Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-231-2825.

1. **Grantee Institution:** Baruch S. Blumberg Institute

2. **Reporting Period (start and end date of grant award period):** June 1, 2012-August 29, 2014

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Patti McAloon

4. **Grant Contact Person's Telephone Number:** 215-489-4911

5. **Grant SAP Number:** 410059194

6. **Project Number and Title of Research Project:** 01 -A New Inhibitor of the Akt/m TOR Pahtway with Remarkable Potency and Selective Anti-Hepatocellular Carcinoma Activity

5. **Start and End Date of Research Project:** June 1, 2012-August 29, 2014

7. **Name of Principal Investigator for the Research Project:** Andrea Cuconati, Ph.D.

8. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $ 910,736.00

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project	Cost
Du	Assistant Professor	38%	68,497
Lu	Research Associate	17%	32,599
Cuconati	Associate Professor	52%	55,316
Goddard	Research Associate	95%	84,423
Rogowskyj	Research Associate	12%	14,454
Liang	Research Associate	82%	108,424
Wang	Research Associate	19%	22,362
Xu	Director of Chemistry	43%	36,073
Ye	Research Associate	82%	39,968
Khan	Research Associate	74%	36,192

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project
None		

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
None		

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_____ No___X_____

If yes, please indicate the source and amount of other funds:

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes_____ No_____X___

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds awarded:
None	☐NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)		$	$

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes_____X____ No_____

If yes, please describe your plans:

We plan to apply for NIH grants

12. Future of Research Project. What are the future plans for this research project?

The project has been licensed by a biomedical start-up, which will pursue proof of concept in in vivo models. We retain the freedom to do research on the basic mechanisms of action of our compounds, and will be applying for grants from the NIH.

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes____X_____ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male	2			
Female				
Unknown				
Total	**2**			

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic	2			
Unknown				
Total	**2**			

	Undergraduate	Masters	Pre-doc	Post-doc
White	2			
Black				
Asian				
Other				
Unknown				
Total	**2**			

14. **Recruitment of Out-of–State Researchers**. Did you bring researchers into Pennsylvania to carry out this research project?

    Yes_____ No_____X_____

    If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. **Impact on Research Capacity and Quality**. Did the health research project enhance the quality and/or capacity of research at your institution?

    Yes____X_____ No_____

    If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

    This project has provided educational opportunities to two visiting undergraduates, and also gave our research staff the funding and purpose to learn new techniques and methods.

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes___X_____ No_____

If yes, please describe the collaborations:

The project has been licensed to a small company for further development.

16(B) Did the research project result in commercial development of any research products?

Yes_____ No_____X_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes____X_____ No_____

If yes, please describe involvement with community groups that resulted from the research project:

Undergraduate students visting from a Delaware Valley College were able to participate.

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending

publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

Reporting Period - July 1, 2011 – August 29, 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

Progress on Aim 1, Milestone 1:
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. Without information on target interaction, 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. A total of 77 new compounds were designed, synthesized, and evaluated for potency, selectivity, and solubility (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 (Figure 2), 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 nitrogens 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 up to IHVR-04042. 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.

Progress on Aim 1, Milestone 1 and Aim 2, Milestone 1:
Optimization guided by metabolic stability. Metabolite ID study on IHVR-04042 revealed thatthere 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 (CC_{50}=0.106 ± 0.01 micromolar on Huh7 cells) and improved selectivity (Scheme 1).

Solubility directed optimization: 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 hydrochloride with basic nitrogens within the molecule. However, this approach only increased aqueous solubility slightly. The second way was to make prodrugs, which will release the parent compounds upon interaction with the enzymes in the body. We synthesized three types of prodrugs, each represented by the compounds IHVR-35132, IHVR-35133, and IHVR-35140. IHVR-35140 demonstrated the equal potency and improved selectivity compared to the parent IHVR-35119. More interestingly, this prodrug shows good solubility in most of the organic solvents which will facilitate the formulation preparation. We predicted that the esterases in the body will cleave the pendant mask group and release the parent IHVR-35119 (Scheme 1). Both parent and prodrug compounds exhibited CC_{50} on HCC-derived Huh7 cells of approximately 0.1 micromolar.

Progress on Aim 2, Milestone 1
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;

Progress on Aim 3, Milestone 2:

In vitro ADME and metabolic 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 (Table 3). 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 37^0C, 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.

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 4). Full identification analysis of the metabolite profile of both IHVR-35119 and the preferred prodrug, IHVR-35140, was later carried out (not shown).

For initial in vivo characterization of pharmacokinetic profile and oral bioavailability in CD-1 mice, 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-04084 versus 2.0 and 10.0 mg/kg IV and PO, respectively, for IHVR-04042). The administration of the compounds was well tolerated, and plasma samples were collected pre-dose, 15 and 30 minutes, and 1, 2, 4, 8 hrs. It was noted that IHVR-04042 had a superior pharmacokinetic profile than IHVR-04084.

After intravenous dosing (IV) in fasting male CD-1 mice at 2.0 mg/kg, IHVR-04042 reached a maximum concentration (C0) of 1423 ±459 ng/mL, with an average half-life of 0.24 ± 0.09 hours. The clearance rate was 4.46 ± 0.30 L/hr/kg. After oral dosing (PO) at 10 mg/kg, maximum concentration (Cmax) of 94.1 ± 51.9 ng/mL was reached between 15 minutes and 1 hour post dose with an average half-life of 1.91 ± 0.26 hours. The average oral bioavailability of IHVR-04042 was 10 ± 5.92%. After IV dosing at 1.0 mg/kg, IHVR-04084 reached a maximum concentration (C0) of 397 ± 206 ng/mL, with an average half-life of 0.22 ± 0.07 hours. The clearance rate was 6.30 ± 1.44 L/hr/kg. After PO dosing at 2 mg/kg, test compound was detectable only in one of the three mice at 15 min (5.26 ng/mL) and 30 min (4.29 ng/mL) post dose. All of the PK parameters were not determined for IHVR-04084 due to a lack of sufficient number of quantifiable data points. Although the results for IHVR-04048 were disappointing, the PK characteristics of IHVR-04042 were better than what we had observed previously for HBF-0079, and represented a good starting point for further development.

As stated in the previous section, the metabolic breakdown of IHVR-04042 in the in vitro LM assay was further examined by identification of the major metabolites by LCMS analysis. These findings guided further optimization of IHVR-04042, yielding IHVR-35119 and its prodrug, IHVR-35140. These were subjected to PK analysis in CD1 mice (Table 5). Both compounds were formulated as per IHVR-04042, and administered by IV and oral PO routes, respectively. It was noted that, as expected, the solubility of IHVR-35140 in the vehicle was at least 10-fold higher than IHVR-35119, and we were able to produce dosing solutions of 1.0 mg/ml and 0.1 mg/ml, respectively. While IHVR-35119 was at its solubility limit at 0.1 mg/ml, IHVR-35140 likely has a higher limit than 1.0/mg/ml, which we did not investigate. Blood samples were collected at 5 mins, 15 mins, 30 mins, and 1, 2 and 4 hours post dose, and livers were also collected at the final time point. Blood and liver concentrations were determined by LC-MS/MS. Following intravenous administration at 1.0 mg/kg, IHVR-35119 reached a maximum concentration (C0) of 634 ± 462 ng/mL at 5 minutes post dosing in all mice, with an average half-life of 0.436± 0.414 hours. The average clearance rate was 9.25± 7.26 L/hr/kg and the average volume of distribution was 3.49 ± 2.62 L/kg. Following oral dosing at 2.5 mg/kg, IHVR-

35119 reached a maximum blood concentration (Cmax) of 48.7 ± 41.7 ng/mL within 1 hour post dosing. The average bioavailability was 15.2 ± 10.5%. When IHVR-35140 was dosed intravenously at 2.0 mg/kg, the majority of the detectable compound at all time points was the parent compound, IHVR-35119, indicating rapid conversion of the pro-drug into the drug. Maximum blood concentrations of IHVR-35119 after the IHVR-35140 IV dose were 470 ±218 ng/mL, and were observed at 5 minutes post dosing in all mice. Following oral dosing of IHVR-35140 at 10 mg/kg, again the only detectable compound was the parent, IHVR-35119. In this dose, IHVR-35119 reached maximum blood concentrations 199 ± 173 ng/mL, as observed by 25 minutes post dosing, with an average half-life of 0.806 hrs. After oral dosing of IHVR-35140, the bioavailability of IHVR-35119 was calculated to be 7.13 ± 5.78%, which is lower than observed after the oral dosing of IHVR-35-119 alone.

