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14. ABSTRACT

Hepatitis B Virus (HBV) causes chronic hepatitis, cirrhosis, liver failure, and liver cancer, but current drugs cannot fully stop disease progression. HBV replicates by reverse transcription. The viral ribonuclease H (RNaseH) removes the viral RNA after it has been copied into DNA so the second strand can be made. Lack of RNaseH activity causes viral DNA replication to fail. Despite being an obvious drug target, the HBV RNaseH is poorly characterized. This project will generate the first in-depth biochemical evaluation of the RNaseH. It will define the interaction between the RNaseH and its Mg^{++} ion cofactor, assess binding between the RNaseH and its substrate, define the mechanism of inhibition induced by RNaseH inhibitors, and provide the first structure-function analysis of the enzyme.

The status as we approach the end of the third year of this project is: i) The affinity of Mg^{++} for the RNaseH is ~0.5 μM; ii) Mg^{++} concentration has no effect on substrate binding; iii) Inhibitor binding to the RNaseH is strongly dependent upon Mg^{++} and inhibition is primarily mixed-mode; iv) Purification for the RNaseH continues to be very challenging; v) A saturation mutagenesis and selection approach has been adopted for structure-function analyses; and vi) A molecular homology model for the RNaseH was refined and used to evaluate inhibitor binding poses; and vii) Inhibitor mechanism of action studies against the related human RNaseH 1 have begun to permit comparative enzymology. Inhibition by α-hydroxytropolone compounds is uncompetitive and strongly Mg^{++}-dependent.

15. SUBJECT TERMS

NONE LISTED

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1. INTRODUCTION:

Hepatitis B Virus (HBV) causes chronic hepatitis, cirrhosis, liver failure, and liver cancer, but current drugs cannot fully control viral replication or stop disease progression. HBV replicates by reverse transcription. The viral ribonuclease H (RNaseH) is needed to remove the viral RNA after it has been copied into the first DNA strand so that the second DNA strand can be made. Failure of RNaseH action causes viral DNA replication to fail. Despite being essential for viral replication and being an obvious drug target, the HBV RNaseH is uncharacterized because it only recently became possible to purify active enzyme. This project will generate the first in-depth biochemical data about function and inhibition of the RNaseH. Specifically, it will define the interaction between the RNaseH and its Mg^{++} ion cofactor, assess binding between the RNaseH and its substrate, define the mechanism of inhibition induced by multiple classes of RNaseH inhibitors, and provide the first structure-function analysis of the enzyme.

2. KEYWORDS:

Hepatitis B virus, reverse transcription, ribonuclease H, enzymology, structure-function analysis, inhibition mechanisms.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Task 1. Investigate affinity of Mg^{++} for the RNaseH. 100% completed.
Task 2. Measure binding of the heteroduplex substrate to the HBV RNaseH. 90% completed.
Task 3. Evaluate the effects of varying Mg^{++} concentration on substrate binding affinity. 75% completed.
Task 4. Measure exonucleolytic RNA degradation at varying Mg^{++} concentrations. 5% completed.
Task 5. Determine impact of key mutations that inhibit substrate binding and/or catalysis on viral replication. 25% completed.
Task 6. Assess how Mg^{++} affects binding of RNaseH inhibitors. 90% completed.
Task 7. Determine how substrate affects inhibitor binding. 25% completed.
Task 8. Assess the affinity and kinetics of inhibitor binding to the RNaseH. 10% completed.
Tasks 9 & 10 (Typo in SOW causing duplication of task 9). Evaluate the mode of RNaseH inhibition. 90% completed.
Task 11. Identify RNaseH residues that affect inhibition efficacy. 35% completed.

What was accomplished under these goals?

1. Major activities. The major activities this reporting period were: i) Continued efforts to stabilize production of the RNaseH purification from *E. coli.* ii) Optimize a molecular model of the HBV RNaseH and use it to evaluate binding of compounds to the active site. iii) Shift to a comprehensive saturation mutagenesis strategy to evaluate structure-function relationships for the HBV RNaseH. iv) Conduct mechanism of action studies on the human ribonuclease H 1 (huRH1) and evaluate the effects of Mg^{++} concentration on efficiency of huRH1 inhibition. Addition of huRH1 to this project was proposed in last year's annual report that was approved on 11-20-2020.

