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14. ABSTRACT

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) removes the viral RNA after it has been copied into the first DNA strand so that the second strand can be made. Lack of RNaseH activity causes viral DNA replication to fail. Despite being essential for viral replication and an obvious drug target, the HBV RNaseH is poorly characterized. 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.

The status as we approach the end of the second year of this project is that we have defined: i) The affinity of the two Mg^{++} for the RNaseH to be ~0.5 µM; ii) Found that Mg^{++} concentration has no effect on substrate binding; iii) Found that inhibitor binding to the RNaseH is strongly dependent upon Mg^{++} and that inhibition of the enzyme is primarily mixed-mode with some effects on catalysis; iv) Continue to improve purification conditions for the RNaseH; v) Have generated mutant RNaseHs for the structure function analyses; and v) generated and validated a molecular homology model for the RNaseH.

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. 5% 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. 5% completed.

What was accomplished under these goals?

1. Major activities. The major activities this reporting period were: i) Efforts to shift production of the RNaseH purification from *E. coli* to *S. cerevisiae*, ii) Define the mechanism of inhibition for 3 chemotypes of RNaseH inhibitors, iii) Evaluate how Mg^{++} concentration affects efficacy of HBV RNaseH inhibitors and binding of substrate, iv) Construct mutant RNaseHs for the structure-function assays, and v) Create a molecular model of the HBV RNaseH and dock inhibitors into its active site.

2. Specific objectives (sorted by *Major activities*). i) Shift production of the RNaseH to yeast cells to increase its specific activity, and use RNaseH-deficient yeast and *E. coli* strains as host strains to reduce cellular contamination. ii) Determine the mechanism of inhibition of the RNaseH using classical mechanism of action studies for inhibitors from the α-hydroxytropolone (αHT), N-hydroxyisoquinolinedieone (HID), and N-hydroxypyridinedione (HPD) chemotypes. iii) Determine the effect of Mg^{++} concentration on inhibition by αHTs, HIDs, and HPDs. iv) Continue to construct mutant RNaseHs for structure-function analyses. vi) Refine a molecular model of the HBV RNaseH and determine if it is of high enough resolution to reveal inhibitor binding poses and possibly guide design of improved inhibitors.

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

i) The RNaseH purification protocol needed to be redesigned again. Details about why this was needed are reported below under "Changes/Problems". To this end, we constructed HBV RNaseH constructs to express the protein in *S. cerevisiae* and obtained an RNaseH-deficient yeast strain to reduce background from cellular contaminants. We also obtained an *E. coli* strain with a temperature-sensitive RNaseH, again with the goal of reducing contamination in the RNaseH preparations. Conditions for induction of the RNaseH in *E. coli* were significantly improved, and expression efforts in yeast are beginning.

ii) The mechanisms of inhibition for the 3 major chemotypes of RNaseH inhibitors were refined. The αHT #110 competitively inhibits the HBV RH (effects are primarily on substrate binding), while the HPD, HID, and HNO compounds exhibit mixed model inhibition (the dominant mode is through interference with substrate binding, but significant effects on catalysis are also present).

iii) The effect of Mg^{++} concentration on inhibition by αHTs, HIDs, and HPDs was refined. Inhibition by compounds from the αHT, HPD, HID, and HNO chemotypes was Mg^{2+}-dependent, with K_i^{app} decreasing as a function of [Mg^{2+}], strongly supporting the proposed binding mechanism by coordination of the two active-site Mg^{2+} ions.

iv) Construct mutant RNaseHs for the structure-function assays. We continued to produce mutant RNaseH constructs. Tod date, we generated a total of 15 mutants. Analysis will commence once the purification issues discussed below are resolved.

v) There is no crystal structure for the HBV RNaseH due to its tendency to aggregate at high concentrations. Because availability of a validated molecular model will vastly ease structure-function analyses and interpretation of inhibition mechanisms, we created homology model for the RNaseH and docked >60 inhibitors into the active site. These studies revealed that there are 2 possible binding poses for αHT and HPD inhibitors in the active site.

In both cases, the metal-binding pharmacophore on the inhibitors was bound to the Mg^{++} ions in the active site, but binding occurred in 2 orientations, with R-groups extending into channels to both sides of the catalytic center. This indicates that bi-winged inhibitors may be able to access both channels, increasing affinity and specificity for the enzyme.