Liver concentrations of IHVR-35119 and IHVR-35140 four hours post dosing were also determined. After IV dosing of IHVR-35-119 only one mouse had quantifiable levels (5.95 ng/g). Following oral dosing of IHVR-35-119 average (n=3) liver concentrations were 33.0 ± 18.5 ng/g (or 0.096 micromolar). IHVR-35-140 was undetectable in liver samples after IV or oral dosing, however, IHVR-35-119 was detectable in one mouse (9.75 ng/g) and in all three orally dosed mice (60.3 ± 20.7 ng/g) (or 0.176 micromolar). (Table 6).

Overall, the PK profile of IHVR-35119 when given orally in its prodrug form was superior to IHVR-35-119 directly given, reaching 4 fold higher blood levels, and 2-fold higher accumulation in the liver, which would be the target organ of these compounds. Interestingly, oral dosing was essential for maximal accumulation of the compound in the liver at the selected time point, likely due to hepatic first pass effect. These advantages over the parent compound are largely due to the increase in solubility of the prodrug, permitting dosing at a higher concentration than the parent compound. This was the express purpose of the prodrug design. It is likely that IHVR-35140 can be dosed at even higher levels, therefore increasing the exposure to the parent compound.

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 8), 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 9). 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 10). 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.

Mechanistic Studies III: Kinase inhibition profiling studies. Based on the fact the aminothiazoles exhibit general structural similarity with kinase inhibitors, we worked with Reaction Biology (Malvern, PA) to run a screen for inhibition of a ~300-kinase panel with HBF-0079, and identified members of the Insulin receptor tyrosine kinase family as being weakly inhibited by 10 micromolar. Chief among these are Insulin receptor, Insulin receptor-related receptor, and Insulin-like growth factor 1 receptor (Figure 11). Interestingly, there was no non-specific inhibition of kinases across the screen, indicating highly selective activity. When we repeated the kinase assays with just the main targets at multiple concentrations, we found HBF-0079 and two other compounds showed IC50 values form 7-~70 micromolar (Table 7), suggesting that these

compounds are indeed moderate inhibitors of this family of kinases. Although the activity is not strong, we surmise that the compounds may be accumulating inside sensitive cells by a selective entry mechanism (see above, and Figure 8), and thus may reach concentrations sufficient to inhibit these signaling receptors. The results are currently being investigated further for confirmation, using a different funding source.

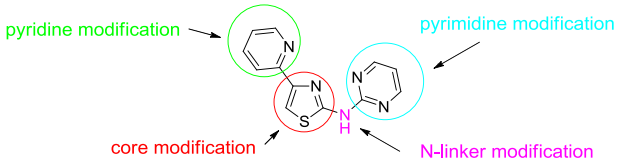
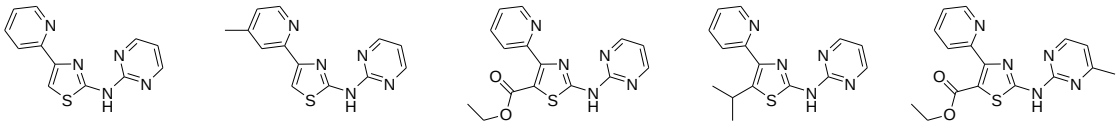


Figure 1. Optimization of lead compound.



HBF-0079
THLE2:Huh7, 35.450
PH5CH:Huh7, 40.294

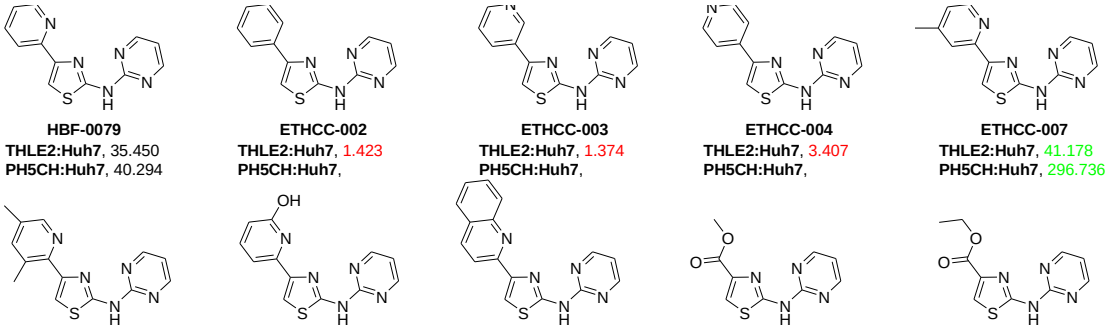
ETHCC-007
THLE2:Huh7, 41.178
PH5CH:Huh7, 296.736

IHVR-04042
THLE2:Huh7, 148.13
PH5CH:Huh7, 157.635

IHVR-04065
THLE2:Huh7, 52.876
PH5CH:Huh7, 80.596

IHVR-04084
THLE2:Huh7, 221.760
PH5CH:Huh7, 218.829

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)