2. Specific objectives (sorted by *Major activities*). i) Modify HBV RNaseH production protocols to provide consistent yields of enzyme with suitable activity for mechanistic analyses. ii) Collaborate with the contract research organization AMRI to optimize the HBV RNaseH model and conduct in-house docking experiments with a wide range of HBV RNaseH inhibitors. iii) Synthesize a pool of HBV RNaseH clones with saturating levels of mutation at each amino acid position with an average of 2-3 mutations/clone and conduct an evolution experiment to determine which variations support HBV RNaseH activity. iv) Use traditional steady-state enzymatic analyses to determine the mechanism of inhibition by α-hydroxytropolone inhibitors of huRH1. Titrate Mg^{++} over a wide range and determine the effects this has on inhibition of huRH1 by α-hydroxytropolone inhibitors.

3. Significant results (sorted by *Major activities*).

i) The RNaseH purification protocol needed to be redesigned yet again. The cause of this extremely frustrating problem is not fully understood because the protocol has been repeatedly reoptimized. After each optimization, enzyme production is stable for a varying period of time (from weeks to up to a year), but then ceases to provide quality enzyme. Two issues were identified this year as contributing to this variability. First, there appears to be a threshold issue in *E. coli* in which small, undefined variations in bacterial growth or metabolism can have enormous effects on efficiency of production of active HBV enzyme. Second, a small minority of subclones within a nominally clonal *E. coli* population were found to be significantly better at producing the HBV enzyme than most of the lineages in this *E. coli* clone. The largest advances this year were a) identifying an HBV genotype B clone whose activity was more stable among repeated purifications than our prior clones, b) eliminating contamination with *E. coli* RNaseH by use of an RNaseH-deficient host strain, and c) identifying and freezing isolates of the RNaseH-deficient bacterial strain that provided more robust HBV RNaseH production. However, issues are still substantial in protein production, with only about a quarter of the preparations yielding useful enzyme. By repeated protein production and quality-control analyses, we have accumulated a stock of the enzyme of high enough quality for analyses that has just begun to be used to assess inhibition by the RNaseH inhibitors.

ii) We collaborated with professional computational biologists at the Albany Molecular Research, Inc. (AMRI) to refine the HBV RNaseH molecular model we generated by homology modeling in our lab and reported last year. This led to correction of 11 defects in the model. Docking compounds at physiological pHs to ensure the correct protonation state did not reveal quantitative correlations between docking score and compound efficacy against HBV replication, indicating that confounding issues such as cellular permeability and compound stability have large effects on efficacy in addition to compound binding. However, comparing the binding poses of the most

effective compounds revealed clear commonalities in predicted binding mechanism that will be useful in designing the next generation of inhibitors.

iii) We altered our approach to the structure-function studies of the HBV RNaseH. The prior plan involved introducing selected site directed mutations into the enzyme and analyzing them individually. Unfortunately, the difficulties in consistently producing enzyme would have rendered interpretation of such studies exceptionally difficult. Consequently, we adopted a genetic approach in which we introduced random mutations into an HBV RNaseH clone at a density that each clone had an average of 2 to 4 mutation, and then selected the mutated population through 15 passages of growth (each with a 10x reduction in bacterial density at the onset of bacterial growth) in RNaseH-deficient *E. coli* cells in which growth of the bacteria is dependent on complementing HBV RNaseH activity. Eight independent lineages plus an unmutated clonal control were selected under varying RNaseH induction conditions, and samples were harvested every 3 passages for subsequent analyses. The selection process has just been completed and 2 analysis are beginning; details are in the future studies section.