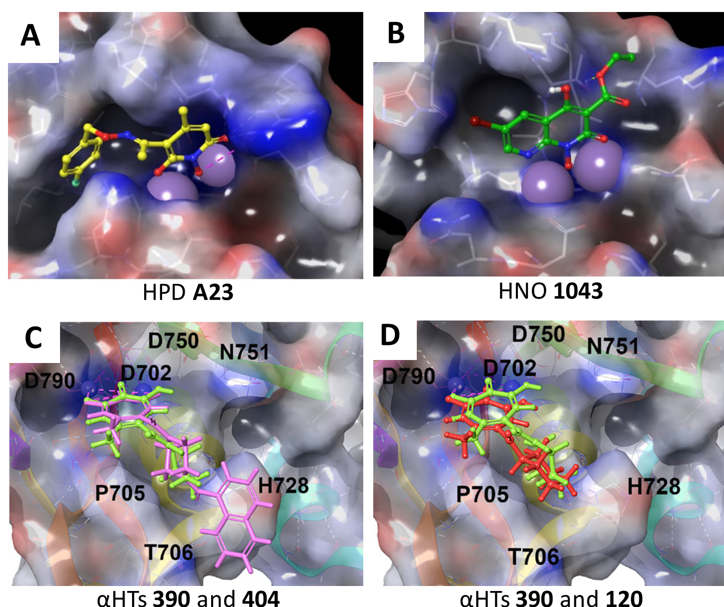

Fig. 5. Docking inhibitors into the HBV RNaseH active site. A. Docking of the HPD **A24** showing interactions with the catalytic Mg^{++} ions and within the active site cleft. **B.** Docked HNO **1043** showing similar interactions with the Mg^{++} ions. **C.** Superposition of αHTs **390** (green) and the longer molecule **404** (magenta) showing binding in an opposite orientation compared to **A23** and indicating key interacting residues. It also reveals minor variations in binding of the αHT pharmacophore to the Mg^{++} ions. **D.** Superposition of the αHTs **120** (red) and **390** (green) showing the unfavorable interaction with P705 stemming from the non-planar nature of the cyclohexyl appendage in **120**. The **A24** and **1043** images are from induced-fit refinement of a primary binding pose prediction, **120**, **390** and **404** are unrefined primary poses predicted by Glide.

The docking studies implied a new class of compounds could bind to the RNaseH active site. To test the hypothesis that this chemotype was an inhibitor, we purchased 6 additional analogs of this chemotype and tested their activity against the RNaseH. The 5 purchased without guidance from the molecular model had IC_{50}s vs the HBV RNaseH of 106-544 µM. One compound was purchased to take advantage of a specific molecular interaction predicted by the model, and it had an IC_{50} of 66 µM. Together, the model was validated by the unsupervised docking poses showing the predicted inhibitor binding to the active site Mg^{++}s, the identification of a new chemotype of HBV RNaseH inhibitors by the model, and the improvement in inhibitory efficacy of the new chemotype from selecting a compound to exploit a predicted interaction.

4. Other achievements

The closest human enzyme to the HBV RNaseH is human ribonuclease H1 (huRH1). Because understanding differences in the enzymology of the HBV RNaseH and huRH1 will be key to achieving sufficient specificity for RNaseH inhibitors, we optimized expression and purification of the full-length huRH1 for expression in *E. coli*. Mechanistic analyses are commencing as of this week for comparative enzymology.

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

This project promoted professional development of five scientists:

-Dr. Mahfuza Akter received training in molecular biology techniques, advanced protein purification, and enzymology.

-Dr. Razia Tajwar received training in molecular biology and advanced protein purification techniques.

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

-Mr. Kaushik Gokul (undergraduate researcher) received training in 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 data were shared as part of two posters at the International Meeting on the Molecular Biology of the Hepatitis B Viruses in Melbourne Australia. See below under "Other publications, conferences…".
- A paper reporting the molecular model and docking studies was published. See below under "Journal Publications".

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

The most critical item is for us to fix the RNaseH purification protocol (see below under "Changes/Problems"). Once this is fixed we will rapidly finalize the paper reporting the mechanism(s) of action for the RNaseH inhibitors.

Next, we will address the affinity and kinetics of RNaseH binding to the RNaseH. Concurrently, we will conduct the structure-function analyses of the enzyme using the new molecular model to guide mutation choice.

Finally, we will address the rate and Mg^{++}-dependence of the exonucleolytic reaction.

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 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.

What was the impact on other disciplines?

The secondary field in this case is RNaseH drug discovery. Comparative docking of compounds between the HBV RNaseH model and the existing crystal structure of the human ribonuclease H will ease design of compounds that inhibit the HBV enzyme but avoid its human homolog. Understanding the mechanisms of action of inhibitors will help guide design of drug combinations to improve therapy for HBV patients.

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 3 significant problems this year.

1) The most serious problem involved the quality of the purified HBV RNaseH. At last year's report, we had just discovered conditions leading to a 20x improvement in specific activity and elimination of detectable DNA contamination. Two back-to-back preps using the new conditions yielded a large stock of enzyme that we used for Major Activities 2 and 3. However, subsequent preparations performed once the high-activity stock was exhausted had suboptimal specific activity and were contaminated with cellular activities. This 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.