HBF-0079
THLE2:Huh7, 35.450
PH5CH:Huh7, 40.294

ETHCC-002
THLE2:Huh7, 1.423
PH5CH:Huh7,

ETHCC-003
THLE2:Huh7, 1.374
PH5CH:Huh7,

ETHCC-004
THLE2:Huh7, 3.407
PH5CH:Huh7,

ETHCC-007
THLE2:Huh7, 41.178
PH5CH:Huh7, 296.736

IHVR-04073
THLE2:Huh7, 0.798
PH5CH:Huh7, 1.035

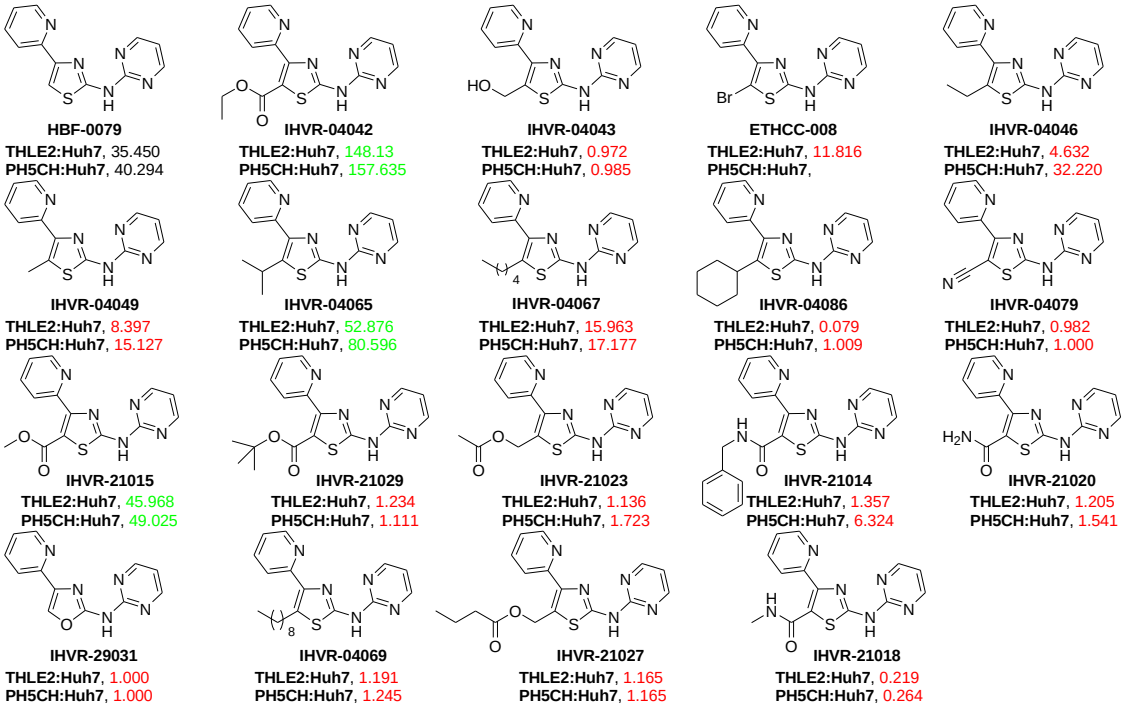
IHVR-04075
THLE2:Huh7, 1.052
PH5CH:Huh7, 1.052

IHVR-04085
THLE2:Huh7, 1.542
PH5CH:Huh7, 10.815

IHVR-22089
THLE2:Huh7, 0.982
PH5CH:Huh7, 1.459

IHVR-22088
THLE2:Huh7, 1.067
PH5CH:Huh7, 1.187

Figure 3. Pyridine side chain optimization



HBF-0079
THLE2:Huh7, 35.450
PH5CH:Huh7, 40.294

IHVR-04042
THLE2:Huh7, 148.13
PH5CH:Huh7, 157.635

IHVR-04043
THLE2:Huh7, 0.972
PH5CH:Huh7, 0.985

ETHCC-008
THLE2:Huh7, 11.816
PH5CH:Huh7,

IHVR-04046
THLE2:Huh7, 4.632
PH5CH:Huh7, 32.220

IHVR-04049
THLE2:Huh7, 8.397
PH5CH:Huh7, 15.127

IHVR-04065
THLE2:Huh7, 52.876
PH5CH:Huh7, 80.596

IHVR-04067
THLE2:Huh7, 15.963
PH5CH:Huh7, 17.177

IHVR-04086
THLE2:Huh7, 0.079
PH5CH:Huh7, 1.009

IHVR-04079
THLE2:Huh7, 0.982
PH5CH:Huh7, 1.000

IHVR-21015
THLE2:Huh7, 45.968
PH5CH:Huh7, 49.025

IHVR-21029
THLE2:Huh7, 1.234
PH5CH:Huh7, 1.111

IHVR-21023
THLE2:Huh7, 1.136
PH5CH:Huh7, 1.723

IHVR-21014
THLE2:Huh7, 1.357
PH5CH:Huh7, 6.324

IHVR-21020
THLE2:Huh7, 1.205
PH5CH:Huh7, 1.541

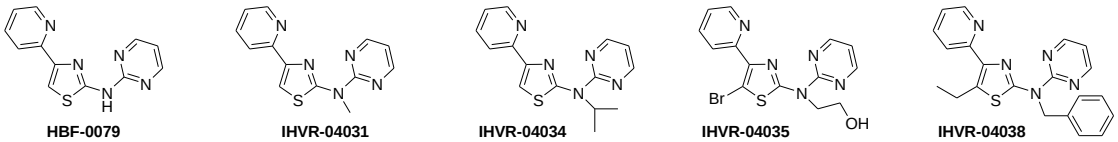
IHVR-29031
THLE2:Huh7, 1.000
PH5CH:Huh7, 1.000

IHVR-04069
THLE2:Huh7, 1.191
PH5CH:Huh7, 1.245

IHVR-21027
THLE2:Huh7, 1.165
PH5CH:Huh7, 1.165

IHVR-21018
THLE2:Huh7, 0.219
PH5CH:Huh7, 0.264

Figure 4. Thiazole central core optimization



HBF-0079
THLE2:Huh7, 35.450
PH5CH:Huh7, 40.294

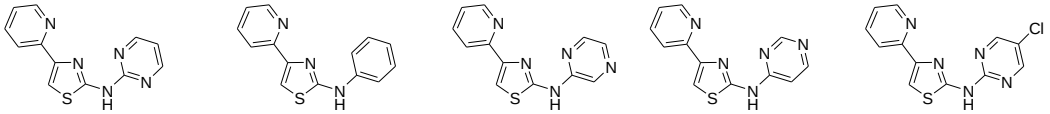
IHVR-04031
THLE2:Huh7, 1.191
PH5CH:Huh7, 1.203

IHVR-04034
THLE2:Huh7, 1.887
PH5CH:Huh7, 1.806

IHVR-04035
THLE2:Huh7, 1.076
PH5CH:Huh7, 1.092

IHVR-04038
THLE2:Huh7, 0.977
PH5CH:Huh7, 1.029

Figure 5. Amino linker optimization



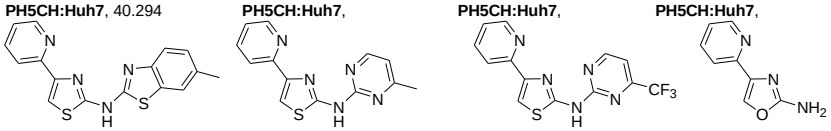
HBF-0079
THLE2:Huh7, 35.450
PH5CH:Huh7, 40.294

ETHCC-005
THLE2:Huh7, 2.725
PH5CH:Huh7,

ETHCC-010
THLE2:Huh7, 0.108
PH5CH:Huh7,

ETHCC-011
THLE2:Huh7, 1.464
PH5CH:Huh7,

ETHCC-012
THLE2:Huh7, 1.428
PH5CH:Huh7,



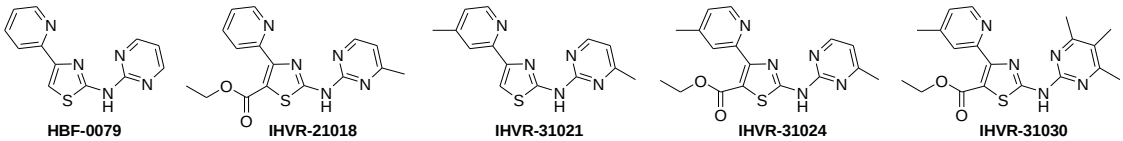
IHVR-04054
THLE2:Huh7, 3.760
PH5CH:Huh7, 0.733

