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TITLE: Antiviral Drug Discovery Targeting Zika Virus Protease

PRINCIPAL INVESTIGATOR: Yongcheng Song, PhD

CONTRACTING ORGANIZATION: Baylor College of Medicine, Houston, TX

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Yongcheng Song

E-Mail: ysong@bcm.edu

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Baylor College of Medicine, 1 Baylor Plaza, Houston, TX 77030

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
Zika virus, transmitted primarily by mosquitos, could become endemic in the tropical and subtropical regions including the southern states and territories of the United States. It could cause catastrophic consequences to the public health, such as microcephaly (small brain/head) of newborns. However, there are no antiviral drugs or vaccines for Zika infection. Zika virus protease (ZVpro) is a viral protein that is essential for viral replication. ZVpro is therefore a drug target. The overall goal of this project is to use a combination of rational inhibitor design, medicinal chemistry, X-ray crystallography and antiviral activity testing to discover small-molecule inhibitors of ZVpro, which are potential drug candidates for Zika infection. During the 3rd funding period, although the overall progress has been delayed due to the COVID-19 pandemic, we have produced good results showing our potent ZVpro inhibitors are non-cytotoxic and have strong anti-ZIKV activity. A total of 5 research articles and 1 patent application have been published for the 3 funding years. We will perform the experiments in accordance with the approved SOW to achieve the goals of the project in the next funding (no-cost-extension) period.

15. SUBJECT TERMS
Zika virus, Antiviral, NS2B-NS3 protease, Small-molecule inhibitor, Medicinal chemistry

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
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1. INTRODUCTION:

Zika virus (ZIKV), transmitted primarily by mosquitos, could become endemic in the tropical and subtropical regions including the southern states and territories of the United States. It could cause catastrophic consequences to the public health, including microcephaly (small brain/head) of newborns and Guillain-Barre syndrome. However, there have been no antiviral drugs or vaccines for the prevention and treatment of ZIKV infection. Zika virus protease (ZVpro) is a viral protein that is essential for viral replication. ZVpro is therefore a drug target for ZIKV infection. The overall goal of this project is to use a combination of rational inhibitor design, medicinal chemistry, X-ray crystallography and antiviral activity testing to discover and develop potent and selective small-molecule inhibitors of ZVpro. These compounds are potential drug candidates to treat and prevent Zika infections.

2. KEYWORDS:

Zika virus, Antiviral, NS2B-NS3 protease, Small-molecule inhibitor, Medicinal chemistry

3. ACCOMPLISHMENTS:

What were the major goals of the project?

There are 4 major goals/tasks of the projects:
The major Task 1 is to use medicinal chemistry to develop potent ZVpro inhibitors, with the milestones (at Month 36) being ~75% accomplished.
The major Task 2 is to use biochemical and X-ray crystallographic methods to characterize ZVpro inhibitors synthesized in Task 1, with milestones (at Month 36) being ~70% accomplished.
The major Task 3 is to test cellular anti-ZIKV activity as well as cytotoxicity of selected ZVpro inhibitors identified in Task 2, with milestones (at Month 36) being ~70% accomplished.
The major Task 4 is to perform pharmacokinetics, toxicity and in vivo antiviral activity studies, with milestones (at Month 36) being ~70% accomplished

What was accomplished under these goals?

Major Task 1: Medicinal Chemistry development of ZVpro inhibitors. The objective of this Task is to use rational inhibitor design, medicinal chemistry and structure activity relationship (SAR) studies to find potent small-molecule inhibitors of ZVpro.

Subtask 1: Docking studies for designing ZVpro inhibitors. We used a drug discovery software package Schrödinger Suite for the modeling studies, with the ZVpro structure (PDB: 5LC0) being the docking template. Experimental procedure has been described in the last progress report. >300 compounds were docked and molecules showing favorable docking results were chemically synthesized in subtask 2.

Subtask 2: Structure activity relationship (SAR)-guided medicinal chemistry. During Year-3, ~40 new compounds were synthesized successfully. In addition to the general synthesis described in the Year-1 progress report, for synthesis of compounds with a 2-amino substituent, mono-substitution of 1,6-dibromo-pyridine or -pyrazine with (*N*-Boc-piperidin-4-yl)methylamine produced an intermediate compound, which was iodized to give 5-iodo product. The target compounds can then be obtained following the general methods. Several other compounds were synthesized according to our published methods (*J. Med. Chem.* **2021**, *64*, 2777–2800)

Subtask 3: Structure-activity relationship (SAR) studies. Inhibitory activity against Zika NS2B-NS3 protease (ZVpro) and Zika virus replication of these synthesized compounds have been tested using the methods described below. Activities of a total of 104 trisubstituted pyrazine and related compounds were analyzed and detailed structure activity relationships can be found in our published article (*J. Med. Chem.* **2021**, *64*, 2777–2800). In brief, a novel series of 2,5,6-trisubstituted pyrazine compounds were found to be potent, allosteric inhibitors of Zika virus protease (ZVpro) with IC_{50} values as low as 130 nM.

