Baruch S. Blumberg Institute

Annual Progress Report: 2011 Nonformula Grant

Reporting Period

July 1, 2013 – June 30, 2014

Nonformula Grant Overview

The Baruch S. Blumberg Institute (formerly Institute for Hepatitis and Virus Research) received $909,170 in nonformula funds for the grant award period June 1, 2012 through August 29, 2014. Accomplishments for the reporting period are described below.

Research Project: Project Title and Purpose

A New Inhibitor of the Akt/mTOR Pathway with Remarkable Potency and Selective Anti-Hepatocellular Carcinoma Activity – This is a proposal for the development into a drug of a novel chemical family with activity against primary liver cancer, known as hepatocellular carcinoma (HCC). HCC is very resistant to chemotherapy, has few therapeutic options for most patients, and is usually fatal. This new drug would be selective for liver cancer cells and would function through a different mechanism than the currently approved anti-HCC chemotherapies. Based on *in vivo* proof-of-concept studies with the parent compound, we predict this series will lead to a drug with higher efficacy, lower toxicity and fewer side effects. At the end of the proposed project, novel compounds will be ready for FDA-sanctioned studies that would lead to an investigational new drug application.

Anticipated Duration of Project

6/1/2012 – 8/29/2014

Project Overview

Our proposal centers on the development of a novel compound for chemotherapy of hepatocellular carcinoma (HCC) patients. HCC is a common consequence of viral hepatitis and fatty liver disease, and its incidence is on the rise in the United States. Globally, it the fourth most common cause of cancer deaths. Current HCC therapies include surgery and liver transplantation, for which only a minority of patients are candidates. Chemotherapy options are limited in both efficacy and availability, as there is only one drug currently approved for use in advanced HCC cases. Our proposal is to test the feasibility of developing a therapeutic compound to selectively target HCC cells. Using cell lines derived from HCC and normal liver tissues, and our "in-house" diverse compound library, we have identified a disubstituted aminothiazole, called HBF-0079 that selectively inhibits growth and viability of HCC-derived cells, while exhibiting minimal effects on normal liver-derived cells. Unlike currently used HCC

drugs, HBF-0079 does not exhibit indiscriminate cytotoxicity, indicating a distinct mechanism of action, and suggesting that it may be useful in cases where resistance to the current drugs has emerged, or where liver damage has rendered the patient sensitive to therapy. We have determined that HBF-0079 inhibits anti-apoptotic and pro-mitotic signaling through the Akt/mTOR axis, with ensuing cell cycle arrest and apoptosis. In addition, the compound also inhibited tumor growth in an *in vivo* xenograft model, constituting proof of concept. Finally, initial chemical optimization of HBF-0079 has already increased potency (CC_{50}) from 1.5 to 0.02 micromolar. The activity of the compound on HCC cell lines with disparate genotypes and oncogenic lesions suggests that it may have broad spectrum activity against HCC, a cancer known for great variability in its response to therapy. This proposal is to perform critical chemical and biological experiments to determine if this approach is practical and feasible. HBF-0079 has several chemical features that offer modification possibilities. Therefore, we will: 1) explore the chemistry and formulation of HBF-0079 to develop an even better analogue; 2) test the active compounds against primary hepatocytes, and a variety of normal hepatocyte, HCC and non-HCC derived cell lines to examine selectivity; 3) determine the absorption, drug metabolism, extraction, and toxicity (ADMET) profiles of two active analogues in the rat; 4) confirm the efficacy of the new compounds in an in vivo model of human HCC; and 5) identify the molecular target of the these compounds to facilitate drug design.

Principal Investigator

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Other Participating Researchers

Yanming, Du, PhD; Huagang Lu; William Kinney, Ph.D.; Nelson Carvalho, MBA; Patti McAloon, MBA – employed by the Institute for Hepatitis and Virus Research (renamed Baruch S. Blumberg Institute)
David L. Horn, MD, FACP, FIDSA consultant
Feng Zhou, MD; Joseph Rager; Lyle Miller – employed by Absorption Systems, LP

Expected Research Outcomes and Benefits

Fulfillment of this proposal will test the feasibility of developing a novel drug-like compound series into a new anti-cancer drug. This project is specifically intended for the treatment of hepatocellular carcinoma, which is a growing problem in Pennsylvania and the US especially among Asian immigrant and disadvantaged urban populations. At completion, the compounds that emerge from this work will 1) be ready for formal FDA required studies to support an investigational new drug (IND)-application, 2) appear to be attractive candidates for further investment and licensing with an industry partner, and 3) open a new avenue toward fulfilling a significant medical need.