IHVR-04082
THLE2:Huh7, 2.348
PH5CH:Huh7, 3.283

IHVR-04098
THLE2:Huh7, 1.782
PH5CH:Huh7, 10.187

IHVR-29030
THLE2:Huh7, 1.112
PH5CH:Huh7, 1.112

Figure 6. Pyrimidine side chain optimization



HBF-0079
THLE2:Huh7, 35.450
PH5CH:Huh7, 40.294

IHVR-21018
THLE2:Huh7, 221.76
PH5CH:Huh7, 218.829

IHVR-31021
THLE2:Huh7, 3.048
PH5CH:Huh7, 3.716

IHVR-31024
THLE2:Huh7, 0.867
PH5CH:Huh7, 1.205

IHVR-31030
THLE2:Huh7, 0.982
PH5CH:Huh7, 1.047

Figure 7. Multiple structural changes

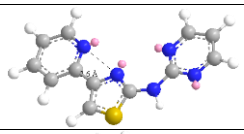
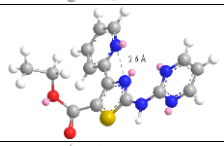
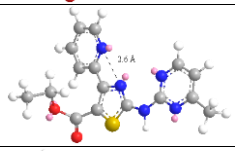
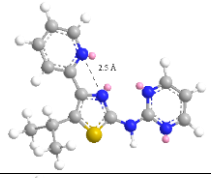
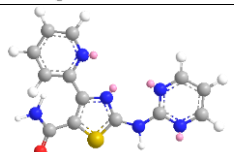
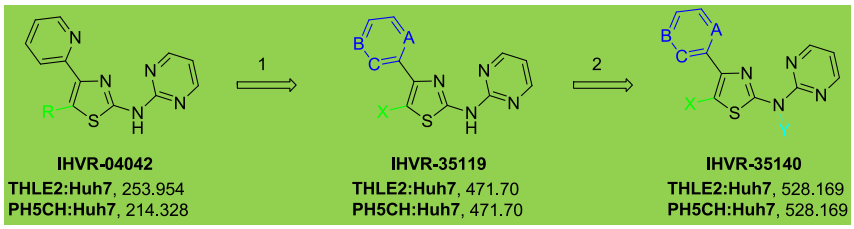
Compound	Conformation	Dihedral angle	Distance between two Ns	clogP	THLE2:Huh7	PH5CH:Huh7
HBF-0079		179.4	2.643	1.851	35.450	40.294
IHVR-04042		172.5	2.581	2.35	148.13	157.635
IHVR-04084		172.3	2.578	2.85	221.76	218.829
IHVR-04065		177.8	2.533	3.48	52.876	80.596
IHVR-21020		171.7	2.572	0.98	1.205	1.541

Table 1. Structural analysis of lead alterations. See text for details.



Scheme 1. Further optimization of IHVR-04042, with selectivity windows indicated.

Compound	Solubility (μM)	
	pH 4.0	pH 7.4
Diclofenac	7.49	296.25
ETHCC-007	147.57	1.79
IHVR-04042	112.84	23.84
IHVR-04065	10.51	0.95
IHVR-04084	28.55	16.64
IHVR-21004	162.27	18.85
IHVR-21015	22.95	12.67

Compound	Solubility (μM)	
	pH 4.0	pH 7.4
Diclofenac	4.57	309.83
HBF-0079	313.98	29.81

Table 2. 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.

Compound	Remaining Percentage @ 60 min (%)		CL_{int} (μL/min/mg proteins)	
	Human	Mouse	Human	Mouse
Verapamil	25.05	8.46	47.06	80.29
HBF-0079	25.44	2.14	44.74	230.30
ETHCC-007	10.45	2.46	74.76	223.88
IHVR-04042	36.33	4.55	33.72	168.49
IHVR-04065	43.05	2.09	27.68	217.18
IHVR-04084	34.52	4.25	34.75	157.43
IHVR-21004	25.91	6.81	45.08	111.88
IHVR-21015	37.19	3.65	31.92	370.92

•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.

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 4. 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.

Individual and Average Blood Concentrations (ng/mL) and Pharmacokinetic Parameters for IHVR-35-119 After Oral Administration in Male CD-1 Mice at 2.5 mg/kg

Oral (2.5 mg/kg; 20% (NMP:Solutol HS15, 1:1); 80% normal saline)					
Time (hr)	Mouse #				
	476	477	478	Mean	SD
Pre-Dose (0)	BLOQ	BLOQ	BLOQ	ND	ND
0.083	14.4	25.4	37.7	25.8	11.7
0.25	23.9	19.7	39.2	27.6	10.3
0.50	11.3	7.59	18.6	12.5	5.60
1.0	5.10	4.90	96.9	35.6	53.1
2.0	1.34	1.86	1.11	1.44	0.384
4.0	BLOQ	BLOQ	1.29	ND	ND
Animal Weight (kg)	27	27	26	27	0.6
Volume Dosed (mL)	0.68	0.68	0.65	0.67	0.02
C_{max} (ng/mL)	23.9	25.4	96.9	48.7	41.7
t_{max} (hr)	0.25	0.083	1.0	0.444	0.488
$t_{1/2}$ (hr)	ND[3]	ND[3]	ND[3]	ND	ND
MRT_{last} (hr)	1.75	2.65	0.908	1.77	0.869
AUC_{last} (hr·ng/mL)	24.9	40.8	95.9	53.9	37.3
AUC_{∞} (hr·ng/mL)	ND[3]	ND[3]	ND[3]	ND	ND
Dose Normalized Values[1]					
AUC_{last} (hr·kg·ng /mL/mg)	10.0	16.3	38.4	21.6	14.9
AUC_{∞} (hr·kg·ng /mL/mg)	ND[3]	ND[3]	ND[3]	ND	ND
Bioavailability (%)[2]	7.03	11.5	27.1	15.2	10.5

C_{max}: maximum blood concentration; t_{max}: time of maximum blood concentration; $t_{1/2}$: half-life, data points used for half-life determination are in bold; MRT_{last}: mean residence time, calculated to the last observable time point; AUC_{last}: area under the curve, calculated to the last observable time point; AUC_{∞}: area under the curve, extrapolated to infinity; ND: not determined; BLOQ: below the limit of quantitation (1 ng/mL); [1]Dose normalized by dividing the parameter by the nominal dose of 10 mg/kg; [2]Bioavailability determined by dividing the individual dose normalized oral AUC_{last} values by the average dose normalized IV AUC_{last} value; [3]not determined because the line defining the terminal elimination phase had an r^2 of less than 0.85 or the terminal elimination phase was not observed.

Individual and Average Blood Concentrations (ng/mL) and Pharmacokinetic Parameters for IHVR-35-119 After Oral Administration of IHVR-35-140 in Male CD-1 Mice at 10 mg/kg

Oral (10 mg/kg; 20% (NMP:Solutol HS15, 1:1); 80% normal saline) IHVR-35-119 after IHVR-35-140 Dosing					
Time (hr)	Mouse #				
	482	483	484	Mean	SD
Pre-Dose (0)	BLOQ	BLOQ	BLOQ	ND	ND
0.083	14.5	189	377	193.5	181
0.25	30.5	**88.6**	186	101.7	78.6
0.50	29.5	**54.1**	112	65.2	42.4
1.0	3.49	**3.12**	**9.33**	5.31	3.48
2.0	BLOQ	**1.58**	**3.47**	2.53	ND
4.0	BLOQ	BLOQ	**1.76**	ND	ND
Animal Weight (kg)	26	29	30	28	2.1
Volume Dosed (mL)	0.26	0.29	0.30	0.28	0.02
C_{max} (ng/mL)	30.5	189	377	199	173
t_{max} (hr)	0.25	0.083	0.083	0.139	0.096
$t_{1/2}$ (hr)	ND[3]	0.292	1.32	0.806	ND
MRT_{last} (hr)	0.405	0.315	0.416	0.379	0.0555
AUC_{last} (hr·ng/mL)	20.1	65.5	142	75.8	61.5
AUC_{∞} (hr·ng/mL)	ND[3]	66.2	145	106	ND
Dose Normalized Values[1]					
AUC_{last} (hr·kg·ng /mL/mg)	2.68	8.74	18.9	10.1	8.20
AUC_{∞} (hr·kg·ng /mL/mg)	ND[3]	8.8	19.4	14.1	ND
Bioavailability (%)[2]	1.89	6.16	13.3	7.13	5.78

