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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 77030**

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**TYPE OF REPORT: Annual**

**PREPARED FOR:** U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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

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**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 first funding period, the overall progress for the project is satisfactory and in line with what we proposed. ~120 potential inhibitors have been designed and synthesized, among which a number of compounds showed potent to good inhibitory activities against ZVpro with IC50 values of 0.2-10 μM. Methods for other biochemical, X-ray crystallography, and biological activity assays have been developed. Testing of the activities of the synthesized compounds are currently on-going.

**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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## TABLE OF CONTENTS

1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

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:** *Provide a brief list of keywords (limit to 20 words).*

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

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

**What were the major goals of the project?**

*List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.*

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 ~30% 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 ~30% 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 ~20% accomplished.
The major Task 4 is to perform pharmacokinetics, toxicity and in vivo antiviral activity studies, with milestones (at Month 36) being ~20% accomplished

**What was accomplished under these goals?**

*For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project*

*progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.*

**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. The protein was prepared and energy-minimized using the program Maestro in Schrödinger. Molecules were designed, constructed and energy-minimized in Maestro, and then docked into the protein using the program Glide. Molecules showing favorable docking results were chemically synthesized in subtask 2.

*Subtask 2: Structure activity relationship (SAR)-guided medicinal chemistry.* The general synthesis for medicinal chemistry studies is described. 6-Chloro-2-aminopyrazine was selectively iodized using *N*-iodosuccinimide, and the 2-amino group was converted to a hydroxyl, which was alkylated using a Mitsunobu reaction to give the product with a protected piperidin-4-yl-methoxy group. Two selective Suzuki reactions were performed to introduce different aryl groups $R^5$ and $R^6$ to produce, after deprotection, the target compounds. With the general methods, ~120 compounds have been synthesized in an overall yield of ~50%.

*Subtask 3: SAR and quantitative SAR (QSAR) studies.* In accordance with the approved SOW, we will start analyzing structure-activity relationships in Year-2.

The overall progress for Task 1 is generally in line with what we proposed and expected, with all of the subtasks being on schedule. The activities and results have been satisfactory, with ~120 compounds being designed and synthesized (out of 300 compounds proposed for the project). The goal and milestone (at Month 36) for the Task have not been met.

**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.* Two plasmids for expression of ZVpro from a 2015 Brazilian strain (GenBank: KU729217) in *E. coli* were constructed for biochemical assays and X-ray crystallography. The first in pET-16b vector contains cDNA (synthesized by Genscript) encoding NS2B (47-95) and NS3 (1-170), interconnected with a $Gly_4$-Ser-$Gly_4$ linker. The second plasmid using pET-Duet-1 vector can produce two separate proteins simultaneously: NS2B(47-95) and NS3(1-170, with an N-terminal His6 tag), which form a complex in *E. coli* and can be purified together.

The two plasmids were used to transform *E. coli* (BL21 Rosetta strain from Agilent) and cultured at 37 °C in LB media containing ampicillin (50 μg/mL) and chloramphenicol (34 μg/mL). Upon reaching an optical density of ~1.3 at 600 nm, protein expression was induced by adding 0.5 mM isopropylthiogalactoside at 18 °C for 20 hours. Cells were harvested, lysed, centrifuged at 20,000 rpm for 20 min and the supernatant was collected and subjected to a nickel affinity and site exclusion column chromatography to achieve >90% purity (SDS-PAGE).

A biochemical assay for ZVpro enzyme activity and inhibition has been developed. The substrate for ZVpro is Bz-Nle-Lys-Lys-Arg-AMC. Upon hydrolysis, the free AMC will produce a significant increase in fluorescence (Ex: 380 and Em: 460 nm). The activity assay was performed in 96-well microplates using the purified ZVpro (1 nM), the substrate (20 μM) in 20 mM HEPES buffer (pH = 7.5). For inhibition assay, triplicate samples of a compound were incubated with the enzyme for 10 min, before adding the substrate to initiate the reaction. The fluorescence of each well was monitored every 30s, using a Beckman DTX-880 microplate reader. The data were imported into Prism 5 (GraphPad) and the $IC_{50}$ values were calculated by using dose response curve fitting. With this biochemical method, inhibitory activities of ~70 compounds (synthesized in Task 1) have been tested, among which a number of these compounds showed strong inhibitory activity against ZVpro with IC50 values of 0.2-10 μM.

*Subtask 2: Enzyme selectivity for ZVpro.* In accordance with the approved SOW, we will start this in Year-2.

*Subtask 3: X-ray crystallography of ZVpro in complex with selected inhibitors.* Recombinant ZVpro was expressed and purified as described above. The protein was concentrated to 15 mg/mL in a buffer containing 20 mM Tris (*p*H 7.2) and 200 mM NaCl. Co-crystallization with an inhibitor (5 mM) was set up by hanging drops with 1:1 ratio mixtures of 1 μL of protein solution and 1 μL of well solution containing 15% PEG 3350, 0.2 M $CaCl_2$ and 0.1 M Tris (pH 8.5). Prism-like single crystals appeared in ~1 week. Crystals were harvested in crystal freezing buffer containing 20% glycerol, 35% PEG 200, 100 mM MES, *p*H 8.5. The crystals were then flash-frozen in liquid $N_2$ for data collection. We have obtained beamline time slots (8-24 hours/month) at the Advanced Photon Source, Argonne National Laboratory. X-ray diffraction data have been collected in beamline 19-ID (or 19-BM). ~10 sets of diffraction data for ZVpro-inhibitor complexes have been collected at a resolution of 2.8-3.3 Å. Processing of these X-ray diffraction data and structural refinement are currently on-going.

*Subtask 4: Other biochemical/biophysical characterization of selected ZVpro inhibitors.* In accordance with the approved SOW, we will start this in Year-3.

The overall progress for the Task 2 is generally in line with what we proposed and expected, with all of the subtasks being on schedule. The activities and results have been satisfactory. The goal and milestone (at Month 36) for the Task have not been 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.* $10^5$ U87 or Vero cells per well were added into 96-well plates and cultured in DMEM media supplemented with 10% fetal bovine serum and penicillin (100 U/mL) and streptomycin (100 μg/mL) overnight. Upon addition of increasing concentrations of a compound, cells were incubated for 5 days. Cell viability was assessed by using an MTT assay (Sigma). ZVpro inhibitors were tested and found to exhibit EC50 values between 5 to >30 μM. Non-toxic compounds (as defined by EC50 >20 μM) were selected for cellular anti-ZIKV activity testing.

*Subtask 2: Perform cellular antiviral activity testing of selected inhibitors.* We have developed antiviral activity testing in U87 and Vero cells. To a monolayer of these cells with ≥80% confluence in 96-well plates were added 0.01 MOI (multiplicity of infection) of ZIKV-FLR and incubated for 2h for virus attachment. Supernatant was removed and cells were washed with PBS. Fresh culture medium (150 µL) containing various concentrations of a ZVpro inhibitor was added. Upon incubation for 48 h, the supernatant from each well containing ZIKV was analyzed for ZIKV RNA copies by qPCR. As the second method, end-point dilution assay was used to determine viral titers to further quantitate antiviral activity. Half-log serial dilutions of the supernatant from each well were added to Vero cells in quadruplicate in 96-well plates. Upon incubation for 7 days, ZIKV infection in each well can be clearly determined by CPE (microscope observation followed by MTT assay). $TCID_{50}$ (tissue culture infective dose) can be calculated based on the highest dilution in which ≥50% (i.e., ≥2 out of 4 quadruplicate wells) of Vero cells are infected with ZIKV. Antiviral activity of selected ZVpro inhibitors are currently on-going.