The overall progress for Task 1 has been delayed due to the COVID-19 pandemic, which caused our institute to essentially shut down all research activities 3/20-5/20/2020 followed by an extended period with restricted access (until July 2021). Despite this, we have produced good results for the project. We have designed and synthesized ~240 compounds (out of 300 compounds proposed for the project) during the 3 years of performance. The goal and milestone for the Task have not been fully met. A no-cost-extension for 1 year has been requested and approved.

Major Task 2: Biochemical and X-ray crystallographic characterization of ZVpro inhibitors. The objective of this Task is to perform enzyme inhibition, X-ray crystallographic and other biochemical studies to characterize compounds made in Task 1, which will be used for rational design and SAR studies in Task 1 to find compounds with improved potency.

Subtask 1: Expression and inhibition of ZVpro. Expression, purification of recombinant ZVpro as well as the biochemical assay to determine the activity and inhibition of ZVpro have been described in the Year-1 progress report. New batches of recombinant ZVpro were expressed and purified. Compounds synthesized in Task 1 were tested for their inhibitory activities against ZVpro, among which potent small-molecule inhibitors were identified with IC_{50} as low as 130 nM. Detailed activity data can be found in our published article (*J. Med. Chem.* **2021**, *64*, 2777–2800).

Following similar methods, we expressed and obtained recombinant NS2B-NS3 proteases of closely related dengue serotype-2 and West Nile viruses in the Flavivirus family, which are also important human pathogens. Activity of selected compounds against these viral proteases were tested and results are shown in Table 1. These ZVpro inhibitors also inhibit the activity of DV2pro and WVpro, and their activities are correlated with those against ZVpro, showing R^2 values of 0.76 and 0.77, respectively. These results suggest these three Flavivirus proteases exhibit similar susceptibility to this series of compounds.

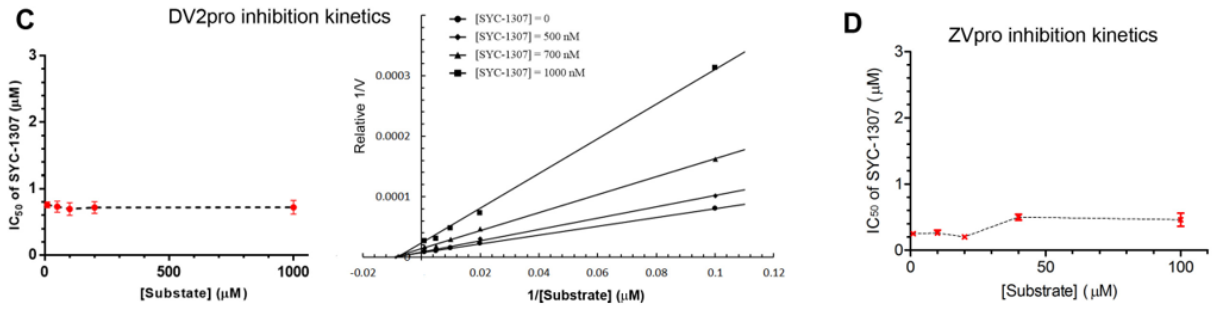
Table 1. Inhibitory activity IC_{50} (μM) against Flavivirus proteases ZVpro, DV2pro and WVpro.

	ZVpro	DV2pro	WVpro
44	32.6	>50	>50
45	26.5	>50	>50
1	21.7	35	>50
20	10.7	>50	>50
10	9.8	13.4	20.0
3	4.6	10.2	1.8
7	3.1	8.4	9.5
16	2.1	6.3	3.6
17	1.6	3.8	3.2
5	1.4	3.6	6.6
55	1.1	0.64	0.93
79	1.1	0.98	1.34
69	0.79	0.86	1.3
46	0.71	0.21	0.12
62	0.53	0.73	0.87
66	0.40	0.29	0.51
47	0.20	0.59	0.78
103	0.13	2.4	0.82

Subtask 2: Enzyme selectivity for ZVpro. As high selectivity is required for these compounds to be less toxic or interfering to normal physiology, selected potent ZVpro inhibitors (IC_{50} < 250 nM) were tested and found not to inhibit 5 selected human proteases at 10 μM, showing a high selectivity.