iv) Inhibition of human ribonuclease H1 by 110 and 404, two α-hydroxytropolones, was Mg^{++}-dependent and inhibition was uncompetitive. However, inhibition of a huRH1 mutant containing only the catalytic RNase H domain was mixed-model. This discrepancy is likely explained by the high substrate-binding affinity imparted by the hybrid binding domain (HBD) in full-length huRH1. Since the compound is assumed to bind the active site in the catalytic domain, substrate binding by HBD is likely unhindered.

v) Inhibition of huRH1 was found to be strongly dependent on Mg^{++} concentration. This provides strong support for the hypothesis that the compound do not bind well to the active site in the absence of saturating Mg^{++}, implying that occupancy of the cation binding site(s) is needed for the inhibitors to work optimally.

4. Other achievements

None of relevance

What opportunities for training and professional development has the project provided?

This project promoted professional development of three scientists:

-Dr. Razia Tajwar received training in molecular biology, advanced protein purification techniques, enzymology, and computational molecular modeling/docking.

-Mr. Nathan Ponzar (Ph.D. graduate student) received training in protein purification and enzymology.

-Ms. Alaina Knier (M.S. graduate student) received training in protein purification and enzymology.

How were the results disseminated to communities of interest?

These results were disseminated by presentation at scientific meetings and discussions with collaborators. These include:

- The data were shared with our medicinal chemist collaborators (Drs. Marvin Meyers, Grigoris Zoidis, Ryan Murelli, and Peng Zhan) to help guide their design of the next batch of experimental RNaseH inhibitors.

- The overall state of the HBV RNaseH project was reviewed in Clark D.N., Tajwar, R., Hu J., and **Tavis, J.E.** The Hepatitis B Virus Polymerase. Chapter 22 in *Viral Replication Enzymes and their Inhibitors, Vol. 49*. Cameron C.E. and Arnold, J.J. eds. In press.

What do you plan to do during the next reporting period to accomplish the goals?

The protein production problem is anticipated to continue to plague this project. Our plan to deal with this will be to use our best production protocol and discard preps that do not meet strict quality control metrics. We have use this strategy to build up a collection of 4 useful preparations. Our experience indicates that this is the best way to get the project progressing faster.

We will finish defining the molecular mechanism of HBV RNaseH inhibition. Most of these studies were completed 1.5 years ago, but the lack of suitable enzyme prevented finalization of the project. That is now feasible and will be done as soon as possible.

The structure-function study on the HBV RNaseH will be completed in two manners. First, we will identify *in vitro* evolved HBV RNaseH variants that are more tolerant of expression in *E. coli* to mitigate the expression problems we have been having. We will characterize the growth kinetics of the selected lineages and examine *E. coli* colony size and number under selective conditions to identify rapidly growing subclones. Variations in HBV RNaseH sequence among these more robust clones will be determined by sequencing, and HBV RNaseH from representative variants will be expressed, purified, and analyzed. Second, we will determine the spectrum of variations that are tolerated in the HBV RNaseH. Each selected lineage and the clonal control will be subjected to next-generation sequencing, the nucleotide sequence will be translated into the amino acid sequences, the variation density at each amino acid position will be evaluated by diversity metrics such as Shannon's entropy, over- and under-represented residues at each position will be identified, and a consensus evolved sequence will be derived.

Molecular modeling efforts will entail: a) Compound docking studies to support the mechanistic studies for the HBV RNaseH and huRH1 enzymes, and b) Attempts to generate the first 2-domain model of the HBV reverse transcriptase (RT):RNaseH catalytic engine for the HBV polymerase protein. These studies will involve computational inter-molecular docking studies and *ab initio* prediction of the linker sequences between the HBV RT and RNaseH sequences within the polymerase protein, and will be guided by the need to maintain a continuous nucleic acid binding groove between the two protein domains and the known inter-active site distance.