The overall progress for the Task 3 is generally in line with what we proposed and expected. The activities and results have been satisfactory, with methods for cytotoxicity and cellular antiviral activity testing being developed. The goal and milestone (at Month 36) for the Task have not been 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.* Since none of our ZVpro inhibitors have met the criteria for proposed PK/Tox evaluation, we will perform this task for qualified compounds in the next funding period.

*Subtask 2: To test in vivo anti-ZIKV activity of selected ZVpro inhibitors.* We have developed a mouse model for testing in vivo antiviral activity of ZVpro inhibitors. Because ZIKV only produces limited replication and does not cause fever/symptoms in immunocompetent adult mice, a useful mouse model for antiviral drug discovery is immunocompromised C57BL/6 mice bearing IFNα/βR-/- and IFNγR-/- (double knockout of interferon receptor α/β and γ genes) from Jackson Lab. $10^2$-$10^3$ $TCID_{50}$ of ZIKV were injected i.p. into these mice. ZIKV replicates rapidly in these mice without interferon-mediated immunity and caused acute disease and death of these animals in ~10 days. Antiviral activity of selected ZVpro inhibitors will be evaluated using this method.

The overall progress for the Task 4 is generally in line with what we proposed and expected. The activities and results have been satisfactory, with experimental methods being developed. The goal and milestone (at Month 36) for the Task have not been met.

**What opportunities for training and professional development has the project provided?**
*If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."*

*Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.*

Nothing to report.

**How were the results disseminated to communities of interest?**
*If there is nothing significant to report during this reporting period, state "Nothing to Report."*

*Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.*

Nothing to report.

**What do you plan to do during the next reporting period to accomplish the goals?**
*If this is the final report, state "Nothing to Report."*

*Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.*

During the second funding period, we will perform the experiments we proposed in accordance with the approved SOW 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:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

**What was the impact on the development of the principal discipline(s) of the project?**
*If there is nothing significant to report during this reporting period, state "Nothing to Report."*

*Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).*

Nothing to report.

**What was the impact on other disciplines?**
*If there is nothing significant to report during this reporting period, state "Nothing to Report."*

*Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.*

Nothing to report.

**What was the impact on technology transfer?**
*If there is nothing significant to report during this reporting period, state "Nothing to Report."*

*Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:*

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report.

**What was the impact on society beyond science and technology?**
*If there is nothing significant to report during this reporting period, state "Nothing to Report."*

*Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:*

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

| |
|---|
| Nothing to report. |

**5. CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

| |
|---|
| Nothing to report. |

**Actual or anticipated problems or delays and actions or plans to resolve them**
*Describe problems or delays encountered during the reporting period and actions or plans to resolve them.*

| |
|---|
| Nothing to report. |

**Changes that had a significant impact on expenditures**

*Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.*

Nothing to report.

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

*Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.*

**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:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  *Report only the major publication(s) resulting from the work under this award.*

**Journal publications.** *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Yao, Y.; Huo, T.; Lin, Y.-L.; Nie, S.; Wu, F.; Hua, Y.; Wu, J.; Kneubehl, A. R.; Vogt, M. B.; Rico-Hesse, R.; Song, Y. Discovery, X-ray Crystallography and Antiviral Activity of Allosteric Inhibitors of Flavivirus NS2B-NS3 Protease. *J. Am. Chem. Soc.* **2019**, *141*, 6832-6836. (Published and Acknowledged this DoD grant award). A copy of reprint is attached.

**Books or other non-periodical, one-time publications.** *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

**Other publications, conference papers and presentations**. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Oral presentation in local societies:
"Discovery, X-ray Crystallography and Antiviral Activity of Allosteric Inhibitors of Flavivirus Protease" in Texas Chemical Biology Conference, Texas A & M University, College Station, TX, May 24, 2019.

- **Website(s) or other Internet site(s)**
  *List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.*

| |
|---|
| Nothing to report. |

- **Technologies or techniques**
  *Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.*

| |
|---|
| Nothing to report. |

- **Inventions, patent applications, and/or licenses**
  *Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.*

| |
|---|
| Nothing to report. |

- **Other Products**
  *Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding,*

*prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:*

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

X-ray crystal structures of dengue serotype-2 virus protease in complex with three inhibitors have been deposited into protein data bank (PDB) as entries 6MO0, 6MO1 and 6MO2.

## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

**What individuals have worked on the project?**

*Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".*

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):
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: Nie, Shen-You
Role: Postdoc
Researcher Identifier (e.g. ORCID ID): 0000-0002-7396-8114
Person Months: 9
Contribution to Project: Dr. Nie worked with Dr. Song to perform organic synthesis, including optimize synthetic route, and medicinal chemistry development of novel inhibitors of ZIKV protease.
Funding Support:

Name: Wu, Jing-Yu
Role: Postdoc
Researcher Identifier (e.g. ORCID ID): 0000-0002-6653-0771
Person Months: 12
Contribution to Project: Dr. Wu worked with Dr. Song to perform computational modeling, inhibitor design and synthesize novel inhibitors of ZIKV protease, perform SAR studies and report results.
Funding Support:

Name: Yao, Yuan
Role: Research Associate
Researcher Identifier (e.g. ORCID ID): 0000-0002-4543-2988
Person Months: 12
Contribution to Project: Dr. Yao performed biochemical and biological activity testing of ZVpro inhibitors.
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?**
*If there is nothing significant to report during this reporting period, state "Nothing to Report."*

*If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.*

| |
|---|
| 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?**
*If there is nothing significant to report during this reporting period, state "Nothing to Report."*

*Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.*

*Provide the following information for each partnership:*
*<u>Organization Name:</u>*
*<u>Location of Organization: (if foreign location list country)</u>*
*<u>Partner's contribution to the project</u> (identify one or more)*

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to report.

## 8. SPECIAL REPORTING REQUIREMENTS

**COLLABORATIVE AWARDS:** *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

**QUAD CHARTS:** *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

**9. APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

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

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# Discovery, X-ray Crystallography and Antiviral Activity of Allosteric Inhibitors of Flavivirus NS2B-NS3 Protease

Yuan Yao,[†,⊥] Tong Huo,[†,⊥] Yi-Lun Lin,[†,⊥] Shenyou Nie,[†,⊥] Fangrui Wu,[†,⊥] Yuanda Hua,[†] Jingyu Wu,[†] Alexander R. Kneubehl,[‡] Megan B. Vogt,[‡,§] Rebecca Rico-Hesse,[‡] and Yongcheng Song*[,†]

[†]Department of Pharmacology and Chemical Biology, [‡]Department of Molecular Virology and Microbiology, [§]Integrative Molecular and Biomedical Sciences Graduate Program, Baylor College of Medicine, 1 Baylor Plaza, Houston, Texas 77030, United States

Supporting Information



**ABSTRACT:** Flaviviruses, including dengue, West Nile and recently emerged Zika virus, are important human pathogens, but there are no drugs to prevent or treat these viral infections. The highly conserved Flavivirus NS2B-NS3 protease is essential for viral replication and therefore a drug target. Compound screening followed by medicinal chemistry yielded a series of drug-like, broadly active inhibitors of Flavivirus proteases with $IC_{50}$ as low as 120 nM. The inhibitor exhibited significant antiviral activities in cells ($EC_{68}$: 300−600 nM) and in a mouse model of Zika virus infection. X-ray studies reveal that the inhibitors bind to an allosteric, mostly hydrophobic pocket of dengue NS3 and hold the protease in an open, catalytically inactive conformation. The inhibitors and their binding structures would be useful for rational drug development targeting Zika, dengue and other Flaviviruses.