Subtask 3: X-ray crystallography of ZVpro in complex with selected inhibitors. Methods for crystallization, data collection and structure determination and refinement have been described in the Year-1 progress report. We continued working on this subtask for our newly synthesized inhibitors, but due to COVID-19 and personnel changes, no new X-ray structures of ZVpro/DV2pro-inhibitor complexes were obtained.

Subtask 4: Other biochemical/biophysical characterization of selected ZVpro inhibitors. Enzyme kinetics studies of ZVpro and DV2pro with an inhibitor (compound **9**) were performed and the results are shown below. (Left panel) IC_{50} values of compound **9** against DV2pro (3 nM) at [S] from 10 to 1000 μM (0.07× to 7×K_m); (Middle panel) Lineweaver-Burke plot using the same raw data. The enzyme kinetics results suggest that compound **9** is a non-competitive inhibitor of DV2pro, which is consistent with our X-ray crystallographic study. IC_{50} values of compound **9** against ZVpro (1 nM) at [S] from 1 to 100 μM (0.07× to 7×K_m) are shown at right. Similar to DV2pro, the IC_{50} values do not linearly increase according to the Cheng-Prusoff equation ($IC_{50} = K_i + K_i/K_m \times [S]$), in which K_m = 14.6 μM. The enzyme kinetics results suggest that compound **9** is not a competitive inhibitor of ZVpro.



The overall progress for Task 2 has been delayed due to the COVID-19 pandemic. Despite this, significant progress has been achieved including obtaining potent and selective inhibitors of Zika and dengue virus proteases with IC_{50} as low as 130 nM. The goal and milestone for the Task have not been fully met.

Major Task 3: To test cellular anti-ZIKV activity and cytotoxicity of selected potent ZVpro inhibitors. The objective of this Aim is to perform cell-based assays to determine cytotoxicity and anti-ZIKV activity of potent ZVpro inhibitors.

Subtask 1: Perform cytotoxicity testing of selected inhibitors. Cytotoxicity assays against mammalian cells Vero and U87 have been described in the Year-1 progress report. We selected ~20 ZVpro inhibitors and tested their cytotoxicity. Most of these compounds do not significantly inhibit proliferation of these cells with EC_{50}s of more than 10 μM.

Subtask 2: Perform cellular antiviral activity testing of selected inhibitors. The assays for evaluating antiviral activity of our compounds have been described in the Year-1 progress report. We tested anti-ZIKV activity of ~20 ZVpro inhibitors without significant cytotoxicity. Several compounds showed strong antiviral activity against ZIKV (FLR strain), as representatively shown in Table 2 with EC_{68} (concentration at which viral replication is inhibited by 68%) values of 0.3-3 μM. In addition, their anti-ZIKV activities are generally correlated with the inhibitory activities against ZVpro, suggesting ZVpro is the cellular target.

Table 2. Antiviral EC_{68} (μM) against ZIKV-FLR in U87 cells.

	ZVpro IC_{50} (μM)	ZIKV-FLR EC_{68} (μM)
1	21.7	>10
50	0.76	>10
7	3.1	5.0
102	1.0	3
79	1.1	2.5
55	1.1	2.5
46	0.71	2.5
69	0.79	1.2
59	0.68	1.2
62	0.53	1.2
66	0.40	1.2
78	0.24	1.2
103	0.13	0.6

47	0.20	0.3-0.6

Although the progress for the Task 3 has been delayed due to the COVID-19 pandemic, our results are satisfactory, showing our potent ZVpro inhibitors are non-cytotoxic and have potent anti-ZIKV activity. The goal and milestone for the Task have not been fully met.

Major Task 4: To test PK/Tox and in vivo anti-ZIKV activity of selected potent ZVpro inhibitors. The objective of this Aim is to perform a series of in vitro and in vivo PK/Tox testing to select good drug candidates and test their in vivo antiviral activity in a mouse model of ZIKV infection.

Subtask 1: PK/Tox and brain distribution testing of selected inhibitors. Due to the COVID-19 pandemic, we will perform this task for qualified compounds in the next NCE period.

Subtask 2: To test in vivo anti-ZIKV activity of selected ZVpro inhibitors. Due to the COVID-19 pandemic, we will perform this task for qualified compounds in the next NCE period.