C_{max}: maximum blood concentration; t_{max}: time of maximum blood concentration; $t_{1/2}$: half-life, data points used for half-life determination are in bold; MRT_{last}: mean residence time, calculated to the last observable time point; AUC_{last}: area under the curve, calculated to the last observable time point; AUC_{∞}: area under the curve, extrapolated to infinity; ND: not determined; BLOQ: below the limit of quantitation (1 ng/mL); [1]Dose normalized by dividing the parameter by the equivalent active compound dose of 7.5 mg/kg; [2]Bioavailability determined by dividing the individual dose normalized oral AUC_{∞} values by the average dose normalized IV AUC_{∞} value; [3]not determined because the line defining the terminal elimination phase was not observed.

Table 5 . PK parameters of IHVR-35119 after oral admistration as either parent compound (Left panel) or the prodrug form, IHVR-35140 (right panel)

Individual and Average Liver Concentrations for IHVR-35-119 after Administration in Male CD-1 Mice

Dose Route	Time Point (hr)	Mouse #	Liver Weight (g)	Liver Homog. Volume (mL)	Liver Homog. Conc. (ng/mL)	Liver Tissue Conc. (ng/g)	Liver Tissue Conc. Average (ng/g)	SD
IV	4	473	1.246	6.23	BLOQ	BLOQ	ND	ND
		474	1.372	6.86	1.19	5.95		
		475	1.311	6.56	BLOQ	BLOQ		
PO	4	476	1.199	6.00	3.88	19.4	33.0	18.5
		477	1.232	6.16	5.11	25.6		
		478	1.260	6.30	10.8	54.0		

BLOQ: below the limit of quantitation (0.5 ng/mL or 2.5 ng/g of tissue); ND: Not determined.

Individual and Average Liver Concentrations for IHVR-35-119 after Administration of IHVR-35-140 in Male CD-1 Mice

Dose Route	Time Point (hr)	Mouse #	Liver Weight (g)	Liver Homog. Volume (mL)	Liver Homog. Conc. (ng/mL)	Liver Tissue Conc. (ng/g)	Liver Tissue Conc. Average (ng/g)	SD
IV	4	479	1.279	6.40	BLOQ	BLOQ	ND	ND
		480	1.254	6.27	1.95	9.75		
		481	1.317	6.59	BLOQ	BLOQ		
PO	4	482	1.132	5.66	14.7	73.5	60.3	20.7
		483	1.272	6.36	7.30	36.5		
		484	1.185	5.93	14.2	71.0		

BLOQ: below the limit of quantitation (0.5 ng/mL or 2.5 ng/g of tissue); ND: Not determined.

Table 6. Hepatic levels of IHVR-35119 after IV and PO admistration as either parent compound (upper panel) or the prodrug form, IHVR-35140 (bottom panel)

Enzyme IC50 Summary:

Compounds tested against 4 kinases, in 10 uM ATP

	Compound IC50* (M)					
Kinases	**ETHCC-007**	**HBF-0079 +2HCl**	**IHVR-04042**	**IHVR-04065**	**IHVR-04084**	**Staurosporine**
EPHA3	**3.11E-05**	**1.96E-05**			**4.80E-05**	**2.14E-08**
IGF1R	**1.39E-05**	**2.23E-05**	**3.72E-05**		**6.57E-05**	**2.14E-08**
IR	**4.82E-06**	**9.03E-06**	**2.39E-05**		**2.29E-05**	**1.60E-08**
IRR/INSRR	**1.14E-05**	**4.19E-05**	**5.05E-05**		**3.40E-05**	**5.79E-09**

Compounds tested against 4 kinases, in 1 uM ATP

	Compound IC50* (M)					
Kinases	**ETHCC-007**	**HBF-0079 +2HCl**	**IHVR-04042**	**IHVR-04065**	**IHVR-04084**	**Staurosporine**
EPHA3	**5.57E-05**	**6.46E-05**				**1.73E-08**
IGF1R	**1.45E-05**	**2.31E-05**	**4.10E-05**		**4.16E-05**	**9.08E-09**
IR	**3.72E-06**	**7.16E-06**	**2.72E-05**		**1.87E-05**	**4.55E-09**
IRR/INSRR	**1.52E-05**	**3.40E-05**	**7.40E-05**		**3.88E-05**	**1.49E-09**

Table 7. HBF-0079 and some related compounds show weak to moderate inhibition of insulin receptor and related tyrosine kinases. Reactions were run at two different concentrations of ATP to discern whether compound binds to ATP site.

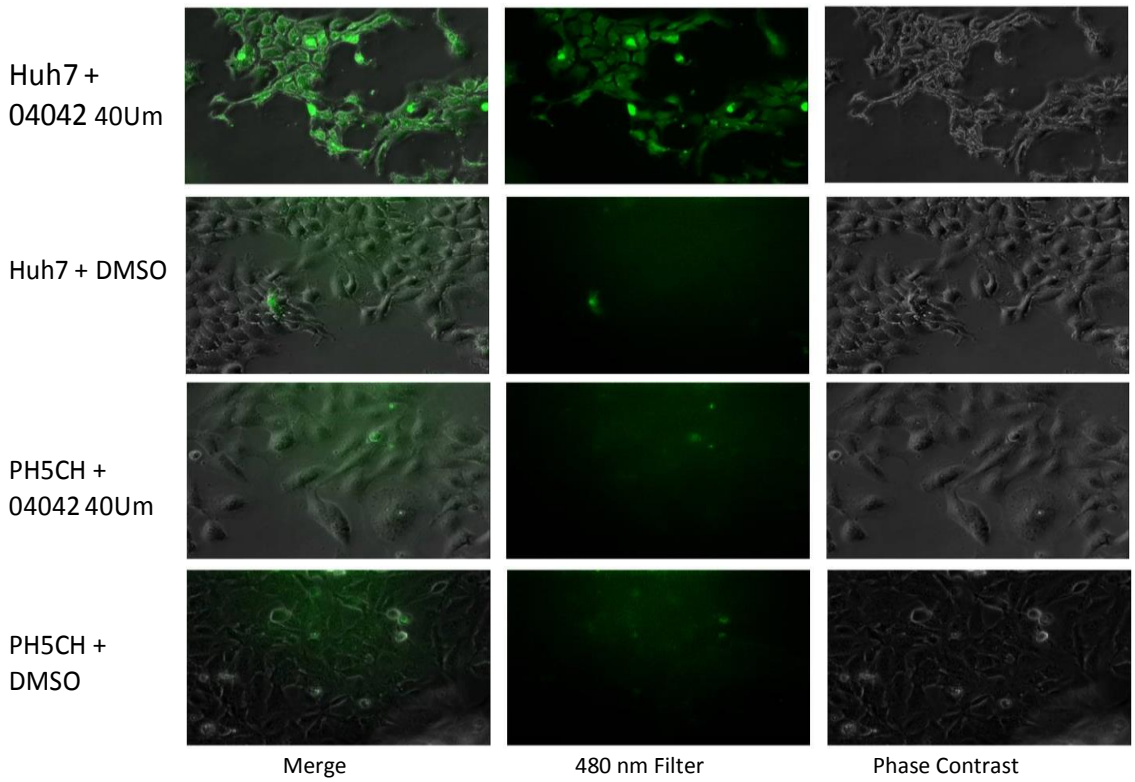


Figure 8. Cell-associated fluorescence of compound IHVR-04042 is specific for aminothiazole-sensitive cells, as imaged by fluorescence microscopy. Huh7 liver cancer cells and PH5CH normal liver cells were each treated with either DMSO or IHVR-04042, as indicated at left. Panels in left column depict 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