Dengue (DENV), West Nile and recently emerged Zika (ZIKV) viruses belong to the genus *Flavivirus* in the Flaviviridae family of RNA viruses. These viruses are transmitted primarily by *Aedes* mosquitos. Four serotypes of DENV infect ∼400 million people each year with 100 million developing dengue fever. ∼500,000 cases develop serious dengue hemorrhagic fever, causing ∼22,000 deaths each year.[1] Moreover, patients recovered from one serotype are still susceptible to other serotypes with an increased likelihood of a more severe disease due to existing antibodies.[2] ZIKV has caused three major outbreaks in Pacific Ocean islands (2007 and 2013), Brazil and other American countries (2015−2016), in which >1 million infections were reported and a large number of patients sought medical treatment.[3] More seriously, ZIKV infection has been correlated with a 20-fold increased incidence of serious neurological disorders, including Guillain-Barré syndrome[4] and >4,000 cases of microcephaly in newborns.[5,6] Since 2015, ZIKV has quickly spread to 48 pan-American countries. Recently, ZIKV was found to be transmitted through sex or body fluids.[7] Despite these serious outcomes as well as possible future outbreaks, there have been no antiviral drugs to prevent or treat ZIKV and DENV infections. A licensed dengue vaccine, Dengvaxia, has raised concerns about efficacy and increased risk of severe disease for seronegative people during clinical trials.[8]

ZIKV/DENV contain a single-stranded, positive-sense RNA with ∼10,800 nucleotides, encoding a viral polyprotein. The polyprotein is site-specifically cleaved by the viral NS2B-NS3 protease and several host proteases to produce functional proteins (Supporting Information Figure S1).[1,3] The NS2B-NS3 protease is essential for viral replication and, therefore, a promising drug target.[1,9,10] A number of peptide-based covalent inhibitors of Flavivirus proteases have been reported,[1,11,12] but they did not demonstrate significant antiviral activities in cells or animal models due to low cell permeability and metabolic stability. Nonpeptidic inhibitors have also been reported, but their inhibitory activities are relatively weak and how these compounds bind to the protease is unknown.[1,13,14]

Homology analysis showed Flavivirus proteases are evolutionally conserved (Figure S2) and highly stable (Figure S3). NS3 contains an N-terminal serine protease domain, but complexation with NS2B is required to become an active enzyme. Previous X-ray[11,15−17] and NMR[18−20] studies show the protease can adopt a "closed" or "open" conformation. In the closed state that is catalytically active, NS2B is fully tied around NS3 (Figure S4), and becomes part of the active site. In the open and inactive conformation, NS2B is partially bound to NS3 and far from the active site.

We produced a $Gly_4$-Ser-$Gly_4$ linked[11] and binary[21] form of recombinant ZIKV protease (ZVpro), containing NS2B (47−95) and NS3 (1−170). ∼1,200 compounds in our laboratory that were synthesized targeting histone modifying enzymes including lysine specific demethylase 1 (LSD1)[22] were screened against the linked-ZVpro. Compounds **1** and **2** were identified to be novel inhibitors with $IC_{50}$ of 21.7 and 3.1 $\mu$M (Table 1).

Scheme 1 shows the general synthesis for medicinal chemistry studies. 6-Chloro-2-aminopyrazine (**10**) was selectively iodized using *N*-iodosuccinimide, and the 2-amino group was converted to a hydroxyl, which was alkylated using a Mitsunobu reaction to give **12** with a protected piperidin-4-yl-methoxy group. Two selective Suzuki reactions were performed to introduce different aryl groups $R^5$ and $R^6$ to produce, after deprotection, compounds **3**−**5**, **7** and **9**. Monosubstitution of 1,6-dibromo-pyridine or -pyrazine (**15**) with (*N*-Boc-piperidin-4-yl)methylamine, followed by iodination produced the intermediate **16**. Selective replace-

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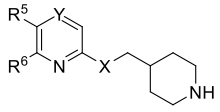
**Table 1. Structures and Activity of Compounds 1−9**

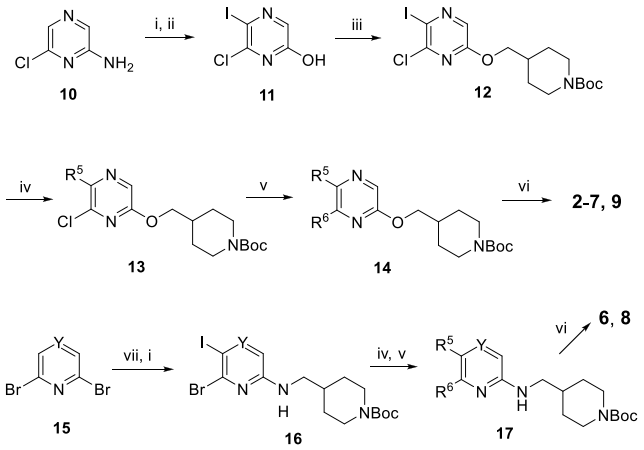



For **1-5**, **7**, **9**: X = O, Y = N
For **6**: X = NH, Y = CH
For **8**: X = NH, Y = N

| Cpd | $R^5$ | $R^6$ | linked-ZVpro $IC_{50}$ ($\mu$M) | ZIKV-FLR $EC_{68}$ ($\mu$M) |
|---|---|---|---|---|
| **1** | 4-Br-Ph | 4-Br-Ph | 21.7 ± 1.5 | >10[a] |
| **2** | 4-*t*-Bu-Ph | 4-*t*-Bu-Ph | 3.14 ± 0.09 | 5.0 |
| **3** | 4-($NH_2CH_2$)-Ph | Indol-5-yl | 1.12 ± 0.07 | 2.50 |
| **4** | 4-($NH_2CO$)-Ph | 4-(furan-3-yl)-Ph | 1.05 ± 0.07 | 2.50 |
| **5** | 4-($NH_2CH_2$)-Ph | 4-(pyrazol-4-yl)-Ph | 0.71 ± 0.07 | 2.50 |
| **6** | 4-($NH_2CH_2$)-Ph | 4-(furan-3-yl)-Ph | 0.79 ± 0.03 | 1.20 |
| **7** | 4-($MeNHCH_2$)-Ph | 4-(furan-3-yl)-Ph | 0.53 ± 0.06 | 1.20 |
| **8** | 4-($NH_2CH_2$)-Ph | 4-(furan-3-yl)-Ph | 0.40 ± 0.05 | 1.20 |
| **9** | 4-($NH_2CH_2$)-Ph | 4-(furan-3-yl)-Ph | 0.20 ± 0.01 | 0.30−0.60 |

[a]$EC_{68}$ cannot be determined because **1** showed cytotoxicity at >10 $\mu$M. All other compounds had no significant cytotoxicity.

**Scheme 1. General Synthesis for Compounds 2−9**[a]




[a]Reagents and conditions: (i) *N*-Iodosuccinimide, DMSO; (ii) $NaNO_2$, $H_2SO_4$(Conc.); (iii) *N*-Boc-piperidin-4-ylmethanol, $PPh_3$, diisopropyl azodicarboxylate, THF; (iv) $R^5$-boronic acid, $Pd(PPh_3)_4$, $Na_2CO_3$, 1,4-dioxane-$H_2O$, 80 °C; (v) $R^6$-boronic acid, $Pd(PPh_3)_4$, $Na_2CO_3$, 1,4-dioxane-$H_2O$, 110 °C; (vi) 4 M HCl, $CH_2Cl_2$, 0 °C; (vii) (*N*-Boc-piperidin-4-yl)methylamine, $K_2CO_3$, DMF, 100 °C.

ment of the 5-iodo and 6-bromo substituent using a Suzuki reaction gave compound **6** or **8**.

