Institute for Hepatitis and Virus Research

Annual Progress Report: 2011 Nonformula Grant

Reporting Period

July 1, 2012 – June 30, 2013

Nonformula Grant Overview

The Institute for Hepatitis and Virus Research received $909,170 in nonformula funds for the grant award period June 1, 2012 through May 31, 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 – 5/31/2014

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**

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Ju-Tao Guo, MD – employed by Drexel University College of Medicine  
Tong Xiao, MS – employed by Enantigen Therapeutics, Inc.  
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

Aim 1, Milestone 1: Complete synthesis and testing of up to 50 derivative compounds.

Progress:

Chemical optimization of lead compound

HBF-0079 is composed of three heterocycles, in which the central thiazole connects to the left pyridine at ortho-position via a carbon-carbon bond, and to the right pyrimidine at 2-position through an amine linker. In the first round of structure-activity relationship (SAR) study, we investigated the role of pyridine side chain, pyrimidine side chain, amine linker, and thiazole core via chemical modifications (Figure 1). The new compounds were evaluated on their potencies, selectivity for liver cancer cells, and solubility improvement in both DMSO and water. Several promising leads have been discovered, as described below. The reasons that drove the selectivity up and down were also analyzed through correlation of the potencies with the physiochemical properties of the molecules.

Pyridine side chain optimization. Pyridine with an ortho-substitution was proven necessary (Figure 3). When nitrogen was replaced with a carbon, or nitrogen was moved to the meta- or para-position, selectivity was decreased. Substitutions were also found to be selective. Polar hydroxyl substitution at 6-position (IHVR-04075) and hydrophobic 3,5-dimethyl (IHVR-04073) or 2,3-benzofused (IHVR-04085) substitutions all failed to improve selectivity. However, when there was a methyl group at 4-position (ETHCC-007), the selectivity was maintained or improved by seven-fold. It was interesting that when this pyridine ring was replaced with either methyl or ethyl ester, to mimic the chelating function of the nitrogen in the pyridine, selectivity decreased although solubility in DMSO was increased (Figure 3).

Thiazole central core optimization. When thiazole was replaced with an oxazole (IHVR-29031), selectivity was dropped more than 35-fold, suggesting the importance of this sulfur atom, which may be involved in interaction with the target (Figure 4). Therefore, the thiazole core was kept during the course of chemical variation. Substitution at 5-position was extensively studied, including the straight alkyl (IHVR-04046, 04049, 04067, 04069), branched or cyclic alkyl (IHVR-04065, 04086), bromo (ETHCC-008), hydroxymethyl (IHVR-04043), cyano (IHVR-04079), ester (IHVR-21015, 04042, 21029), reversed ester (IHVR-21023, 21027), and amide (IHVR-21014, 21018, 21020). Among these variations, methyl ester (IHVR-21015) retained the selectivity of HBF-0079, while ethyl ester (IHVR-04042) improved it 4-5 fold. However, when the more bulky tert-butyl ester (IHVR-21029) was introduced, the potency and selectivity were decreased; straight alkyls did not bring in higher potencies, but a branched isopropyl group (IHVR-04065) maintained potency and selectivity with a moderate increase, although a more bulky cyclohexyl (IHVR-04086) group caused a decrease; Methyl amide (IHVR-21018) did not achieve the same favorable effect as the methyl ester (IHVR-21015) (Figure 4), indicating the hydrogen bond donor may be not preferred at this position. Nevertheless, the potency and selectivity data were hard to correlate simply with cLogP, a parameter to represent hydrophobicity. Because an immediate change introduced by 5-substitution alters the dihedral angle between the pyridine ring and the thiazole ring, and also due to the distance between the pyridine and the thiazole nitrogens, these changes would influence the conformation of the molecules and thus target biding. More detailed analysis is discussed in the structural analysis section below.
Amino linker optimization. The amino group was alkylated with methyl (IHVR-04031), branched isopropyl (IHVR-04034), hydroxylethyl (IHVR-04035), and a benzyl group (IHVR-04038) (Figure 5). However, all these changes led to the losses of potency and selectivity, demonstrating that the presence of the hydrogen bond donor here is favored.

Pyrimidine side chain optimization. Similar to the pyridine ring, the pyrimidine ring also proved irreplaceable and sensitive to the substitutions. Misplacement of the nitrogen in the pyrimidine reduced potency and selectivity (ETHCC-010, 011); both electron withdrawing (IHVR-04098, ETHCC-012) and electron donating groups (IHVR-04054, 04082) that were examined were not able to improve potency and selectivity (Figure 6).