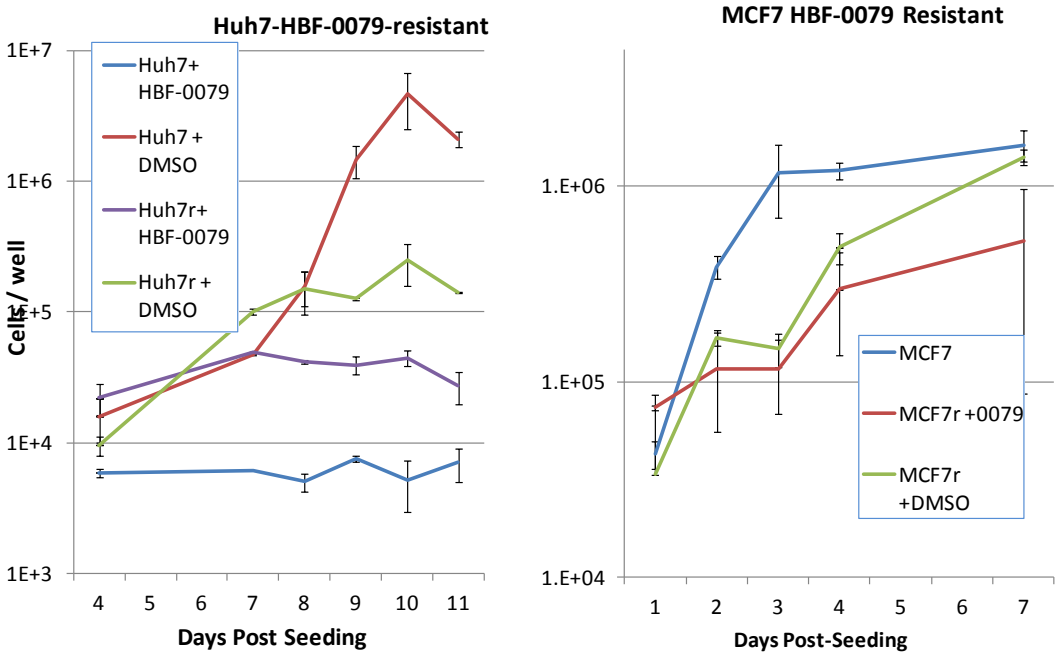


Figure 9. Aminothiazole-resistant clones of Huh7 liver cancer cells (Huh7r) and MCF7 breast cancer cells (MCF7r) exhibit reduced proliferation compared to the sensitive parental cell lines. The resistant clones were derived as described in the text, and along with the parent cell lines were cultured 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.

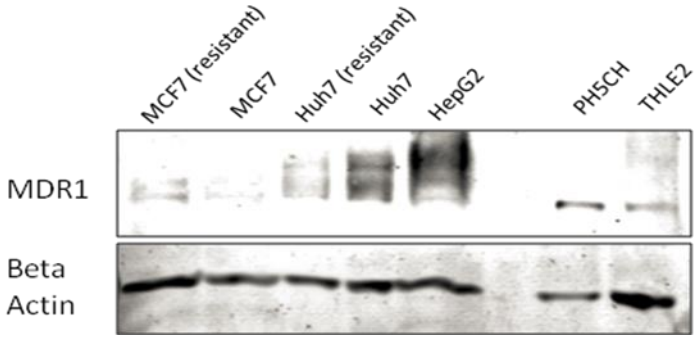


Figure 10. Expression of multiple drug resistance marker 1 (MDR-1) in aminothiazole-resistant vs parent Huh7 liver cancer and MCF7 breast cancer lines, in the HepG2 liver cancer line, or the PH5CH and THLE2 normal liver cell lines, does not corollate with sensitivity to the compounds. Depicted are Western 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.

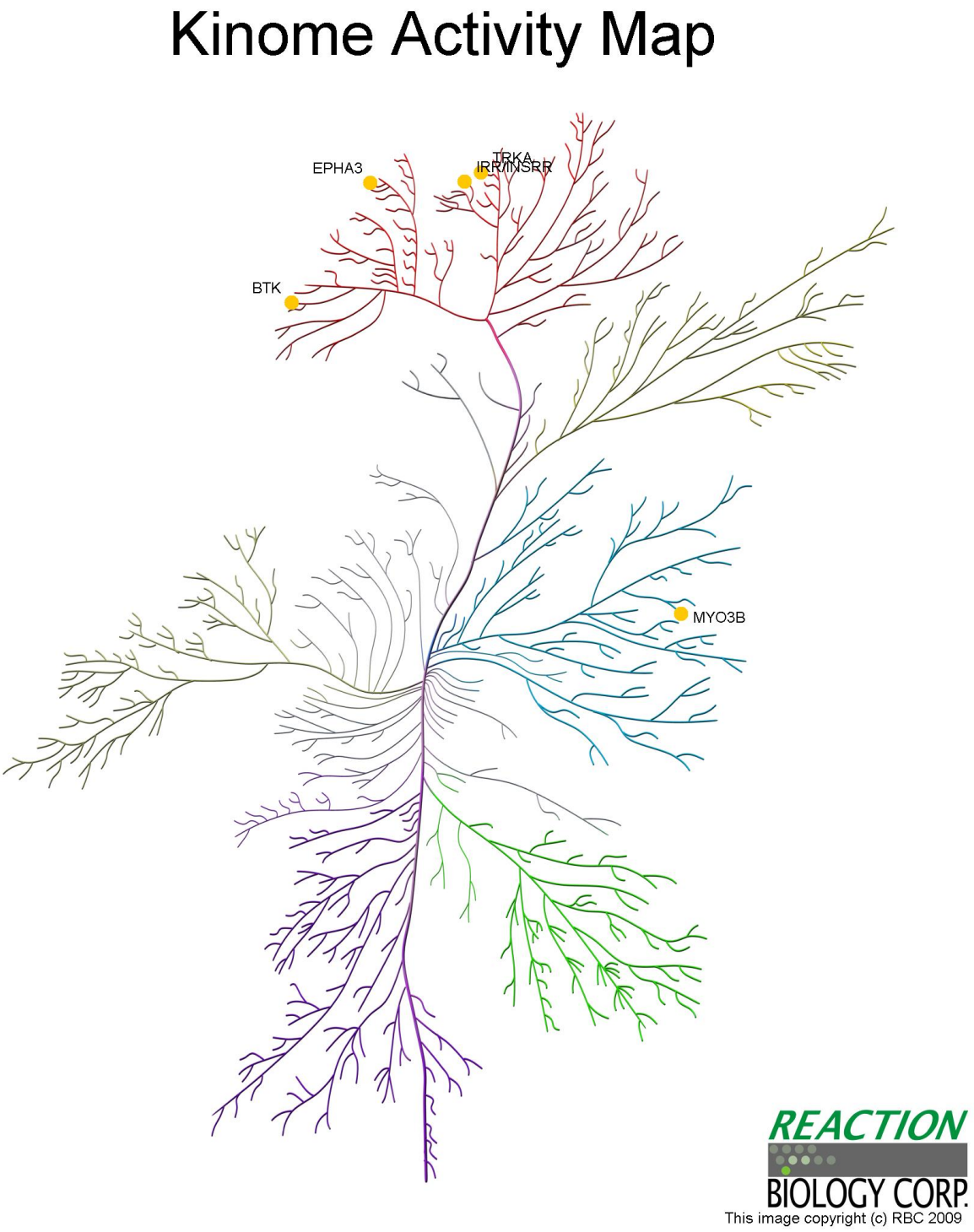


Figure 11. Kinome screen results of HBF-0079 depicted on the kinase family tree suggest specific inhibition of insulin-receptor related tyrosine kinases. Specific enzymes indicated by name showed a greater than 20% inhibition at 10 uM compound.