Compound **9** was found to be a potent inhibitor of the linked- and binary-ZVpro with $IC_{50}$ of 200 and 220 nM, respectively (Figure S5). Tables 1 and S1 summarize the inhibitory activities of selected analogs **3**-**8**. Changing the −O− linkage at 2-position to an −NH− in **8** ($IC_{50}$: 400 nM) resulted in a 2-fold activity reduction. Changing the central pyrazine ring in **8** to a pyridine in **6** ($IC_{50}$: 790 nM) further reduced the potency. As compared with **7** ($IC_{50}$: 530 nM) with a *N*-methyl secondary amine or **4** ($IC_{50}$: 1.1 $\mu$M) with an amide at the 5-position, the primary amine in **9** is more favored. Changing the furan-3-yl group in **9** to a pyrazol-4-yl in **5** ($IC_{50}$: 710 nM) or a fused pyrrole ring in **3** ($IC_{50}$: 1.1 $\mu$M) also decrease the inhibitory activity.

Compounds **3**−**9** also inhibited DENV serotype-2, -3, and West Nile protease (DV2pro, DV3pro and WVpro)[15,16] with $IC_{50}$ values of 120−1340 nM (Table 2). However, **9** exhibited negligible activities against several human serine-, cysteine-, aspartic- and metallo-proteases (Table S2). These

**Table 2. $IC_{50}$ ($\mu$M) of 3-9 against Flavivirus Proteases**

| | linked-ZVpro | DV2pro | DV3pro | WVpro |
|---|---|---|---|---|
| **3** | 1.12 ± 0.07 | 0.64 ± 0.03 | 0.54 ± 0.01 | 0.93 ± 0.03 |
| **4** | 1.05 ± 0.07 | 0.98 ± 0.02 | 0.93 ± 0.02 | 1.34 ± 0.01 |
| **5** | 0.71 ± 0.07 | 0.21 ± 0.04 | 0.20 ± 0.07 | 0.12 ± 0.02 |
| **6** | 0.79 ± 0.03 | 0.86 ± 0.01 | 0.86 ± 0.02 | 1.27 ± 0.03 |
| **7** | 0.53 ± 0.06 | 0.73 ± 0.02 | 0.53 ± 0.01 | 0.87 ± 0.02 |
| **8** | 0.40 ± 0.05 | 0.29 ± 0.06 | 0.21 ± 0.07 | 0.51 ± 0.04 |
| **9** | 0.20 ± 0.01 | 0.59 ± 0.02 | 0.52 ± 0.06 | 0.78 ± 0.02 |

results show **9** is a broadly active inhibitor of Flavivirus proteases with a high selectivity.

We determined X-ray structures of DV2pro in complex with compounds **5**, **8** and **9** at 2.7−3.0 Å. Statistics for diffraction data and structure refinement are shown in Table S3. The three structures are very similar to each other, with each asymmetric unit containing two inhibitor-bound proteins (Figure 1a−c, Figure S6 and S7). Similar to the apoprotein,[16] the DV2pro-inhibitor complexes adopt an open conformation, with NS2B binding partially to NS3 (Figure S8a). The inhibitor-bound NS3 does not deviate significantly from the apo- or substrate-bound protein, except that the residues 152−164 are disordered (with no observed electron density) upon inhibitor binding. In contrast, the U-shaped peptide segment is well organized in both the apo- and substrate-bound NS3 (Figure S8b,c). In the latter case, residues 152−164 constitute part of the S1 and S2 pockets of the active site and have interactions with the substrate.[15] Inhibitor binding pushes the loops 71−75 and 117−122 outward by ∼1.3 and 3 Å (Figure S8d). All of these movements remodel the surface of NS3 and create a deep, L-shaped pocket (Figures 1d and S8e) that accommodates the inhibitors. The compounds are allosteric inhibitors, which do not occupy the substrate binding site (Figure 1e). Mechanistically, these inhibitors bind to and stabilize DV2pro in the open conformation, which prevents NS2B from folding into the active site as well as the binding of the substrate.

The inhibitor-protein interactions are illustrated in Figures 1d,f and S9. The central pyrazine ring of **9** is located at the junction of the L-shaped pocket. The furanylphenyl group is deeply inserted into the pocket with favorable hydrophobic interactions. The 2- and 5-substituents occupy a deep surface groove, having mostly hydrophobic interactions. The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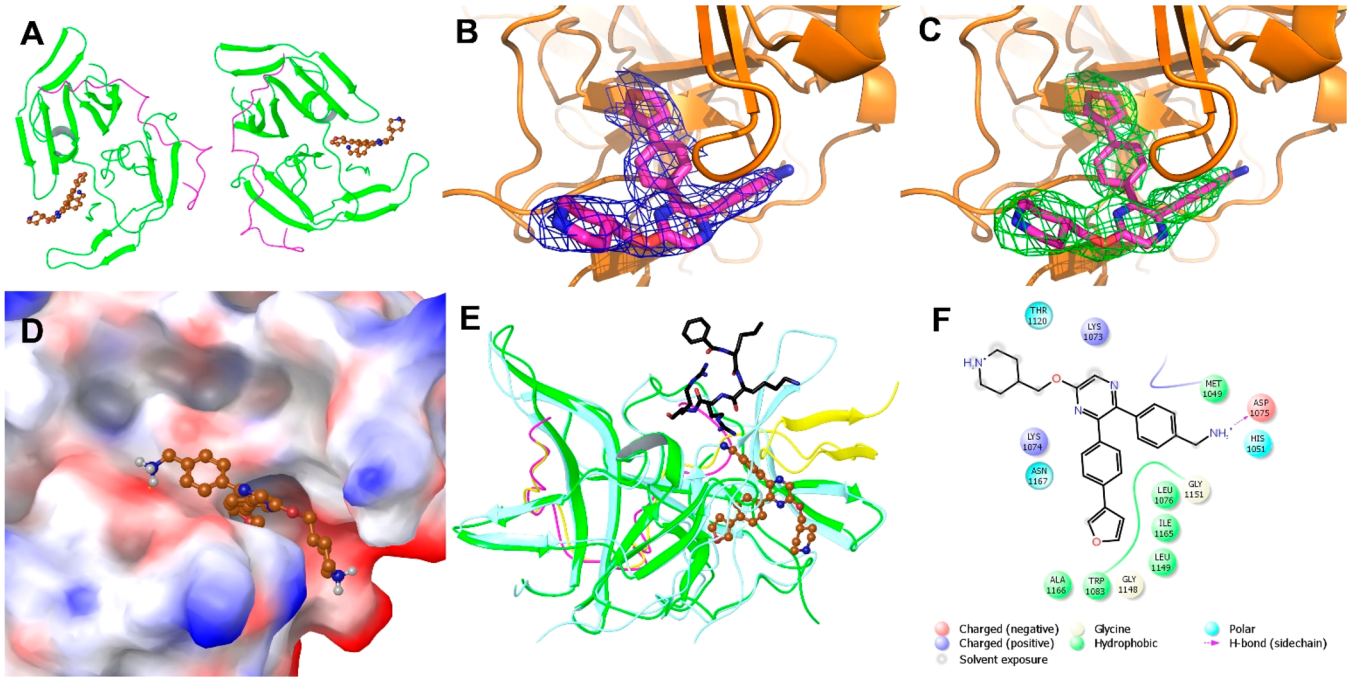


**Figure 1.** X-ray structure of the DV2pro-**9** complex. (**A**) The overall structure of DV2pro in complex with compound **9** (ball and stick model with C atoms in brown). NS3 is shown in green and NS2B in purple; (**B**) The $2F_o$-$F_c$ electron density map of DV2pro-**9** at the inhibitor binding site (contoured at 1$\sigma$) and (**C**) the Fo-Fc omit map (at 3$\sigma$); (**D**) Compound **9** is located in an L-shaped, deep pocket of NS3 (shown as an electrostatic surface) that is mostly hydrophobic; (**E**) Aligned structures of DV2pro-**9** with the substrate-bound DV3pro (PDB: 3U1I, NS3 in cyan and NS2B in yellow), showing **9** occupies a different binding site from the substrate (tube model with C atoms in black); (**F**) Interactions between compound **9** and DV2pro.