Multiple structural changes. Combinations of multiple substitutions were also explored to pursue synergetic effects. When the ethyl ester in the thiazole core was coupled with the methyl group at 4-position of the pyrimidine (IHVR-21018), an improvement that was better than from either one of them was observed. Interestingly, additional methyl group at 4-position of the pyridine (IHVR-31021) or 5,6-dimethyl groups in the pyrimidine (IHVR-31030) reduced potency and selectivity (Figure 7).

Structural analysis of lead alterations. As we have discussed, the introduction of a substitution group at 5-position of the thiazole core will influence the dihedral angle between the pyridine and thiazole rings, and the distance between the pyridine and thiazole nitrogens. These two numbers are important in some areas, such as coordination and catalyst chelation chemistries. Therefore, we examined the changes of these parameters in some molecules using Chemdraw 3D modeling tool to calculate the minimized energy conformation, then measuring the corresponding dihedral angles and the distances between the two nitrogens (Table 1), in comparison to the cLogP. From the preliminary comparison, introduction of the ethyl ester (IHVR-04042, 04084) reduced the dihedral angle and shortened the distance between the two nitrogens, while the isopropyl group (IHVR-04065) reduced the dihedral angle to a less extent, but shortened the distance between the two nitrogens to an even higher degree. The amide substitution (IHVR-21020) had very similar dihedral angle and distance changes, however, the presence of the hydrogen bond donor affected activity.

Aim 2, Milestone 1: Complete in vitro selectivity profiling of up to 10 active compounds
Progress: Five of the most promising compounds (HBF-0079, ETHCC-007, and IHVR-04042, -21015, -21004) have been tested for activity against a spectrum of HCC-derived cell lines (SNU-449, SNU-475, SNU-387, and SNU-423, and Huh7), as compared to the non-HCC derived liver lines THLE-2 and PH5CH. All have shown HCC-specific cytotoxicity and SI similar to those reported in Table 1, and are currently being evaluated in additional HCC and non-HCC lines; results will be presented in the final report.

Aim 3, Milestone 2: Complete ADMET profiling of up to 5 compounds in vivo
Progress:
In vitro and in vivo ADME analysis. The seven compounds that were identified by SAR studies as having the highest activity and selectivity for HCC cells were examined for aqueous solubility at two different pH values (Table 2). This parameter is useful for two reasons: 1) Solubility level at neutral pH permits assessment of whether data from tissue culture and biochemical experiments is valid (i.e. the measured CC50 value of a compound cannot be higher than its measured upper solubility limit), and 2) solubility level at low pH can be predictive whether a
compound is likely to be in solution in the gastrointestinal tract, and therefore more likely to be absorbed. The compounds were dissolved in 3% DMSO in aqueous buffer (PBS) at either pH 4.0 or 7.4, to a final of 300 uM. After filtration, concentration was determined by LCMS. All the compounds were significantly more soluble at pH 4.0 than pH 7.4, suggesting that absorption after oral administration should be efficient. Not surprisingly, solubility at pH 7.4 tended to be highest for compounds containing polar groups (esters, methyl esters; IHVR-04042, -04084, -21004, and -21015). All the measured solubility values were higher than the measured CC50 values, giving us confidence in our ranking by potency in the tissue culture assay. Somewhat disappointing is the observation that overall solubility for the parent compound (HBF-0079) was actually better than for any of our derivatives; this suggests that aqueous solubility will be need to be further optimized.

The same set of compounds was tested for solubility in preparations of microsomes from CD-1 mouse and human livers. This assay is highly predictive of the rate at which the liver will metabolize a compound in human patients or a rodent model, a major factor in its overall pharmacodynamic profile. The need to test stability in mouse liver microsomes (LM) is necessitated by our future plans to use a mouse cancer xenograft model for efficacy testing. Compounds were dissolved in DMSO and mixed with LM at final concentration of 2 uM, and 1.0% DMSO, with or without 1 mM NADPH. Mixtures were incubated for 1 hr at 37ºC, with sampling at 0, 15, 30, 45 and 60 minutes, and remaining concentration of compound was determined by acetonitrile extraction and LCMS analysis. With the exception of ETHCC-007, most of the compounds were moderately stable in human LM, with a percentage remaining after incubation ranging from ~25-43%, which is similar to the stable positive control compound (~25%). However, stability was much lower in the mouse LM, ranging from ~2-7% remaining, which is also similar to the control (~8%); this characteristic will inform the practicality of doing efficacy studies in a mouse xenograft model. Interestingly, the limited degradation undergone by four of the compounds (IHVR-04042, -04084, -21004, and -21015), was independent of NAPDH presence, indicating that the ester-containing compounds are acted upon by different metabolic pathway than the non-esters; given that all seven compounds have selective anti-HCC activity, there is the possibility that in vivo stability may be optimized independently of potency, making it more likely that we will eventually arrive at a potent and stable compound.