Finally, we will finalize determination of the mechanism(s) of action of the RNaseH inhibitors against huRH1 and evaluate the role of Mg^{++} and the nucleic acid substrate on inhibitor binding. These studies are ~70% complete.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

The primary field is HBV RNaseH enzymology. The development of a validated HBV RNaseH model and defining the inhibition mechanisms of the compounds will permit a detailed understanding of how the inhibitors interact with the enzyme active site. This will advance understanding of how ribonucleases H can be inhibited. Understanding the range of permissible HBV RNaseH amino acid variations will advance understanding of the enzyme's mechanism and also help define potential fitness costs of resistance mutations that may arise against the HBV RNaseH inhibitors.

What was the impact on other disciplines?

There are two secondary fields. The first is RNaseH drug discovery. Comparing the inhibitor binding poses on huRH1 and the HBV RNaseH will permit design of more specific anti-HBV RNaseH inhibitors in our ongoing drug development program. The second is huRH1 inhibition. Comparing the inhibitory mechanisms of the compounds and inhibition by individual compounds against the HBV RNaseH and huRH1 will permit better interpretation of selectivity studies between the two enzymes and may also help in the design of more selective inhibitors.

What was the impact on technology transfer?

We have a collaboration with Casterbridge Pharmaceuticals, a startup biotechnology company seeking to develop and commercialize HBV RNaseH inhibitors as anti-HBV drugs. We will use our newly validated molecular model of the RNaseH in collaboration with Casterbridge to advance our joint drug development efforts on the α-hydroxytropolones that they have licensed from Saint Louis University.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

We faced 2 significant problems this year.

1) The most serious problem continues to be the variable quality of the purified HBV RNaseH. Production problems left us with a paper reporting the Mg^{++}-binding and inhibition mechanism that is 90% written but cannot be rigorously finalized until the protein purification issues are resolved. We believe this is manageable now through a combination of identifying a more robust HBV RNaseH expression clone, discovering that there are vast differences in the yield and specific activity of the enzyme when expressed from different subclones of the nominally clonal *E. coli* strain we employ, and through a brute-force approach of making many protein preparations and using only those that pass rigorous quality control metrics. This has permitted us to re-start the inhibition and mechanism studies with the HBV RNaseH.

2) COVID-induced supply chain disruptions and intermittent need for precautionary self-isolation of staff have reduced efficiency of our efforts on the project. All members of the Tavis lab are now fully vaccinated, Saint Louis University has authorized a return to normal operating procedures as of he start of the fall semester, and the supply chain disruptions are moderating, so it is anticipated that the impact of COVID on this project will continue to wane.

Changes in approach and reasons for change

Two major changes were adopted in this project this reporting period.

i) Problems with consistency of the HBV RNaseH preparations rendered the previous approach to structure-function analysis of making defined mutations and testing them individually uninterpretable and technically unfeasible. Consequently, we shifted to a saturation mutagenesis/selection/deep sequencing approach as described under the "What was Accomplished" section, point iii).

ii) We added mechanistic and inhibition studies of huRH1 to this project for two reasons. First, understanding the details of inhibition of huRH1 will permit design of more selective HBV RNaseH inhibitors. Second, progress was too slow on the HBV analyses and the difficulties were too great with the HBV enzyme to provide an optimal training platform for my PhD student Nathan Ponzar, whereas neither problem existed with huRH1.

Actual or anticipated problems or delays and actions or plans to resolve them

The major remaining issue is the quality of the recombinant HBV RNaseH. This will be addressed by using our best-available protocol to make many preparations of the enzyme and then discarding all of them that do not meet rigorous quality metrics.

Changes that had a significant impact on expenditures

None

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Not applicable.

Significant changes in use or care of vertebrate animals

Not applicable.

Significant changes in use of biohazards and/or select agents

None.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

None this cycle.

Books or other non-periodical, one-time publications.

The hepatitis B virus polymerase. Clark, DN, Tajwar, R, Hu, J, and Tavis, JE. 2021. Chapter 22 in *The Enzymes, Vol. 49*. ISBN 1874-6047. Https://doi.org/10.1016/bs.enz.2021.06.010. In production.

Other publications, conference papers and presentations.

None.

Website(s) or other Internet site(s)

None.