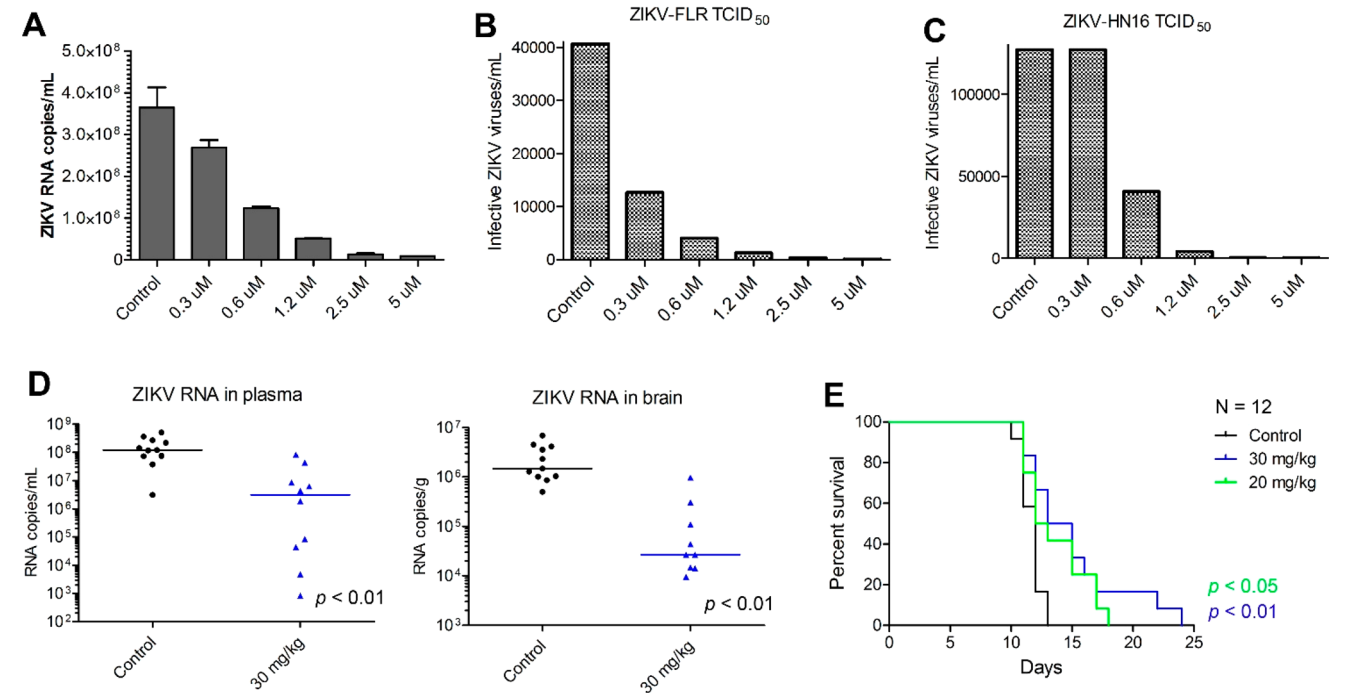


**Figure 2.** Cellular and in vivo antiviral activities of compound **9**. (**A**–**C**) Treatment of U87 cells with **9** caused dose-dependent reduction of (**A**) RNA copies of ZIKV (FLR strain), (**B**) infectious ZIKV (FLR strain) and (**C**) infectious ZIKV (HN16 strain). (**D**) Treatment with compound **9** significantly reduced ZIKV RNA in plasma (left) and brains (right) of ZIKV-infected mice. (**E**) Treatment with compound **9** significantly prolonged the survival of ZIKV-infected mice.

positively charged $-NH_2$ of **9** has hydrogen-bond and electrostatic interactions with Asp75, one of the protease catalytic triad.

High structural and sequence similarities between DV2pro and ZVpro, particularly for the inhibitor-interacting residues (Figure S10a,b), suggest compound **9** binds to ZVpro



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similarly. Enzyme kinetics studies showed that **9** is a noncompetitive inhibitor of DV2pro (Figure S10c), consistent with its X-ray structure. Similar enzyme kinetics results support **9** is also an allosteric inhibitor of ZVpro (Figure S10d).

Anti-ZIKV activity of **9** was evaluated in U87 glioma cells.[23,24] The passage-3 stock of ZIKV FLR strain[25] was used for clinical relevance. Upon infection with ZIKV-FLR, U87 cells were incubated with **9** for 48 h. Newly generated ZIKV viruses in the media were determined quantitatively. Compound **9** significantly reduced ZIKV RNA in a dose-dependent manner (Figure 2a). Because ~$1/10^4$ of RNAs represent infectious viruses,[25] an end-point dilution assay was used to determine viral titers more accurately. Half-log (0.32×) serial dilutions of the media were added to Vero cells in quadruplicate. Upon incubation for 7 days, ZIKV infection in each sample was determined with cytopathic effects. $TCID_{50}$ (tissue culture infective dose) was calculated based on the highest dilution in which ≥50% of the quadruplicate samples were infected with ZIKV. As shown in Figure 2b for a representative experiment, compound **9** reduced infectious ZIKV viruses by 68% at 300 nM, 90% at 600 nM, 97% at 1.2 $\mu$M, 99% at 2.5 $\mu$M, and 99.7% at 5 $\mu$M. Multiple experiments showed that $EC_{68}$ of **9** was 300 or 600 nM. Compound **9** exhibited similar antiviral activities against ZIKV HN16 strain[26] (Figure 2c). **9** also showed significant activity against DENV-2 (strain K0049),[27] inhibiting viral replication in Vero cells by 97% at 5 $\mu$M. These results demonstrate compound **9** has potent cellular antiviral activity against Zika and dengue viruses. Moreover, cellular antiviral activities of compounds **1**–**9** are generally correlated with their biochemical activities against ZVpro (Table 1). Compound treatment also dose-dependently inhibited the viral proteins capsid, NS3 and NS5 in infected Vero cells (Figure S11). These results support ZVpro is the cellular target of these compounds.

In vivo anti-ZIKV activity of **9** was evaluated in C57BL/6 mice with both interferon-$\alpha/\beta$ and -$\gamma$ receptor genes knocked out.[28] We found that intraperitoneal (ip) injection of 100 $TCID_{50}$ of ZIKV-FLR caused rapid viral replication and death of the mice in ~10 days. Started 1 h before inoculation of ZIKV, ip treatment with **9** (15 mg/kg/12 h) for 24 h (when ZIKV replication is in a rapidly growing phase) reduced ZIKV RNA copies in both plasma and brains of the mice by 96% and 98% (Figure 2d). Treatment at 30 and 20 mg/kg/day for 3 days significantly prolonged the survival of ZIKV-infected mice, with the average values for the control, 20 and 30 mg/kg groups ($N$ = 12) being 11.7, 13.7 and 15.1 days (Figure 2e). These results show that **9** can effectively inhibit ZIKV replication in vivo.