Summary of Research Completed

Reporting Period - July 1, 2013 – June 30, 2014:
Aim 1, Milestone 1: Completed Synthesis and testing of up to 50 new derivative compounds[#]
Aim 2, Milestone 1: Completed in vitro selectivity profiling of up to 10 active compounds[#]
Aim 3, Milestone 1: Completed MTD studies of up to 5 compounds in vivo*
Aim 3, Milestone 2: Completed ADMET profiling of up to 5 compounds in vivo[#]
Aim 4, Milestone 1: In vivo proof of concept for up to 2 compounds*
Aim 5, Milestone 1: Preliminary ID of compound binding targets, and mechanism of action
Aim 5, Milestone 2: Progress in confirmatory studies of binding targets, and mechanism of action

* no progress during current reporting period
[#] progress reported during previous reporting period

Progress on Aim 1, Milestone 1 and Aim 2, Milestone 1 during current reporting period:
Metabolic stability guided optimization. Metabolite ID study on IHVR-04042 (see previous reporting period) revealed that there were two major metabolites, in which one was an oxidized derivative and the other was a partial fragment of the parent 04042, indicating the parent 04042 was not stable enough in liver. To reduce the propensity of the oxidation and fragmentation, eleven more metabolically stable bioisosteres were employed to replace the heterocyclic ring and other labile groups. It turned out that IHVR-35119 with a less basic heterocycle and more sterically hindered group maintained the potency and improved selectivity.
Solubility directed optimization 2. A common feature associated with all three previous lead compounds is the poor solubility, not only in water but also in organic solvents. This problem has resulted in unsuccessful preparation of high concentration of the compound in a formulation. Two strategies have been used to improve the solubility. The first one was to make salt by reaction of inorganic acid with basic nitrogens within the molecule. However, this approach only increased aqueous solubility slightly. The second way was to make prodrugs, which were designed to release the parent compounds upon interaction with the enzymes in the body. We synthesized three types of prodrugs, including IHVR-35132, IHVR-35133, and IHVR-35140. IHVR-35140 demonstrated the equal potency and improved selectivity compared to the parent IHVR-35119. More interestingly, these prodrugs show improved solubility in most of the organic solvents which will facilitate the formulation preparation. We expect that the enzymes in the body will cleave the pendant mask group and release the parent IHVR-35119. Both parent and prodrug compounds exhibited CC50 on HCC-derived Huh7 cells of approximately 0.1 micromolar.

Progress on Aim 3, Milestone 2 during current reporting period:
In vitro metabolic analysis. IHVR-35119 was predicted to be less metabolically labile, while we hoped that the different prodrug approaches would increase solubility of the compounds in either aqueous or nonpolar solvents, thus improving formulation and possibly boosting oral bioavailability. Indeed, in the liver microsome assay, IHVR-35119 displayed somewhat increased metabolic stability compared to IHVR-04042. One of the prodrugs that was tested in the same assay, IHVR-35132, was able to be metabolized in a non NADPH-dependent manner, suggesting that the prodrug moiety is readily removed as predicted (results not shown).

Moreover, further analysis indicated that the IHVR-35132 was indeed converted to IHVR-35119, again as predicted (Table 1). Full identification analysis of the metabolite profile of both IHVR-35119 and the preferred prodrug, IHVR-35140, is currently being carried out.