The overall progress for the Task 4 involving mouse studies has been particularly affected and delayed due to the COVID-19 pandemic, we will perform this task for qualified compounds in the next funding period. The goal and milestone for the Task have not been fully met.

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

Nothing to report.

How were the results disseminated to communities of interest?

Nothing to report.

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

During the next no-cost-extension (NCE) period, we will perform the experiments we proposed in accordance with the approved SOW (NCE period) to achieve the goals of the project, using a combination of rational inhibitor design, synthetic medicinal chemistry, biochemistry, X-ray crystallography and in vitro and in vivo testing of biological activities and toxicities of potent ZVpro inhibitors.

4. **IMPACT:**

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

Nothing to report.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Nothing to report.

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

Due to the COVID-19 pandemic, which caused our institute to essentially shut down all research activities 3/20-5/20/2020 followed by an extended period with restricted access (until July 2021), our overall progress has been delayed. Despite this, our results have been satisfactory as described in the Accomplishment section. With the approval of the NCE, we expect there would be no negative impact to our overall accomplishment of this project.

Changes that had a significant impact on expenditures

Nothing to report.

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

Nothing to report.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:

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

Journal publications.

Nie, S.; Yao, Y.; Wu, F.; Wu, X.; Zhao, J.; Hua, Y.; Wu, J.; Huo, T.; Lin, Y.-L.; Kneubehl, A. R.; Vogt, M. B.; Ferreon, J.; Rico-Hesse, R.; Song, Y.* Synthesis, Structure-Activity Relationships and Antiviral Activity of Allosteric Inhibitors of Flavivirus NS2B-NS3 Protease. *J. Med. Chem.* **2021**, *64*, 2777–2800. (Published and acknowledged this DoD grant award). A copy of reprint is attached.

Wu, F.; Nie, S.; Yao, Y.; Huo, T.; Li, X.; Wu, X.; Zhao, J.; Lin, Y.-L.; Zhang, Y.; Mo, Q.; Song, Y.* Small-molecule inhibitor of AF9/ENL-DOT1L/AF4/AFF4 interactions suppresses malignant gene expression and tumor growth. *Theranostics*, **2021**, 11(17):8172-8184. (Published and acknowledged this DoD grant award). A copy of reprint is attached.

Li, X.; Song, Y.* Structure, function and inhibition of critical protein–protein interactions involving mixed lineage leukemia 1 and its fusion oncoproteins. *J. Hematol. Oncol.* **2021**, 14(1):56. (Published and acknowledged this DoD grant award). A copy of reprint is attached.

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

Nothing to report.

Other publications, conference papers and presentations.

Nothing to report.

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Song, Yongcheng
Role: PI
Researcher Identifier (e.g. ORCID ID): 0000-0003-2611-2476
Person Months: 4
Contribution to Project: As PI, Dr. Song is responsible for all aspects of the studies proposed, including experimental design, data analysis, postdoc training and manuscript preparation.
Funding Support:

Name: Rico-Hesse, Rebecca
Role: co-Investigator
Researcher Identifier (e.g. ORCID ID): 0000-0001-6216-1000
Person Months: 1
Contribution to Project: Dr. Rico-Hesse helped the PI design experiments for Aims 3 and 4, train personnel and analyze results.
Funding Support:

Name: Wu, Fangrui
Role: Research Faculty
Researcher Identifier (e.g. ORCID ID): 0000-0001-8141-3584
Person Months: 2
Contribution to Project: Dr. Wu performed biochemical activity testing of ZVpro inhibitors.
Funding Support:

Name: Yao, Yuan
Role: Research Associate
Researcher Identifier (e.g. ORCID ID): 0000-0002-4543-2988
Person Months: 4
Contribution to Project: Dr. Yao performed biochemical and biological activity testing of ZVpro inhibitors.
Funding Support:

Name: Wu, Xiaowei
Role: Postdoc
Researcher Identifier (e.g. ORCID ID): 0000-0002-2157-2847
Person Months: 2
Contribution to Project: Dr. Wu performed molecular modeling and organic synthesis of novel inhibitors of ZIKV protease.
Funding Support:

Name: Ashraf Uz Zaman, Md
Role: Postdoc
Researcher Identifier (e.g. ORCID ID): 0000-0003-1327-3166
Person Months: 2
Contribution to Project: Dr. Ashraf performed molecular modeling and organic synthesis of novel inhibitors of ZIKV protease.
Funding Support:

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

See attached updated Other Support forms (with changes noted) for the PI and co-Investigator. These changes have no impact on the funded project.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES:

1. A copy of the published article.
2. Other Support forms for the PI and co-Investigator.