In summary, compound **9** is a broadly active inhibitor of Flavivirus proteases and exhibits significant cellular and in vivo activities against Zika virus. In addition, X-ray studies reveal that it binds to an allosteric pocket of NS3 and provide, for the first time, a druggable pocket of the Flavivirus protease, as contrasted to the shallow active site recognizing polar and positively charged Arg or Lys of the substrate. Rational inhibitor development based on the pharmacological leads and structural platform could lead to compounds with improved potency.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.9b02505.

Experimental details (PDF)

## ■ AUTHOR INFORMATION


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### Author Contributions

⊥These authors contributed equally.

### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

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## Previous, Current and Pending Support

**SONG, Yongcheng (PI)**

Previous (within the past 5 years)

R01NS080963 (PI: Song)
Agency: NIH/NINDS
Title: Chemical Probes Targeting Gliomas with IDH Mutation
The major goals of this project are to use structure guided medicinal chemistry and biological activity testing to develop novel inhibitors of mutant IDH1 and use these compounds to probe the biological functions of mutant IDH1 gene in glioma.
Aim 1. Structure-guided medicinal chemistry to develop potent inhibitors of IDH1(R132H).
Aim 2. In vitro activity and ADMET evaluation
Aim 3. In vivo activity evaluation and investigation of mechanism
Role: PI
Dates: 9/1/2012 – 8/31/2018
Level of effort: 25%
Contact: Jane Fountain, fountai@ninds.nih.gov
OVERLAP: There are no scientific or budgetary overlaps.
Note: This grant has closed during Year-1.

RP140469
Agency: Cancer Prevention and Research Institute of Texas (CPRIT)
Title: Chemical Probes Targeting Cancer-Associated Mutation
The major goals of this project are to use structure guided medicinal chemistry and biological activity testing to develop 2-thiohydantoin and other related compounds as novel inhibitors of mutant IDH1.
Aim 1 is to use structure-based inhibitor design, medicinal chemistry, X-ray crystallography and QSAR (quantitative structure activity relationship) to develop two classes of novel inhibitors of IDH1(R132H);
Aim 2 is to evaluate the *in vitro* biological activities of our mutant IDH1 inhibitors as well as the ADMET (absorption, distribution, metabolism, elimination and toxicity) properties of selected compounds;
Aim 3 is to test the *in vivo* anticancer activities of selected compounds and investigate their mechanisms of action, using our clinically relevant mouse models of human GBMs with IDH1(R132H) mutation.
Role: PI
Dates: 9/1/2014 – 2/28/2018
Level of effort: 25%
Contact: Patty Moore, PhD, Senior Program Manager, pmoore@cprit.texas.gov
OVERLAP: There are no scientific or budgetary overlaps.

DAMD W81XWH-13-1-028 (PI: O'Malley)
Agency: DOD/CDMRP
Title: Development of Coactivator-dependent first-in-class therapies for breast cancer
The overall goal of this project is to develop SRC-3 SMIs that will go on to find a place as first-in-class chemotherapeutic agents that, in combination with existing endocrine, targeted and chemotherapeutic agents, can be used to treat BC patients. Through the combined use of novel preclinical BC models, a deep exploration of the underlying genomic biology, and identified coactivator-related escape mechanisms associated with late recurrence, we will identify accurate predictors and novel therapeutic drug approaches to prevent and overcome the development of late recurrence and hormone-resistant metastases of BCs.
Role: co-Investigator
Dates: 9/1/2013 - 8/14/2018
Level of effort: 8% (Note: it will be reduced to 0% on the start date of the project PR170692)
Contact: Julia M. Huiberts, Email: julia.m.huiberts.ctr@mail.mil

OVERLAP: There are no scientific or budgetary overlaps.
Note: This grant has closed during Year-1.

Promise grant 221410 (PI: O'Malley)
Agency: Susan G. Komen for the Cure
Title: Targeting to Restore Endocrine Therapy Sensitivity in Recurrent Breast Cancers
The overall goals of this project are to develop new treatments for ER-positive, tamoxifen-resistant breast cancer (BC) targeting steroid receptor coactivator (SRC). Dr. Song is a Co-Investigator and will be responsible for medicinal chemistry, SAR/QSAR and ADMET studies in Specific Aim 1.
Specific Aim 1: To define the roles of SRC Coactivators (SRC-1 and 3) as "personalized" markers and therapeutic Targets for Recurrent BC.
Specific Aim 2: To interrogate novel escape mechanisms identified at the transcriptional or signaling pathway levels in preclinical models of resistance.
Specific Aim 3: To discover and validate predictive biomarkers associated with late recurrence and hormone therapy-resistant metastasis.
Role: co-Investigator
Dates: 7/1/2012 - 6/30/2015
Level of effort: 8.3%
Contact: Kenda DeLeon-Fanzo, Email: KDeLeonFanzo@komen.org
OVERLAP: There are no scientific or budgetary overlaps.

Collaborative Research Project (PI: David Lonard)
Agency: Adrienne Helis Malvin Medical Research Foundation
Title: SRC-3 small molecule stimulator based treatment for GBM
The goal of this research is to investigate the molecular events underlying SRC-3's role in glioblastoma multiformae and to use SRC-3 small molecule stimulators to treat this form of cancer.
AIM 1: To interrogate the molecular events underlying MCB-613 mediated SRC hyper-stimulation and the consequences of this hyper-stimulation on the cellular homeostasis of pediatric GBM tumor cells.
AIM 2: To assess the therapeutic efficacy of MCB-613 and MCB-613 derivatives in blocking GBM tumor growth using a large panel of patient-derived orthotopic xenograft (PDX) model lines.
Role: Co-Investigator
Dates: 05/01/2015 – 04/31/2018
Level of effort: 0.01%
Contact: Shari Yepez, Email: sharir@bcm.edu
OVERLAP: There are no scientific or budgetary overlaps.

<u>Current</u>

W81XWH-18-1-0368
Agency: DOD/CDMRP
Title: Antiviral Drug Discovery Targeting Zika Virus Protease
The major goals of this project are to use a combination of rational inhibitor design, medicinal chemistry, X-ray crystallography and biological activity testing to discover and develop potent and selective inhibitors of Zika virus (ZIKV) protease (ZVpro), which is essential for the viral replication and a drug target for ZIKV infection. In vivo anti-Zika activity and pharmacodynamics of these compounds will be evaluated.
Aim 1. To perform medicinal chemistry studies to develop potent ZVpro inhibitors.
Aim 2. To perform biochemical and X-ray crystallographic characterization of ZVpro inhibitors.
Aim 3. To perform cellular anti-ZIKV activity and cytotoxicity testing of selected ZVpro inhibitors.
Aim 4. To perform pharmacokinetics/toxicity and in vivo anti-ZIKV activity testing of selected ZVpro inhibitors.
Role: PI
Dates: 7/15/2018 – 7/14/2021
Level of effort: 35%
Contact: Joshua Disbennett, joshua.l.disbennett.civ@mail.mil