The HBV RNaseH is notoriously difficult to work with (this is why the Tavis lab is the only one that has ever studied it in detail). This problem stems from the necessity for post-translational activation of both the native enzyme and our recombinant RNaseH by cellular chaperones. The activation step requires purification of the enzyme at room temperature in the presence of Mg^{++} and ATP to stimulate the *E. coli* chaperone DnaK. This is hyper-sensitive to purification condition.

2) We again faced staffing problems that constrained expenditure slowed progress. Dr. Mahfuza Akter, the postdoc leading this project, abruptly resigned on June 10, 2019 to take an industrial position. The first person recruited to replace Dr. Akter declined the position six weeks after he had accepted it, forcing re-initiation of the personnel search. This issue was solved with recruitment of Dr. Razia Tajwar on December 2, 2019 to lead the project.

3) COVID forced Saint Louis University to close its research labs from the middle of March till late May, 2020, with substantial restrictions to laboratory access for 2-3 weeks before and after the closure. This led us to lose about 3 months of benchwork. This time was used to re-evaluate the purification mechanisms, identify and order the RNaseH-deficient *E. coli* and *S. cerevisiae* strains, and to redesign the HBV RNaseH expression constructs.

Changes in approach and reasons for change

We are taking two parallel approaches to solve the protein production problem. First, we acquired an *E. coli* strain with a temperature-sensitive RNaseH and supplemented the chaperones in the bacterial cells with various combinations of mammalian and bacterial chaperones. Chaperone supplementation and including glucose during induction significantly improved HBV RNaseH induction, and we are currently re-optimizing the activation step. Second, we are developing a *Saccharomyces cerevisiae* expression system for the HBV RNaseH to take advantage of the eukaryotic chaperones in the yeast cells.

We are beginning efforts to conduct comparative enzymology between the human RNaseH1 (huRH1) and the HBV RNaseH because this will greatly enhance understanding of the HBV RNaseH and advance drug discovery efforts. To date, those efforts have been supported by departmental and discretionary funds, but I will seek approval to incorporate them into this DoD project once this annual review has been processed.

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. Solving this issue (as we have done twice before when it cropped up) is our primary focus, as described under "changes in approach" above.

Changes that had a significant impact on expenditures

The resignation of Dr. Mahfuza Akter in June 2019 led to a reduction in salary and consumables expenditures that lasted until Dec. 2, 2019 when her replacement, Dr. Razia Tajwar, started work.

Mr. Nathan Ponzar, the graduate student who worked on this project for the full preceding reporting cycle, was fortunate enough to be supported by an NIH pre-doctoral training grant at Saint Louis University from July 1, 2018 to June 30, 2019. Per University policy, our Core Program in the Biomedical Sciences then picked up his stipend and health insurance until June 30, 2020. This reduced salary expenses on this DoD project. Consequently, he is reported below as only being supported financially by this project for 2 months despite committing 12 months effort.

The research labs at Saint Louis University were shut down to control the COVID-19 pandemic from mid-March to late May, halting supply expenditures.

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.

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.

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

None.

Other publications, conference papers and presentations.

Poster presentation. Ponzar NP, Kukla C, Akter M, Pozzi N, **Tavis JE.** (Oct. 3, 2019). Inhibitors of the HBV Ribonuclease H act by multiple Mg^{2+}-dependent mechanisms. Molecular Biology of the Hepatitis B Viruses 2019, Melbourne, Australia.

Poster presentation. Edwards T, Li Q, Ponzar N, O'Dea A, Kukla C, Akter M, Donlin M, Cao F, Zoidis B, Zhan P, Meyers M, Murelli R, **Tavis J**. (Oct. 3, 2019). Advances in HBV ribonuclease H drug development. Molecular Biology of the Hepatitis B Viruses 2019, Melbourne, Australia.

- **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
Contribution 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:	8
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:	6
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:	4
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:	2
Contribution to Project:	He led the enzymatic analyses and determined the Mg^{++} binding parameters and mechanisms of inhibition for the RNaseH inhibitors.

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:

R01 AI148362 (Tavis, P.I.) 12/02/2019 – 12/01/2024
NIH/NIAID 3 CM
HBV RNaseH inhibitors: Effects on HBV biology and resistance development

The goal of this project is to determine the precise effects of inhibiting the HBV RNaseH on viral reverse transcription, identify mutations to the RNaseH domain that promote resistance to HBV RNaseH inhibitors, and to define the impact(s) that inhibiting the RNaseH during HBV replication may have on the host cell.

75N93020C00044 (Rogers, P.I., Tavis, subcontract P.I.) 08/21/2020 to 08/20/21 0.6 CM
NIH/HIAID
Optimization of amide-based hydroxytropolones for HBV drug development

The goal of this study is to conduct hit to lead optimization of R^4 amide α-hydroxytropolones as HBV ribonuclease H inhibitors.

What other organizations were involved as partners?

None.

8. SPECIAL REPORTING REQUIREMENTS

None.

9. APPENDICES: None.