following liver cirrhosis, wherein the liver has been damaged to the point of losing functionality. This process causes dramatic genetic and epigenetic changes in hepatocytes, which can potentially result in a cancerous phenotype. Liver cirrhosis can be caused from alcohol abuse, autoimmune diseases, viral hepatitis B or C, long term inflammation, and hemochromatosis (El-Serag et al, 2007). In the United States, 30% to 40% of HCC is the result of chronic hepatitis B or C (Caselmann, 1996). The available treatment options for HCC are limited; currently there is only one FDA-approved chemotherapy for the disease: sorafenib. This treatment is only moderately effective in hampering HCC progression. Because of the lack of treatment options, research has been largely concentrated on elucidating the metabolic differences between normal and cancerous hepatocytes in order to identify potential therapeutic targets.
A hallmark of cancer is the deregulation of normal cellular energetics (Weinberg et al., 2011). The purpose of this deregulation is to support excessive growth and proliferation. The functionality of various intracellular signaling pathways and enzymes have been altered in HCC cells as a result. Previous research indicates that HCC cells have a higher capacity to take up the amino acid glutamine from the extracellular environment, primarily through the plasma membrane amino acid transporter ASCT2 (SLC1A5) (Wise et al., 2010). Glutamine is being utilized for two purposes: the synthesis of metabolic precursors and intermediates required for cellular proliferation, and the LAT1-coupled transport of leucine. Glutamine has the highest blood plasma concentration of any amino acid because it is the primary carrier of nitrogenous functional groups in the blood. These nitrogenous groups are incorporated into the synthesis of immense quantities of nucleotides and proteins required for cancerous proliferation. Glutamine can also be used to synthesize other amino acids and α-ketoglutarate, an important, bidirectional TCA cycle intermediate (Nelson et al., 2012). ASCT2 is found to be significantly over-expressed in a variety of epithelial cancers, and is thought to be the fundamental mediator of increased glutamine uptake in these cancers (Bode et al., 2002). ASCT2 is a sodium-dependent, neutral amino acid transporter which utilizes the sodium concentration gradient to energetically couple the import of certain amino acids into the cell, including glutamine and asparagine. The mere presence of glutamine has been linked to the overexpression of SLC1A5, the gene sequence for the ASCT2 transporter (Brasse-Lagnel et al., 2009). The gene for the LAT1 transporter, SLC7A5, is also over-expressed in HCC. LAT1 is a sodium-independent transporter that uptakes large, branched, neutral amino acids, particularly leucine (Qiang et al., 2013). An important function of LAT1 is utilizing intracellular glutamine to uptake leucine via an exchange mechanism. The
overexpression of both of these amino acid transporters contributes greatly to the increased influx of glutamine and leucine into the cell.

Figure 1. ASCT2 and LAT1 tertiary amino acid transporter system. Bracketed amino acids are transported by both ASCT2 and LAT1 (Image: Paige Bothwell).

While both glutamine and leucine are important for biosynthesis, these amino acids serve another role in HCC cells: sustained activation of mTOR growth signaling. The mTORC1 signaling protein, an acronym for mammalian target of rapamycin complex one, is a crucial serine/threonine kinase component of cell signaling implicated in both adjusting growth rates to nutrient availability and inhibiting autophagy (Villanueva et al, 2008). Leucine, transported by LAT1, is the primary stimulator of mTORC1 (Stipanuk, 2007). High intracellular glutamine concentrations have been shown to activate mTORC1 signaling, albeit through a different mechanism than leucine (Fumarola et al, 2005). ASCT2 and LAT1 are classified as a tertiary
transporter system because the ASCT2-imported glutamine is utilized by LAT1 to uptake leucine, which subsequently activates mTORC1 (Bode et al, 2005). Some of the downstream targets of mTORC1 signaling include the transcription and subsequent translation of proteins involved in ribosome biogenesis, nutrient transport, and the inhibition of autophagy (Nicklin et al, 2009). The activation of translational machinery, enhanced nutrient import for biosynthesis, and downregulation of protein turnover greatly enhances HCC cell survival.

Figure 2. The complexity of mTOR growth signaling (Image: http://www.novusbio.com/mTOR-pathway).