RP180177 (PI: Song)
Agency: Cancer Prevention and Research Institute of Texas (CPRIT)
Title: Novel Small Molecule Probes Targeting Histone Acetyltransferase p300/CBP
The major goals of this project are to use a combination of rational inhibitor design, medicinal chemistry, X-ray crystallography and biological activity testing to discover and develop novel inhibitors of histone acetyltransferase p300/CBP. In vivo antitumor activity and pharmacodynamics of these compounds will be evaluated.
Aim 1: We will use rational inhibitor design, medicinal chemistry and structure activity relationship (SAR) studies to develop our novel p300-HAT inhibitors, based on our lead compounds. Execution of this Aim will be in parallel with that of Aims 2 and 3, in which biological activities and X-ray structures of these compounds are to be determined.
Aim 2: We will perform biochemical, X-ray crystallographic and other studies to characterize compounds made in Aim 1, which will be used in Aim 1 to find compounds with improved activity.
Aim 3: We will evaluate cellular biological activities of potent inhibitors of p300-HAT identified in Aims 1 and 2. Activities of potent p300-HAT inhibitors will be tested on histone acetylation and proliferation of several cell models related to p300-HAT. Moreover, activities of these compounds in combination with a p300-Bromodomain inhibitor will be evaluated.
Aim 4: We will perform a series of in vitro and in vivo PK/Tox testing to select chemical probes for in vivo studies. In vivo antitumor activity and pharmacodynamics of these compounds will be evaluated in a systemic SKNO-1 leukemia mouse model.
Role: PI
Dates: 3/1/2018 – 2/28/2021
Level of effort: 25%
Contact: Patty Moore, PhD, Senior Program Manager, pmoore@cprit.texas.gov
OVERLAP: There are no scientific or budgetary overlaps.

RP150129 (PI: Song)
Agency: Cancer Prevention and Research Institute of Texas (CPRIT)
Title: Drug Discovery and Mechanistic Studies of Protein Methylation Targeting Leukemia
The first goal of this project is to use a combination of rational inhibitor design, medicinal chemistry and biological activity testing to develop novel inhibitors of LSD1 as potential therapeutics for acute myeloid leukemia. The second goal is to perform mechanistic studies to investigate the molecular interactions of LSD1 in Runx1-ETO leukemia.
Aim 1: To use rational design and medicinal chemistry to find potent, drug-like LSD1 inhibitors.
Aim 2: To test the biological, pharmacokinetic (PK) and toxicity (Tox) of these compounds in vitro and in vivo.
Aim 3: To use molecular biology and biochemical methods to find LSD1's molecular role in RUNX1-ETO leukemia.
Role: PI
Dates: 3/1/2015 – 2/28/2020
Level of effort: 20%
Contact: Patty Moore, PhD, Senior Program Manager, pmoore@cprit.texas.gov
OVERLAP: There are no scientific or budgetary overlaps.

P01 AI057788 (PI: Estes)
Agency: NIH/NIAID
Molecular Dissection of Norovirus Replication and Pathogenesis to Develop Therapeutics
This program project aims to develop new diagnostics, culture systems and antivirals for noroviruses based on atomic resolution structural data on viral capsids and virus-encoded proteins. Dr. Song is a Co-Investigator of Project 1 and Co-Director for Core C (Development of inhibitors of human NoV protease).
Aim 1. Provide purified proteins and virus-like particles (VLPs) to project investigators.
Aim 2. Synthesize small molecule compounds to be evaluated as inhibitors of the norovirus protease.
Aim 3. Determine binding affinity for protein-protein and protein-ligand binding interactions.
Aim 4. Perform site-directed mutagenesis on genes encoding proteins of interest to facilitate functional studies.
Role: co-Investigator
Dates: 4/15/2010 - 5/31/2020

Level of effort: 13%
Contact: Alarcon, Rodolfo (NIH/NIAID), email: rodolfo.alarcon@nih.gov
OVERLAP: There are no scientific or budgetary overlaps.

DAMD W81XWH-16-1-007 (PI: Zhang)
Agency: DOD/CDMRP
Title: Targeting Breast Cancer Micrometastases: to eliminate the seeds of evil
The overall goal of this project is to find the mechanism of breast cancer metastasis to bone and compounds that can inhibit the metastasis and metastatic breast cancer cells in bone. Dr. Song's role is to use medicinal chemistry to develop lead compounds.
Aim 1. To assess the differential responses of bone micrometastases to adjuvant therapies as compared to their parental tumors in mammary glands and dissect how the difference is attributable to the interaction with the microenvironment niche.
Aim 2. To establish the bone-in-culture array (BICA) platform, which aims to faithfully recapitulate the molecular profile, cell-biological behaviors, microenvironment niche, and therapeutic responses of bone micrometastases in vivo, and is amenable to medium-to-high throughput drug discovery/screening.
Aim 3. To identify and mechanistically investigate therapies against bone micrometastases by analyzing the omics data obtained from previous goals, and by screening pre-established libraries of FDA-approved drugs or small molecule inhibitors (SMIs).
Role: co-Investigator
Dates: 3/15/2016 - 3/14/2021
Level of effort: 5%
Contact: Jamie Shortall, jamie.a.shortall.civ@mail.mil
OVERLAP: There are no scientific or budgetary overlaps.

R33AI122418 (PI: Q. Feng)
Agency: NIH/NIAID
Title: An epigenomic approach to reactivate latent HIV
Although Combination Antiretroviral Therapy is very potent in suppressing HIV replication and therefore life-prolonging, it can't eradicate HIV infection. HIV can persist in long-lived resting memory CD4+ T cells. This reservoir of latent HIV proviruses is the principal impediment to eradication of HIV infection. The goal of this project is to uncover new mechanisms of regulation of HIV latency at the epigenomic level, and to develop a novel class of epigenetic compounds for reactivation of the latent HIV.
Aim 1. To dissect the mechanisms of how HIV transcription is regulated by histone modifications including H3K27ac and H3R26me2.
Aim 2. To determine the synergistic effect of CARM1 inhibitor 7g and other latency reversing agents (LRAs) in multiple latency models, including a primary CD4+ T cell model.
Aim 3. To synthesize and characterize CARM1 inhibitors with higher specificity and affinity.
Aim 4. To test CARM1 inhibitors on resting CD4+ T cells isolated from cART-treated HIV patients.
Role: co-Investigator
Dates: 12/01/2017-11/30/2020
Level of effort: 1%
Contact: Roger Miller, rmiller@niaid.nih.gov
OVERLAP: There are no scientific or budgetary overlaps.
Note: This was a new subcontract during Year-1.

<u>PENDING</u>

R01CA247927-01
Agency: NIH/NCI
Title: Novel Small-Molecule Probes Targeting Critical Protein-Protein Interactions in MLL-rearranged Leukemia

The major goal of this project is to use a combination of rational inhibitor design, medicinal chemistry, biochemical and biophysical studies to develop the first potent inhibitors of the AHD domain of AF9/ENL, whose interaction with DOT1L or AF4 is critical to MLL-rearranged leukemia. Cellular and in vivo anti-leukemia activities of selected compounds will be evaluated.
Aim 1: Rational inhibitor design and medicinal chemistry will be used to develop inhibitors with improved potency. Structure activity relationship analysis will be applied to guide inhibitor design.
Aim 2: Biochemical assays, X-ray crystallography and NMR studies will be used to characterize AF9 AHD inhibitors.
Aim 3: Cell-based assays will be performed to test biological activities of selected potent inhibitors.
Aim 4: In vitro and in vivo pharmacokinetics and toxicity will be evaluated for selected AF9 AHD inhibitors to find compounds suited for animal studies, whose antitumor activity will be tested in a mouse model of MLL-rearranged leukemia.
Role: PI
Dates: 9/1/2019 – 8/31/2024
Level of effort: 25%
Contact: LEOTA HALL, hallle@mail.nih.gov
OVERLAP: There are no scientific or budgetary overlaps.