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__X___No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X__No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics

_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
___X__No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
____ Yes
_____No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, and an abbreviated title of the

publication. For example, if you submit two publications for Smith (PI for Project 01), one publication for Zhang (PI for Project 03), and one publication for Bates (PI for Project 04), the filenames would be:

Project 01 – Smith – Three cases of isolated
Project 01 – Smith – Investigation of NEB1 deletions
Project 03 – Zhang – Molecular profiling of aromatase
Project 04 – Bates – Neonatal intensive care

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1. None				☐Submitted ☐Accepted ☐Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes___X_____ No_____

If yes, please describe your plans:

We plan to prepare and submit for publicaiotn one paper detailing the advances in chemistry and biological studies that were on these compounds, and another which (after further research), will discuss the possible mechanism of action of these compounds as kinase inhibitors.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None. This project is much too early stage at the present.

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

This project is designed to produce novel drug candidates for the treatment of primary liver cancer, which has very low survival rate, and for which there is no real cure. As such, we believe that new molecules that were developed have promise for advancement to the clinic, especially now that a commercial partner has been identified. If we are ultimately successful, a new therapeutic option for patients will become available which currently does not exist.

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes__X____ No_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention: Substituted Aminothiazoles as Inhibitors of Cancers, Including Hepatocellular Carcinoma, and as Inhibitors of Hepatitis Virus Replication

b. Name of Inventor(s): Andrea Cuconati, Yanming Du, Huagang Lu, and William Kinney

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

There is a long felt need for new drugs that are both disease-modifying and effective in treating patients with primary liver cancer,including but not limited to hepatocellular carcinoma, hepatoblastoma, and cholangiocarcinoma. There is also a clear and present need for new therapeutic agents that are both disease modifying and effective in treating patients that are infected with a hepatitis virus. The present invention addresses the need for new drugs that are both disease-modifying and effective in treating patients suffering from primary liver cancer and hepatocellular carcinoma. Because the present invention targets the cell types that have been demonstrated to support viral infection in the liver,the present invention addresses also the need for new antiviral drugs that are both disease-modifying and effective in treating patients that are infected with hepatitis A virus, hepatitis B virus, hepatitis C virus, hepatitis D virus, and hepatitis E virus., as well as other viral species that infect the liver.

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____X_____ No____

If yes, indicate date patent was filed: will be filed in November 2014

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____X_____
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes__X_____ No____

If yes, how many licenses were granted?_____1_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes__X_____ No____

If yes, describe the commercial development activities: Project was licensed to a small company for development into a drug for the marketplace

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes___X_____ No_____

If yes, please describe your plans: see above

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages. *For Nonformula grants only – include information for only those key investigators whose biosketches were not included in the original grant application.*

Investigator biosketches which are not included in the grant application include:

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Du, Yanming	Associate Professor
eRA COMMONS USER NAME (credential, e.g., agency login) YANMINGDU1	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
Jilin University, Changchun, Jilin, China	B.S.	07/85	Polymer Chemistry
Wuhan University, Wuhan, Hubei, China	M.S.	07/88	Biopolymer Chemistry
The University of Iowa	Ph.D.	03/02	Organic Chemistry
Johnson & Johnson Pharmaceutical Research and Development	Postdoctoral	08/04	Medicinal Chemistry

A. Personal Statement

My current research is to design and synthesize novel compounds that can be effective against liver related diseases including HBV, liver cancer, and viral hemorrhagic fevers. Our goal is to deliver compounds not only active in in-vitro assays, but also efficacious in in-vivo models with good ADMET profiles.

I have training in organic chemistry, and more than ten years of expertise in medicinal chemistry and drug discovery working on both natural product modification and rationally designed molecules. As a postdoctoral fellow at Johnson & Johnson Pharmaceutical Research and Development, I discovered a new rearrangement reaction and provided two novel scaffolds which were applied in CNS and antibacterial therapeutic projects. At Rib-X Pharmaceuticals, I conducted research in the lead generation and optimization of new molecules derived from a natural product, macrolides and TAN-1057, against multi-drug resistant bacteria employing enabling tools, such as parallel synthesis and information from SAR analysis, X-ray crystallography, molecular modeling, pharmacokinetic and efficacy data. From this work, a preclinical candidate (gram positive) and a lead compound (gram-negative) were generated. And this lead compound series later became part of a deal in partnership with Sanofi-Aventis. At Fox Chase Chemical Diversity Center, I designed and synthesized a novel family of compounds demonstrating excellent anticonvulsant activity both in vitro and in mouse Maximal Electroshock Seizure (MES) test. At current position targeting virus infection, we have successfully developed a novel family of benzamide compounds with sub-micromolar antiviral efficacy against HBV and novel N-alkyl imino sugars effective against filovirus infection in mouse model.

B. Positions and Honors

Positions and Employment

1988-1990, Teaching Assistant, Department of Chemistry, Hainan Normal University, Haikou, Hainan, China
1990-1996, Research Engineer, Hunan Research Institute of Chemical Industry, Changsha, Hunan, China
2004-2009, Research Scientist II, Rib -X Pharmaceuticals, New Haven, Connecticut
2009-2010, Senior Scientist, Fox Chase Chemical Diversity Center Inc., Doylestown, PA
2010-2013, Assistant Professor, Institute for Hepatitis and Virus Research, Doylestown, PA
2013-present, Associate Professor, Blumberg Institute, Doylestown, PA

Other Experience and Professional Memberships

2014- Editorial board member for the journal *Current Topics in Medicinal Chemistry*
2010- Guest Editor for the journal *Current Topics in Medicinal Chemistry*
1999- Member of the American Chemical Society
2011- Member of American Association for the Study of Liver Diseases
2013- Member of International Society for Antiviral Research
2008- Reviewer for *Journal of Organic Chemistry, Organic Letters, Tetrahedron Letters, Bioorganic and Medicinal Chemistry Letters, European Journal of Medicinal Chemistry, and Medicinal Chemistry Communications.*

Honors

2013 26th International Conference on Antiviral Research young investigator poster 1st place award
2004-2009 Numerous Rib-X Spot awards for innovative design, synthesis, and service
2004 Johnson & Johnson Vision I Award for Discovery of a Novel Class of Bicyclic Piperazine Derivatives
2000 University of Iowa, Graduate School Summer Fellowship
1999 University of Iowa, Graduate School Summer Fellowship

C. Selected Peer-reviewed Publications

List available upon request

D. Research Support

Ongoing Research Support

R01AI104636 (Timothy Block PI)
Small molecule for management of filovirus hemorrhagic fevers.
Role: Sub-award (Chemistry) PI

R01AI113267 (Ju-Tao Guo PI)
Evaluation of therapeutic benefits of HBV nucleocapsid assembly inhibitors.
Role: Co-PI