Technologies or techniques

None new.

Inventions, patent applications, and/or licenses

None supported directly by this project.

Other Products

None.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	John E. Tavis, Ph.D.
Project Role:	PI
Nearest person month worked:	4
bution to Project:	He helped design the experiments, analyze the data, train laboratory personnel, and set research priorities.
Name:	Nicola Pozzi, Ph.D.
Project Role:	Co-Investigator
Nearest person month worked:	2
Contribution to Project:	He provided expert guidance for both the redesign of the protein purification protocol and the design/interpretation of the enzymatic analyses.

Name:	Razia Tajwar, Ph.D.
Project Role:	Postdoctoral Research Associate
Nearest person month worked:	12
Contribution to Project:	She led the efforts to redesign the protein purification, constructed most of the mutant RNaseH constructs generated this cycle, and optimized the exonuclease activity conditions.
Name:	Qilan Li, Ph.D.
Project Role:	Lab Manager/Technician
Nearest person month worked:	3
Contribution to Project:	She provided general support such as ordering and laboratory management, ran enzymatic analyses, and assisted with basic analyses of the RNaseH preparations during redesign of the purification protocol.
Name:	Austin O'Dea
Project Role:	Research Assistant
Nearest person month worked:	2
Contribution to Project:	He assisted with redesign of the protein purification protocol and performed some of the enzymatic analyses.
Name:	Nathan Ponzar
Project	Role: Graduate student
Nearest person month worked:	10
Contribution to Project:	He led the enzymatic analyses and of huRH1 and assisted ongoing efforts with the HBV RNaseH.
Name:	Mariah Hamm
Project Role:	Research Assistant
Nearest person month worked:	3
Contribution to Project:	Ms. Hamm assisted Dr. Tajwar on identifying the more robust HBV RNaseH clone and improving the purification protocol.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Newly funded grant and sub-contract:

None this reporting cycle.

What other organizations were involved as partners?

None.

8. SPECIAL REPORTING REQUIREMENTS

Collaborative Awards: None.

Quad Charts: See attachment.

9. APPENDICES: None**.**

W81XWH1810307, Hepatitis B Virus Ribonuclease H: Mechanisms of Catalysis and Inhibition

PI: John Tavis, Ph.D., St. Louis University

Budget: $ 1,530,631 **Topic Area:** Hepatitis B and Hepatitis C **Mechanism:** W81XWH-17-PRMRP-IIRA

Research Area: 0109 and 0801 **Award Status:** 01 Aug 2018 – 31 July 2021

Study Goals: The goal of this project is to conduct an in-depth biochemical assessment of the Hepatitis B Virus ribonuclease H and its interaction with ribonuclease H inhibitors in support of anti-ribonuclease H drug discovery. .

Specific Aims:

Aim 1. How do Mg^{++} ions and the heteroduplex substrate interact with the RNaseH?
Aim 2. How do RNaseH inhibitors interact with the RNaseH?

Key Accomplishments:

Publications:

Li Q., Lomonosova E., Donlin M.J., Cao F., O'Dea A., Milleson B., Berkowitz A.J., Baucom J.C., Stasiak J.P., Schiavone D.V., Abdelmessih R.G., Lyubimova A., Fraboni A.J., Bejcek L.P., Villa J.A., Gallicchio E., Murelli R.P., Tavis J.E. (2020). Amide-Containing α-Hydroxytropolones as Inhibitors of Hepatitis B Virus Replication. Antiviral Res. 177:104777. Support from the NIH was acknowledged but I forgot to acknowledge the DoD for its support of the modeling within this drug discovery publication.

The hepatitis B virus polymerase. Clark, DN, Tajwar, R, Hu, J, and Tavis, JE. 2021. Chapter 22 in The Enzymes, Vol. 49. ISBN 1874-6047. Https://doi.org/10.1016/bs.enz.2021.06.010. In production.

Molecular model: Predicted, refined, and partially validated model of the HBV RNaseH.

Patents: None to date.
Funding Obtained: None to date.