Progress on Aim 5, Milestones 1 and 2

Mechanistic Studies I. Basis for cell type selectivity of aminothiazoles. Due to the presence of sequential aromatic rings, the structure of all the compounds in this series are predicted to fluoresce when excited by an appropriate wavelength. We ran excitation scans of HBF-0079 and IHVR-04042 in a fluorometer, and found a relatively broad peak of excitation centered around 330 nm, with emission at 460 nm. We exploited this property to determine whether the compound could be detected in cell culture conditions, and possibly visualized by fluorescence microscopy. Interestingly, we indeed found that Huh7 cells, one of the HCC-derived cell lines most sensitive to these compounds, was actually accumulating fluorescent signal over the background when incubated with IHVR-04042 which was chosen for study over HBF-0079 due to its higher emission. We also noted that one cell line that is comparatively insensitive to the compound, PH5CH (which is derived from normal non-cancerous hepatocytes), did not seem to fluoresce under treatment as intensely as the Huh7 cells (Figure 1), suggesting that the compound did not associate with these cells. The level of cell-associated fluorescence over background could be quantified by treating cells with IHVR-04042, washing with phosphate-buffered saline (PBS) to remove compound that is not associated with the cells, harvesting by trypsinization, and measuring level of emission in a fluorometer, with the background of each cell line measured in treatment with dimethyl sulfoxide (DMSO) only. Treatment of Huh7 cells with IHVR 04042 results in approximately 40% more emission over background. When equivalent numbers of PH5CH or THLE2 (another insensitive hepatocyte-derived cell line), are treated, emission is only approximately 12-16% over background (results not shown). We are currently working on full quantification of the amount of cell associated compound based on the level of fluorescence. These results strongly suggest that physical association of the compound with cells is a determinant of sensitivity. Although the general diffuse pattern of fluorescence in micrographs suggests that the compound is internalized in sensitive but not insensitive cell lines, we have not confirmed that that is the case. Other possibilities for the differential fluorescence that we observe include 1) internalization in both types of cells, but rapid efflux in only the insensitive cells, and 2) extracellular association with a cell-surface receptor. This last possibility as evidenced by our fluorometer assay is perhaps unlikely given that the emission signal in Huh7 cells can survive trypsin digestion which would likely hydrolyze cell surface proteins bound to the compound and cause its release. We are currently investigating the various possibilities.

Mechanistic Studies II. Phenotype of resistant cell line isolates. To determine the potential for resistance to the aminothiazoles, cultures of two highly sensitive cell lines, Huh7 and MCF7 (a breast- cancer derived line) were treated with 10 micromolar IHVR-04042 for extended periods (3-4 weeks), and dead or dying cells in the culture were washed away with media periodic media replacements. It was noted that although the majority of cells died, some cells did remain attached under treatment, and some even proliferated into colonies. Compared to the total number of cells seeded at the start of treatment, resistant colonies emerged at a rate of approximately 1.0 % in the Huh7 culture, and 0.01 % in the MCF7 culture, as quantified by

crystal violet staining and counting (results not shown). Several clones were isolated from each culture, and were amplified and studied. Interestingly, growth curves of some of the resistant clones and the parental cell lines, with or without treatment with IHVR-04042, indicated that the resistant clones are attenuated in growth compared to the parental lines (Figure 2). This observation strongly suggests that in a therapeutic setting, the emergence of tumor cell resistance to an aminothiazole could be mitigated by a reduction in growth fitness of those cells, with a more favorable clinical outcome. The basis for resistance in these clones is under investigation. We have already confirmed that in the MCF-7 cells, they are most likely resistant due to having acquired overexpression of the multiple drug resistance marker 1 (MDR1), which is responsible for small molecule drug efflux. In the Huh7 resistant clones, MDR-1 levels appear equivalent to the parental cells, which have similar levels to THLE2 and PH5CH cells (Figure 3). Thus it is unlikely that resistance in the non-hepatocellular carcinoma (HCC) cells, and in the Huh7 clones resistant to IHVR-04042, is due to increased drug efflux.

Incubation Time (min)	Remaining percentages of IHVR-35-132 (Prodrug) (%)		Released percentages of IHVR-35-119 (Parent Drug) (%)**	
	With NADPH	Without NADPH	With NADPH	Without NADPH
0	100.00	100.00	2.037	3.739
15	27.88	31.88	2.141	6.691
30	6.22	10.60	1.061	9.053
45	1.07	1.78	0.354	8.671
60	0.24	0.40	0.219	8.196

*MMLM means pooled male mouse liver microsomes.
**Released percentage of IHVR-35-119
= $PAR_{\text{IHVR-35-119 in each time point sample}}$ / $PAR_{\text{2 µM IHVR-35-119 standard in mouse liver microsomes}}$ *100%
PAR means peak area ratio of the analyze to the internal standard.