For initial in vivo characterization of pharmacokinetic profile and oral bioavailability, we picked IHVR-04042 and IHVR-04084; this was based on their superior potency in the tissue culture assay, acceptable stability in the LM assay, and acceptable solubility profile. These compounds were subjected to development of DMSO-free formulation, and the best excipient combination was found to include Solutol HS-15/NMP/normal saline (ratios varying with specific compound). Not surprisingly, we found that the ester compound with the methylated pyrimidine (IHVR-04084) was significantly harder to dissolve in this standard excipient combination, forcing a lower level of dosing than IHVR-04042 (1.0 and 2.0 mg/kg IV and PO respectively for IHVR-04042 versus 2.0 and 10.0 mg/kg IV and PO, respectively, for IHVR-04084). The administration of the compounds was well tolerated, plasma samples were collected pre-dose, 15 and 30 minutes, and 1, 2, 4, 8 hrs, and results of bioanalysis are still pending. More compounds to be analyzed will be determined after further SAR.
Kinase profiling and cell culture resistance studies.
We have run a screen for inhibition of a ~300-kinase panel with HBF-0079, and identified members of the receptor tyrosine kinase family as being weakly inhibited (IC50=10 micromolar). These represent candidate targets.
A screen for identification of HBF-0079-resistant clones of Huh7 was also run by long term incubation with compound and selection of survivors, but the isolates were very slow-growing and could not be propagated. This indicates that resistance to these compounds may be difficult to elicit.

Aim 5, Milestone 1: Preliminary ID of compound binding targets
There was no progress during the reporting period.
Figure 1. Optimization of lead compound.

Figure 2. New lead derivatives. Selectivity is shown as ratio of potency in Huh7 versus either THLE2 or PH5CH cells (higher number=more selective for liver cancer cell line)

Figure 3. Pyridine side chain optimization

Figure 4. Thiazole central core optimization
Compound | Conformation | Dihedral angle | Distance between two Ns | clogP | THLE2:Huh7 | PH5CH:Huh7
--- | --- | --- | --- | --- | --- | ---
HBF-0079 | | 179.4 | 2.643 | 1.851 | 35.450 | 40.294
IHVR-04042 | | 172.5 | 2.581 | 2.35 | 148.13 | 157.635
IHVR-04084 | | 172.3 | 2.578 | 2.85 | 221.76 | 218.829
IHVR-04065 | | 177.8 | 2.533 | 3.48 | 52.876 | 80.596
IHVR-21020 | | 171.7 | 2.572 | 0.98 | 1.205 | 1.541

Table 1. Structural analysis of lead alterations. See text for details.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Solubility (μM)</th>
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<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>pH 4.0</td>
<td>pH 7.4</td>
<td></td>
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<tr>
<td>Diclofenac</td>
<td>7.49</td>
<td>296.25</td>
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<tr>
<td>ETHCC-007</td>
<td>147.57</td>
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<tr>
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<tr>
<td>IHVR-21004</td>
<td>162.27</td>
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<td>IHVR-21015</td>
<td>22.95</td>
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</table>

Table 2 (A and B). The solubility data of 7 compounds and control compound diclofenac in PBS pH 4.0 and PBS pH 7.4. HBF-0079 was tested in a different experimental trial from the remainder.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solubility (μM)</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>pH 4.0</td>
<td>pH 7.4</td>
<td></td>
</tr>
<tr>
<td>Diclofenac</td>
<td>4.57</td>
<td>309.83</td>
<td></td>
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<tr>
<td>HBF-0079</td>
<td>313.98</td>
<td>29.81</td>
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Table 2B

<table>
<thead>
<tr>
<th>Compound</th>
<th>Remaining Percentage @ 60 min (%)</th>
<th>CL\textsubscript{int} (μL/min/mg proteins)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Human</td>
<td>Mouse</td>
</tr>
<tr>
<td>Verapamil</td>
<td>25.05</td>
<td>8.46</td>
</tr>
<tr>
<td>HBF-0079</td>
<td>25.44</td>
<td>2.14</td>
</tr>
<tr>
<td>ETHCC-007</td>
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<td>2.46</td>
</tr>
<tr>
<td>IHVR-04042</td>
<td>36.33</td>
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</table>

*IHVR-04042, IHVR-04084, IHVR-21004 and IHVR-21015 were also metabolized by non-NADPH-dependent enzymes.

Table 3. Stability of 7 compounds and control compound verapamil in human and mouse liver microsome preparations.