Within a tumor, areas become nutrient-deprived due to the lack of an adequate blood supply. This is primarily a result of restricted blood vessel circulation due to intra-tumoral cell density,
termed the necrotic center. Limited vascularization leads to very low oxygen concentrations, a hypoxic environment, and hypoxia induces the expression of a transcription factor known as hypoxia-inducible factor-1α (HIF-1α). HIF-1α has many transcriptional targets including vascular endothelial growth factor (VEGF). VEGF is responsible for a variety of growth signals including angiogenesis, the development of blood vessels. A common characteristic of HCC is to constitutively activate HIF-1α, sustaining growth signaling (Moon et al., 2003). In addition to limiting the oxygen supply, limited vascularization in dense regions of a tumor constrains the delivery of glutamine via the blood stream. In such cases, cancerous tissue will upregulate glutamine synthetase (GLUL) to produce glutamine from glutamate, ammonia, and ATP. In fact, GLUL is shown to be overexpressed in HCC, particularly under the previously described hypoxic conditions (Kuramitsu et al., 2006). These are two clever mechanisms HCC can evolve to promote desired signaling and maintain intracellular glutamine concentrations.

Another critical metabolic difference between HCC and normal hepatocytes is the significant upregulation of glycolysis, the first ATP-yielding step converting sugar to energy. Normally, hepatocytes acquire most of their ATP through oxidative phosphorylation. The uncoupling of glycolysis from oxygen tension, despite the efficiency of oxidative catabolism, is known as the “Warburg effect” (Heiden et al., 2009). The hypoxic environment in the tumor, coupled with beneficial VEGF signaling, selects for cells that can thrive on means other than oxygen through a Darwinian mechanism. The purpose of the transition is not for energy generation, but to exploit the pentose-phosphate shunt and other feeder pathways of glycolysis and the TCA cycle to produce metabolic precursors.
Figure 3. Cancer metabolism in contrast to normal metabolism. Enhanced glucose uptake and upregulated glycolysis results in the accumulation of lactate, a byproduct that is subsequently expelled from the cell, acidifying the extracellular environment (Image: http://www.sabiosciences.com/pathwaymagazine/minireview/metareprogram.php).

While HCC and other cancers acquire these characteristics to sustain proliferative capacity, there are a number of important defense mechanisms established as well. One of these mechanisms is the over expression of glutathione (GSH), a crucial protein complex whose role is to eliminate reactive oxygen species produced by the cell. Interestingly, GSH also provides drug resistance by binding chemotherapies and facilitating their expulsion from the cell (Batist et al, 1986). Altered metabolism coupled with defensive capabilities fortifies the ability of HCC to survive and persist within the body. The purpose of describing these key biochemical changes is to provide background for the various pathways targeted for therapeutic effectiveness in this project. The following table displays the names, concentrations, and functions of the treatments used:
Table 1: Names, concentrations, and functions of the inhibitors used throughout the project. Metformin, a drug used for modulating blood glucose levels in patients with type II diabetes, is of particular interest. Epidemiological research indicates the incidence of various cancers decreases in patients prescribed metformin; however, the mechanism of growth inhibition and cell death is unknown.
III. Methodology:

Cell Lines and Treatments:

Epithelial and mesenchymal-derived HCC cell lines were utilized throughout the experiments. Epithelial cell lines include: Huh1, Huh7, HepG2, Hep3B, and P5. Mesenchymal cell lines include: Focus, 2237, 2238, 2234, SK-Hep1, and 2236. SK-Hep1 LAT1 and ASCT2 knockdown cell lines were also used; they were generated by Dr. Barrie Bode’s doctoral student Paige Bothwell using a lentiviral vector encoding several specific short hairpin RNAs (shRNA) targeting the mRNA transcripts of ASCT2 and LAT1. Puromycin resistance and Green Fluorescent Protein (GFP) were indicators of successful plasmid integration. Cell lines were maintained at 37°C in a humidified atmosphere of 5% CO$_2$-95% air in Dulbecco’s Modified Eagle Medium (DMEM, 4.5 mg/ml D-glucose) supplemented with 10% triple 0.1 μm filtered fetal bovine serum (FBS), 2 mM L-glutamine, 1% antibiotic/anti-mycotic solution (100x stock: 10,000 U/ml Penicillin G, 10,000 μg/ml Streptomycin, and 25 μg/ml Amphotericin). The culture vessels used include 25 cm$^2$ Falcon T-flasks with 0.2 μm vented blue plug seal caps, 100mm plates, 12-well plates, and 48-well plates. All cell count measurements in this study were performed using a hemacytometer. Treatments were filter-sterilized to prevent bacterial contamination.
MTT Cell Proliferation Assay:

The MTT cell proliferation assay is a colorimetric, metabolic assay utilized to determine cell number. The concept is that the quantity of oxidoreductase enzymes in a well will be proportional to the number of cells; these enzymes reduce tetrazolium dye to formazan, a purple crystal (Fig 5.). After treatments were completed, the tetrazolium dye solution was added to the wells and incubated to promote the enzymatic reaction. Next, the plates were dumped and the formazan was solubilized using a 0.04 N HCl solution in absolute isopropanol. Absorbance data was collected from the spectrophotometer and exported to Microsoft Excel to be displayed graphically.
Figure 5. After solubilizing formazan crystals, cell number can be qualitatively assessed based on the purple hue; however, this is assuming the number of oxidoreductase enzymes are consistent between cells (Image: http://www.iivs.org/scientific-services/laboratory-services/ocular-irritation/human-3d-tissue/step-by-step/).

Tali™ Image-Based Cytometer:

In contrast to the MTT assay, the Tali™ Image-Based Cytometer counts the cells directly. This eliminates a confounding variable: assuming the number of oxidoreductase enzymes is consistent between cells. Cells were washed in phosphate buffered saline (PBS), TrypLE, an enzymatic solution for dissociating adherent cells from a growth surface, and then suspended in supplemented DMEM. Cells were subsequently loaded into capillary plates and examined using the Tali™ Image-Based Cytometer and associated programming. Data was exported to Microsoft Excel to be displayed graphically.
**SDS-PAGE:**

Extracted protein lysate concentration was measured using the Nanodrop 2000™ Spectrophotometer. After determining protein concentrations, 20 µg of each protein sample was prepared using 1 M dithiothreitol (DTT), 4x sample Laemmli buffer (LSB), and 1x lysis buffer. Samples were subsequently denatured and separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 150 V for 45 minutes. The electrical field generated in the gel allows denatured proteins to separate based on molecular weight. Biotinylated protein ladder and three color molecular weight marker were also loaded; they are used to confirm protein band location.

**Western Blotting:**

Before SDS-PAGE was completed, 1x transfer buffer was prepared and refrigerated. PVDF membranes were equilibrated for fifteen seconds in methanol, two minutes in nanopure water, and five minutes in 1X transfer buffer. After SDS-PAGE was completed, the gels were removed and incubated in transfer buffer for ten minutes. A transfer “sandwich” was then constructed and ran at 75V for 90 minutes (Fig 6.). Ice surrounds the rig to lower the temperature, facilitating a successful transfer. Afterwards, the western blots were hung to dry for one hour. After drying, the blots were incubated in blocking buffer composed of 5% bovine serum albumin (BSA) in tris-buffered saline and 1% tween (TBST) for one hour. This is to prevent non-specific binding between the antibody and PVDF membrane instead of the intended protein. After the blocking step, the membranes were further incubated in the primary antibody of interest for twelve hours. The primary antibody specificity depends on the protein of interest;
antibodies can also be monoclonal or polyclonal. The blots were then washed 3 times for 5 minutes in TBST. After the washes, the blots were incubated in anti-rabbit, HRP-conjugated secondary antibody for one hour. The secondary antibody binds specifically to the primary antibody used in the previous step (Fig 7.)

![Diagram 1: Illustration of Western Blot Setup.](http://www.antikoerper-online.de/resources/17/1224/Western+Blot+Hintergrundinformationen/)

Figure 6. Western blot setup, including the contents of the “sandwich” (Image: http://www.antikoerper-online.de/resources/17/1224/Western+Blot+Hintergrundinformationen/).

**Membrane Imaging:**

After three, five minute washes in TBST, membranes were incubated for one minute in Lumiglo chemiluminescent substrate. Lumiglo is comprised of luminol and horseradish peroxidase (HRP), where the luminol is converted to an intermediate dianion in the presence of
hydrogen peroxide. This dianion emits light when reverting to its ground state energy stability, which can be captured on x-ray film using Genesnap software. The membrane was then re-probed for a particular loading control depending on the protein of interest.