Table 1. Conversion of prodrug IHVR-35-132 to IHVR-35-119 in male mouse liver microsomes (MMLM). IHVR-35-132 was incubated in liver-derived microsome preparations, and levels of prodrug and parent compound (IHVR-35-119) were monitored by high performance liquid chromatography at the indicated timepoints. Incubation was carried out with or without nicotimamide adenine dinucleotide phosphate (NADPH), an enzyme cofactor involved in metabolic degradation of small molecules in the liver. The results indicate that the prodrug is converted to the parent drug over time, and that the parent drug can be degraded by both NADPH-requiring and non-requiring enzymatic mechanisms.

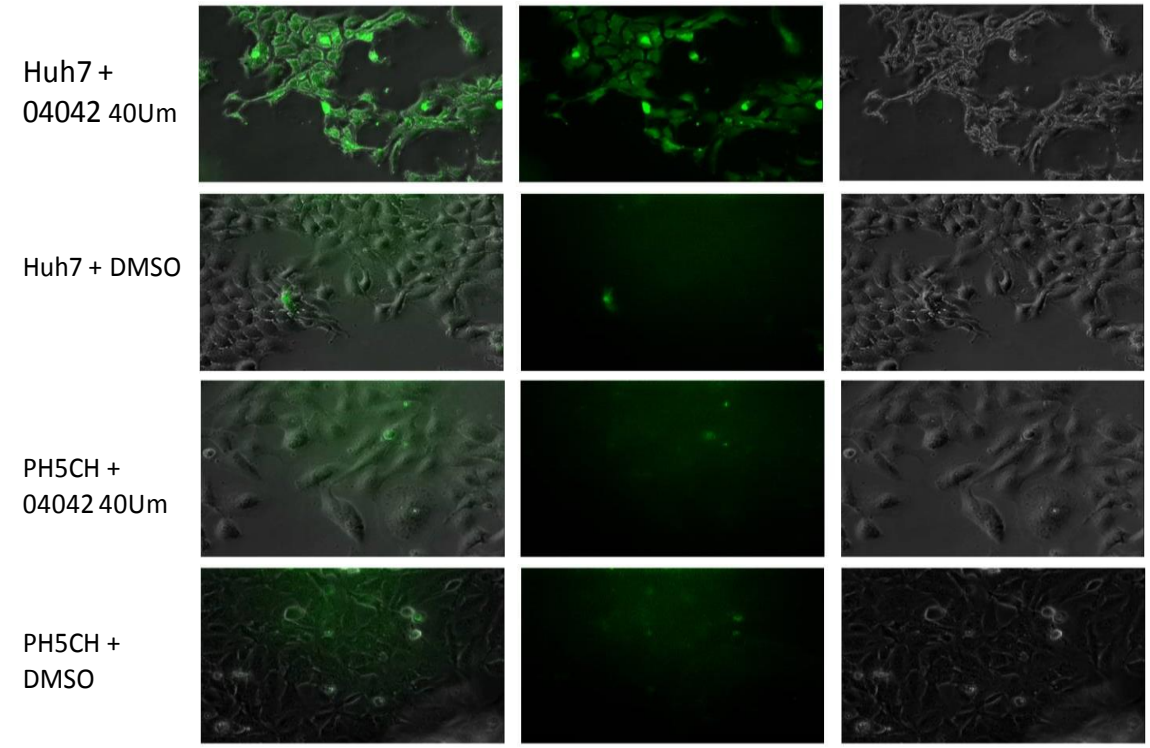

Figure 1. Cell-associated fluorescence of compound IHVR-04042 is specific for aminothiazole-sensitive cells, as imaged by fluorescence microscopy. Huh7 liver cancer cells and PH5CH normal liver cells were each treated with either DMSO or IHVR-04042, as indicated at left. Panels in left column depict merged phase contrast imaging and fluorescent (compound) signal, middle column depicts fluorescent signal, and right column depicts phase contrast imaging (whole cells). Results indicate that fluorescent signal emitted by the compound is only associated with the cancer cells, and not the normal cells. Therefore, the selectivity of the aminothiazole compound family may be based on selective entry or intracellular retention.