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME WILLIAM A. KINNEY, Ph.D.	POSITION TITLE Director of Natural Products and Medicinal Chemistry
eRA COMMONS USER NAME (credential, e.g., agency login) WKINNEY1	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Ohio State University, Columbus, Ohio	Ph.D.	1984	Organic Chemistry (Prof. Leo A. Paquette)
Ohio State University, Columbus, Ohio	BS.	1979	Chemistry

A. Personal Statement

Dr. Kinney began his industrial career in the CNS group at Wyeth, where he invented the cyclobutenedione-containing NMDA antagonist perzinfotel that progressed to phase II clinical trials for pain. At Magainin Pharmaceuticals, he directed projects on trodusquemine (phase II, obesity) and squalamine (phase II, eye disease), two shark-derived natural products. A variety of labeling (tritium, deuterium) strategies were utilized for squalamine to track distribution of the new chemical entity in animals, patients, and in the laboratory. In 2000, he joined Johnson & Johnson, where he pursued peptide-mimetic integrin antagonists and urotensin-II receptor modulators for cardiovascular indications. He also made seminal discoveries with respect to self-assembling collagen-mimetic peptides that stimulate platelet aggregation. Currently, Dr. Kinney is a faculty member within the Baruch S. Blumberg Institute (BSBI). He directed the transfer and utilization of the former Merck Research Laboratories Natural Product Collection at the BSBI. He also initiated a structure based drug design project on a new anti-viral target in collaboration with Prof. Peter Katsikis at Drexel University to develop PI3K p110δ kinase inhibitors that block host cell signaling pathways required for influenza infection and pathogenesis. This project was funded by a Pennsylvania DCED Innovation Grant C000048686. He is an adjunct faculty member at Drexel University and Founder of IteraMed, a company focused on internal and out-sourced organic synthesis, consulting, and drug discovery. His scientific contributions include 70 publications, invited lectures, and oral presentations and inventorship on 39 issued U.S. patents.

B. Positions

Adjunct Professor 2010 – Present Drexel College of Medicine
Founder 2008 – Present IteraMed
Professor, Medicinal Chemistry 2010 – Present Baruch S. Blumberg Institute
Research Fellow, Drug Discovery 2000 – 2007 Johnson & Johnson P.R.D
Director of Chemistry 1993 – 1999 Magainin (Genaera) Pharmaceuticals
Principal Scientist 1984 – 1993 Wyeth Pharmaceuticals

C. Selecteed Peer-reviewed Publications

Over 50, list available upon request.

D. Selected US Patents (of 39 issued US patents)

"Collagen-Related Peptides and Uses Thereof" W. A. Kinney, M. A. Cejas, B. E. Maryanoff, T. Matalenas, C. Yang, U.S. Patent 8,076,294, December 13, **201**1.

"Urotensin-II Receptor Antagonists" B. E. Maryanoff, W. A. Kinney, E. C. Lawson, D. K. Luci, N. Marchenko, S. Sviridov, S. Ghosh, *U.S. Patent* 7,915,260, March 29, **2011**.

"Piperidinyl Compounds that Selectively Bind Integrins" B. DeCorte, W. A. Kinney, B. E. Maryanoff, S. Ghosh, L. Liu, *U.S. Patent 7,879,881*, Feb. 1, **2011**.

"Therapeutic Uses for Aminosterol Compounds" M. Zasloff, J. Williams, W. Kinney, M. Anderson, M. McLane, *U.S. Patent 7,410,959*, Aug. 12, **2008**.

"Treatment of Neovascularization Disorders with Squalamine" M. Zasloff, A. Shinnar, W. Kinney, S. Jones, *U.S. Patent 6,962,909*, Nov. 8, **2005**.

"Regioselective and Stereoselective Oxidation of Fused Ring Systems Useful for the Preparation of Aminosterols" W. A. Kinney, X. Zhang, R. Michalak, *U.S. Patent 6,933,383*, Aug. 23, **2005**.

"Aminosterol Compounds and Uses Thereof" M. Rao, B. Feibush, W. Kinney, M. Zasloff, L. Noecker *U.S. Patent 6,388,108*, May 14, **2002**.

"Stereoselective Synthesis of 24-Hydroxylated Compounds Useful for the Preparation of Aminosterols, Vitamin D Analogs, and Other Compounds" W. A. Kinney, S. Jones, X. Zhang, M. N. Rao, M. Bulliard, H. Meckler, N. Lee, *U.S. Patent 6,610,866*, Aug. 26, **2003**; *U.S. Patent 6,262,283*, July 17, **2001**.

"Method of Inhibiting Proliferation of Cells by Administering an Aminosterol Compound" W. A. Kinney, *et al. U. S. Patent 5,994,336*, Nov. 30, **1999**.

"Process for the Preparation of [2-((8,9)-Dioxo-2,6-diazabicyclo[5.2.0]-non-1(7)-en-2-yl)ethyl]phosphonic Acid" A. A. Asselin, W. A. Kinney, J. Schmid, *U. S. Patents 6,011,168*, Jan. 4, **2000***,* and *5,990,307*, Nov. 23, **1999**.

"Certain Aminosterol Compounds and Pharmaceutical Compositions Including These Compounds" M. Zasloff, A. Shinnar, W. Kinney, S. Jones, *U. S. Patent 5,847,172*, Dec. 8, **1998**.

"Aminosterol Compounds Useful as Inhibitors of the Sodium/Proton Exchanger (NHE)" M. Zasloff, A. Shinnar, M. Rao, W. Kinney, *U. S. Patent 5,840,936*, Nov. 24, **1998**.

"Aminosterol Compounds and a Method of Treating Infection Using the Aminosterol Compounds" M. Zasloff, A. Shinnar, W. Kinney, M. Rao, *U. S. Patent 5,840,740*, Nov. 24, **1998**.

"Method of Inhibiting Proliferation of Cells by Administering an Aminosterol Compound" M. Zasloff, A. Shinnar, W. Kinney, M. Anderson, J. Williams, M. McLane, *U. S. Patent* 5,795,885, Aug. 18, **1998**.

"Method for Treating Infection Using Steroid Based Pharmaceutical Compositions" L. L. Frye, M. A. Zasloff, W. A. Kinney, R. Moriarty, D. C. Collins, *U. S. Patent* 5,733,899, Mar. 31, **1998**.

"Method for Inhibiting Angiogenesis Using Squalamine and Squalamine Steroid Derivatives" L. L. Frye, M. A. Zasloff, W. A. Kinney, R. Moriarty, D. C. Collins, *U. S. Patent* 5,721,226, Feb. 24, **1998**.

"Steroid Derivatives, Pharmaceutical Compositions Containing them, and their Use as Antibiotics or Disinfectants" L. L. Frye, M. A. Zasloff, W. A. Kinney, R. M. Moriarty, *U. S. Patent* 5,637,691, June 10, **1997**.

"[[2-(Amino-3,4-dioxo-1-cyclobutene-1-yl)amino]alkyl]-Acid Derivatives" W. A. Kinney, D. T. Garrison, *U. S. Patents* 5,240,946, Aug. 31, **1993**, and 5,168,103, Dec. 1, **1992**.

"4-Azatricyclo[4.3.1.1(3,8)]undecylarylpiperazines with Anxiolytic Activity" W. A. Kinney, N. E. Lee, *U. S. Patent* 5,021,569, June 4, **1991**.

E. Research Support

Seed money ($10,000) was received from the PA Department of Community and Economical Development Innovation Grant C000048686 to identify PI3K p100δ kinase inhibitors that block host cell signally pathways important for viral replication.