![Western blot and imaging mechanism](http://info.gbiosciences.com/blog/bid/159273/Which-Blocking-Agent-for-Western-Blotting)

**Figure 7. Western blot and imaging mechanism.**

**Protein Quantification:**

In order to quantify and normalize protein quantities to the loading controls used, Image-J and Microsoft Excel software was utilized. The ratio of black pixels in the bands can be compared and displayed in a bar graph, providing a quantitative and normalized representation of the results.
IV. Results:

Graph 1. Epithelial HCC cell lines HepG2, Hep3B, Huh1, Huh7, P5, and Tong under the treatment conditions of metformin, sorafenib, rapamycin, GPNA, MeAIB, and BCH. MTT proliferation assays were performed at 24, 48, 72, and 96 hours. DMSO was used as a vehicle control for sorafenib and rapamycin. The x-axis represents time while the y-axis is the ratio of control absorbance.
Graph 2. Mesenchymal HCC cell lines SK-Hep1, Focus, 2234, 2236, 2237, 2238, and Mahlavu under the treatment conditions of metformin, sorafenib, rapamycin, GPNA, MeAIB, and BCH. THLE5B is an immortalized hepatocyte cell line and MIA-PACA-2 is a pancreatic cancer cell line. MTT proliferation assays were performed at 24, 48, 72, and 96 hours. DMSO was used as a vehicle control for sorafenib and rapamycin. The x-axis represents time while the y-axis is the ratio of control absorbance.
Graph 3. Mesenchymal HCC cell line SK-Hep1 treated with various concentrations of buthionine sulfoximine, cobalt chloride, 2-deoxy-D-glucose, and 3-bromopyruvate. MTT proliferation assays were performed after 72 hours in order to establish an ED$_{50}$, which is the median effective dosage of treatment. The estimated ED$_{50}$’s are: 0.60 mM (2DG), 0.28 mM (CoCl$_2$), 0.07 mM (BSO), and 0.05 mM (3BP). The x-axis is treatment concentration and the y-axis is the absorbance measured.
Graph 4. SK-Hep1-derived ASCT2 (A2) and LAT1 (L1) knockdowns were incubated in differing concentrations of the amino acid glutamine for 72 hours. Media was changed daily for the “fed” cell lines, while the “un-fed” cell lines retained the same media from time zero. The nonsense control refers to SK-Hep1 that was manipulated via RNAi; however, purposely no knockdown is exhibited. The Tali™ image-based cytometer and MTT assay were performed at the 72 hour time point and compared to determine differences between metabolic and concentration centered growth assays. The x-axis is the differing glutamine concentrations, while the y-axis is average absorbance for the MTT assay and cell concentration for the Tali™ image-based cytometer.
Figure 8. Mesenchymal HCC cell line SK-Hep1 was treated with normal media, vehicle control (DMSO), sorafenib, rapamycin, metformin, and lactate for 24 hours (C, V, S, R, M, L labels respectively). Lactate was used because it is a large byproduct of the “Warburg Effect”. The images were derived from western blot analysis. Beta-actin and Cox IV were used as loading controls for ASCT2 and LAT1 respectively. Protein quantification and normalization were accomplished through Image-J software. The x-axis represents the different treatments and the y-axis is the ratio of the control protein quantity.
Figure 9. Epithelial HCC cell line Hep3B was treated with normal media, vehicle control (DMSO), sorafenib, rapamycin, metformin, and lactate for 24 hours (C, V, S, R, M, L labels respectively). Lactate was used because it is a large byproduct of the “Warburg Effect”. The images were derived from western blot analysis. Beta-actin and Cox IV were used as loading controls for ASCT2 and LAT1 respectively. Protein quantification and normalization were accomplished through Image-J software. The x-axis represents the different treatments and the y-axis is the ratio of the control protein quantity.
V. Discussion