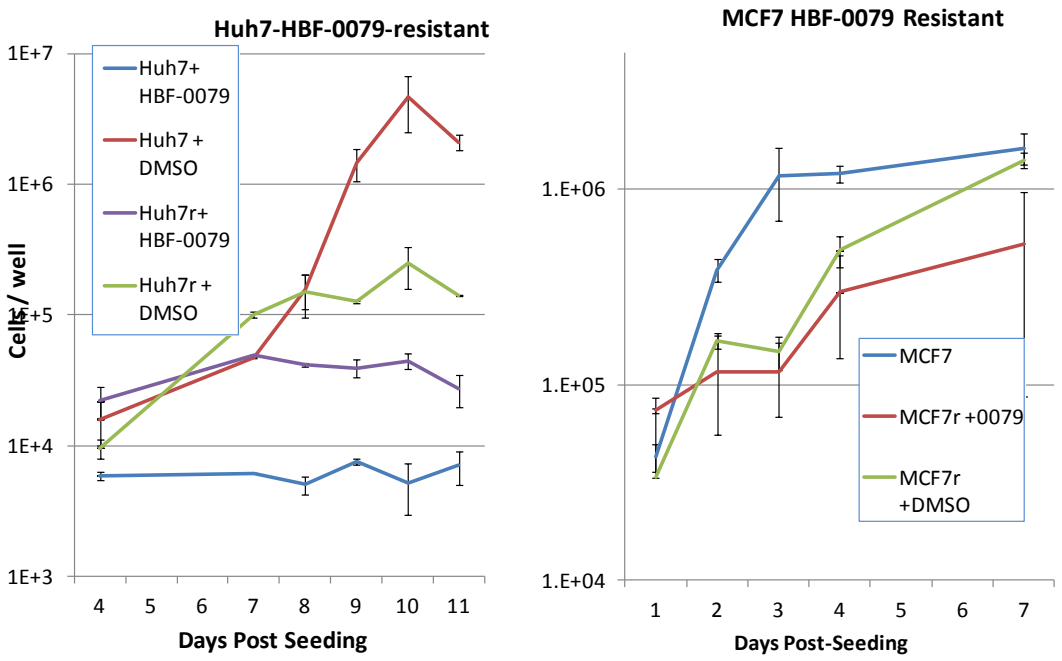

Figure 2. Aminothiazole-resistant clones of Huh7 liver cancer cells (Huh7r) and MCF7 breast cancer cells (MCF7r) exhibit reduced proliferation compared to the sensitive parental cell lines. The resistant clones were derived as described in the text, and along with the parent cell lines were cultured either in the presence or absence of the aminothiazole HBF-0079 at 10 uM concentration. Cell numbers per well were determined at time points indicated. The results indicate that the resistant clones grow more slowly than the parental lines, suggesting that resistant tumors arising from treatment with these compounds would also be impaired in growth.

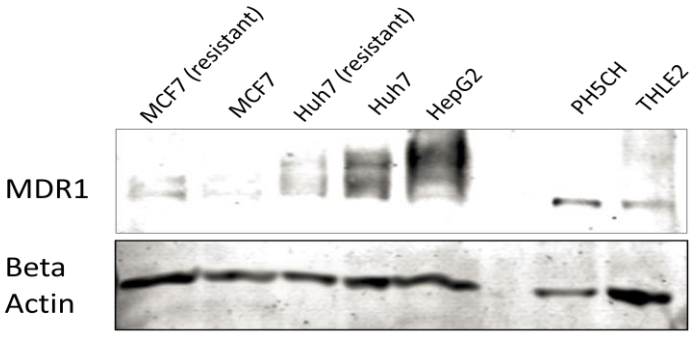

Figure 3. Expression of multiple drug resistance marker 1 (MDR-1) in aminothiazole-resistant vs parent Huh7 liver cancer and MCF7 breast cancer lines, in the HepG2 liver cancer line, or the PH5CH and THLE2 normal liver cell lines, does not corollate with sensitivity to the compounds. Depicted are Western blotting detection signals for MDR-1 (top panel) in each cell line, and Beta actin (bottom) panel as a control to indicate equivalent amounts of cellular protein was loaded in each lane.