RP200179
Agency: CPRIT
Title: Novel Small-Molecule Probes Targeting Critical Protein-Protein Interactions in MLL-rearranged Leukemia
The major goal of this project is to use a combination of rational inhibitor design, medicinal chemistry, biochemical and biophysical studies to develop the first potent inhibitors of the AHD domain of AF9/ENL, whose interaction with DOT1L or AF4 is critical to MLL-rearranged leukemia. Cellular and in vivo anti-leukemia activities of selected compounds will be evaluated.
Aim 1: Rational inhibitor design and medicinal chemistry will be used to develop inhibitors with improved potency. Structure activity relationship analysis will be applied to guide inhibitor design.
Aim 2: Biochemical assays, X-ray crystallography and NMR studies will be used to characterize AF9 AHD inhibitors.
Aim 3: Cell-based assays will be performed to test biological activities of selected potent inhibitors.
Aim 4: In vitro and in vivo pharmacokinetics and toxicity will be evaluated for selected AF9 AHD inhibitors to find compounds suited for animal studies, whose antitumor activity will be tested in a mouse model of MLL-rearranged leukemia.
Role: PI
Dates: 3/1/2020 – 2/28/2024
Level of effort: 25%
Contact: Patty Moore, PhD, Senior Program Manager, pmoore@cprit.texas.gov
OVERLAP: There are no scientific or budgetary overlaps.

**RICO-HESSE, R.** (Co-Investigator)

PREVIOUS (within the past 5 years)

R01 AI098715-05 (PI: Rico-Hesse)
Agency: NIH/NIAID
Title: Therapy of Dengue with Modified Antibodies in Humanized Mice
This is a project to test three genetically modified neutralizing monoclonal antibodies (mAbs), for efficacy in treating the clinical signs of DF or DHF in humanized mice, caused by all four DV (1-4) serotypes. The specific aims are: 1. Antibody production. The fusion loop mAb E60 has already been humanized, Fc modified, and tested in an immunocompromised mouse model with DV2 infection. Two previously described mouse mAbs (1A1D-2 and 4E11) will be humanized, using chimerization and CDR-grafting techniques and produced in therapeutic levels and quality. 2. Antibody therapy in a mouse model of dengue fever. Each of the mAbs will be tested individually for reduction of clinical signs of DF in humanized mice infected with 4 DV2 strains

representing 4 genotypes, and DV1, DV3 and DV4 representatives. Three test doses of each mAb will be given at 3 days post-infection, as previously demonstrated to be effective in other mouse models, or day 7, as when peak viremia occurs in our mouse model and humans after mosquito bite. 3. Antibody therapy in a cocktail. We will test cross-protection by a cocktail of two selected mAbs (hu-E60 + hu-1A1D2 or hu-E60 + hu-4E11), against all 4 serotypes of DV.
Role: PI
Dates: 06/05/2012 - 05/31/2017
Level of effort: 41.7%
Contact: Cristina Cassetti, ccassetti@niaid.nih.gov
OVERLAP: There are no scientific or budgetary overlaps.

R01 AI099483-05 (PI: Rico-Hesse)
Agency: NIH/NIAID
Title: Mosquito Saliva in Dengue Virus Pathogenesis
This is a 5-year project to study the effect of mosquito saliva on dengue virus pathogenesis.
The specific aims are: 1). Definition of determinants of dengue pathogenesis in humanized mice, including pinpointing specific genome regions involved in viral replication and tropism, measurement of human antibodies and cellular immunity after mosquito infection, and the possibility of inducing typical DHF, with hemorrhagic signs of disease, after serial infection; and 2). Measure the effect of specific saliva components on pathogenesis, using injection of recombinant saliva proteins and deletion or knockout of some of these saliva proteins, in engineered mosquitoes.
Role: PI
Dates: 12/12/12-11/30/18
Level of effort: 41.7%
Contact: Mark Challberg, mchallberg@niaid.nih.gov
OVERLAP: There are no scientific or budgetary overlaps.
Note: This grant has closed during Year-1.

CURRENT
W81XWH-18-1-0368
Agency: DOD/CDMRP
Title: Antiviral Drug Discovery Targeting Zika Virus Protease
The major goals of this project are to use a combination of rational inhibitor design, medicinal chemistry, X-ray crystallography and biological activity testing to discover and develop potent and selective inhibitors of Zika virus (ZIKV) protease (ZVpro), which is essential for the viral replication and a drug target for ZIKV infection. In vivo anti-Zika activity and pharmacodynamics of these compounds will be evaluated.
Aim 1. To perform medicinal chemistry studies to develop potent ZVpro inhibitors.
Aim 2. To perform biochemical and X-ray crystallographic characterization of ZVpro inhibitors.
Aim 3. To perform cellular anti-ZIKV activity and cytotoxicity testing of selected ZVpro inhibitors.
Aim 4. To perform pharmacokinetics/toxicity and in vivo anti-ZIKV activity testing of selected ZVpro inhibitors.
Role: Co-Investigator
Dates: 7/15/2018 – 7/14/2021
Level of effort: 10%
Contact: Joshua Disbennett, joshua.l.disbennett.civ@mail.mil

PENDING

2 R01 AI099483-06 (PI: Rico-Hesse)
Agency: NIH/NIAID $396,509
Title: Mosquito Saliva in Dengue Virus Pathogenesis
This is a 4-year, continuing project to study the effect of mosquito saliva on dengue, Zika and chikungunya virus pathogenesis. The specific aims are: 1) Produce 6 recombinant mosquito saliva proteins with proper glycosylation. 2) Measure the immunogenicity of 6 mosquito saliva proteins in DRAG humanized mice that contain human innate and adaptive immune system components; and 3). Test the effect of individual saliva

proteins on arbovirus (DENV, ZIKV, CHIKV) infection and pathogenicity, by addition (injection with virus) and subtraction (infection via KO mosquito bite).
Role: PI
Dates: 07/01/19 - 06/30/23
Level of effort: 30%
Contact: Mark Challberg, mchallberg@niaid.nih.gov
OVERLAP: There are no scientific or budgetary overlaps.

1 R01 AI146942-01 (PI: Rico-Hesse)
Agency: NIH/NIAID $495,659
Title: Human immunity to mosquito-bite delivery of dengue and Zika viruses
This project has three aims: Produce 6 recombinant mosquito saliva proteins with proper glycosylation; measure the human immunogenicity of these 6 mosquito saliva proteins in BLT humanized mice that contain human innate and adaptive immune system components; and test the effect of individual saliva proteins on arbovirus (DENV2, ZIKV) infection and pathogenicity, by addition (injection with virus) and subtraction (infection via KO mosquito bite).
Role: PI
Dates: 7/01/2019 - 6/30/2023
Level of effort: 30%
Contact: Mark Challberg, mchallberg@niaid.nih.gov
OVERLAP: There are no scientific or budgetary overlaps.

1 R21 AI144858-01-01 (PI: Rico-Hesse)
Agency: NIH/NIAID $150,000
Title: Zika virus innate immune responses in the prostate
The long-term goal is to define ZIKV urogenital tract tropism and viral persistence mechanisms. The two specific aims are: Identify male urogenital tract cell types permissive to ZIKV infection and assess resulting viability, and characterize ZIKV innate immune response in permissive male urogenital tract cells.
Role: PI
Dates: 7/01/2019 - 6/30/2021
Level of effort: 10%
Contact: Mark Challberg, mchallberg@niaid.nih.gov
OVERLAP: There are no scientific or budgetary overlaps.