The MTT and cytometric analysis demonstrated that the treatments that were the most successful in inhibiting cellular proliferation were sorafenib, rapamycin, and metformin (Graphs 1 & 2). Sorafenib’s efficacy was expected because it is currently the only FDA-approved medication for HCC. Inhibition of the growth signaling pathways mediated by VEGFR/PDGFR was certainly effective in vitro. Rapamycin, a drug commonly employed in transplant patients, was also effective in many of the cell lines. Rapamycin inhibits mTORC1, the growth signaling pathway stimulated by leucine. Remarkably, metformin was similarly as effective as sorafenib in many of the cell lines. Metformin is a gluconeogenic inhibitor; this is a component of the mechanism by which it modulates blood sugar concentrations. This may interfere with the “Warburg Effect” described previously, preventing proliferation and causing cell death. Metformin’s impact on growth appears to be more significant in the mesenchymal cell lines versus the epithelial. Perhaps hampered gluconeogenesis affects aggressive cell lines more because they require relatively more biosynthetic precursors than a slower, epithelial cell line. Another inhibitor that worked moderately was GPNA; P5, HepG2, SK-Hep1, and Mahlavu were all significantly affected. GPNA inhibits ASCT2, the transport protein responsible for the upregulated influx of glutamine. Insufficient glutamine hampers the cells’ ability to proliferate by limiting biosynthesis and indirect mTORC1 stimulation. This may suggest a greater dependency on the glutamine uptake activity of ASCT2 for survival in these cell lines.

The next objective was determining the effective median doses of four inhibitors using secondary HCC cell line SK-Hep1. The results were as follows: 0.60 mM (2DG), 0.28 mM (CoCl$_2$), 0.07 mM (BSO), and 0.05 mM (3BP) (Graph 3). Buthionine sulfoximine, 3-bromopyruvate, and cobalt chloride were effective at significantly lower concentrations than 2-
deoxy-D-glucose. Buthionine sulfoximine inhibits glutathione synthesis, which would promote the accumulation of reactive oxygen species. Reactive oxygen species can be beneficial to cancer by increasing the incidence of mutation; however, reaching a critical threshold would render the cell incapable of functioning and stimulate apoptosis. Inhibition of glycolysis by 3-bromopyruvate prevents utilization of the “Warburg Effect” to drive growth. Cobalt chloride effectiveness is interesting because it induces a hypoxic response. Hypoxia typically stimulates HIF1-α, which is pro-growth. The mechanism of action is unknown; other effects could be the cause of the growth inhibition.

Glutamine is a significant player in the stimulation of growth signaling; its availability in the extracellular environment differentially affects the growth of the ASCT2 knockdown cell line versus the nonsense control. The results from the glutamine dilution study indicate that when media is not changed daily over 72 hours, at every glutamine concentration the ASCT2 knockdown cell line exhibits decreased proliferation (Graph 4). Since ASCT2 is the primary channel for glutamine influx, reduced ASCT2 expression may manifest in a slower proliferative phenotype. The second, side objective of this particular study was to compare cell growth data received from the MTT assay to the data obtained via the Tali™ Image-Based Cytometer. Both results appear to be quite similar; however, the Tali™ Image-Based Cytometer provides other morphological characteristics of the cells, such as size, and eliminates a confounding variable: the number of oxidoreductase enzymes between cells. This variable appears to be constant from this experiment, and the results from both the MTT and cytometric assays are in accord.

The final study assessed the protein expression of ASCT2 and LAT1 in SK-Hep1 and Hep3B under the treatment conditions of sorafenib, rapamycin, metformin, and lactate. The most significant result concerns metformin: ASCT2 expression is higher in both the primary and
secondary HCC cell lines (Figures 8 & 9). Upregulation of ASCT2 may be a stress response to the induction of oxidative metabolism. However, further experimentation must be completed to accurately assess metformin’s mechanism of action. Another interesting result is that lactate stimulated down-regulation of ASCT2 in SK-Hep1. Since lactate is acidifying the extracellular environment, this decrease in pH may trigger hypoxic responses in the cell. This response may involve downregulating the expression of ASCT2 because there is no purpose to having a transporter for something that is absent in the extracellular environment.

The greatest obstacle in treating cancer is that the molecular and physiological functions utilized by normal cells and tissues are commandeered and exploited; how does one specifically target biochemical signatures that are utilized by the 100 trillion other healthy cells in the human body? Despite this inherent dilemma, novel chemotherapies are being developed constantly to target critical differences that manifest from cancerous transformation. This project was a small step towards assessing the importance of glutamine, ASCT2, LAT1, and the effectiveness of multiple inhibitors on human hepatocellular carcinoma, a cancer that bears dismal prognoses due to the lack of viable therapies. Although cancer incidence is surging, the awareness of this disease is also growing. This increased awareness is manifesting in the development of effective, specialized treatments that have the potential to make lethal cancer a relic of the past.

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