PI: **Domashevskiy, Artem**	Title: Development of a Novel Inhibitor of Ricin: A Potential Therapeutic Lead against Deadly Shiga and Related Toxins	
Received: 06/22/2016	FOA: PA16-200	Council: 01/2017
Competition ID: FORMS-D	FOA Title: Academic Research Enhancement Award (Parent R15)	
1 R15 AI124044-01A1	Dual:	Accession Number: 3950045
IPF: 1605024	Organization: JOHN JAY COLLEGE OF CRIMINAL JUSTICE	
Former Number:	Department: Sciences	
IRG/SRG: MSFB	AIDS: N	Expedited: N
Subtotal Direct Costs (excludes consortium F&A) Year 1: [redacted]	Animals: N Humans: N Clinical Trial: N Current HS Code: 10 HESC: N	New Investigator: N Early Stage Investigator: N
Senior/Key Personnel:	*Organization:*	*Role Category:*
Artem Domashevskiy Ph.D	Research Foundation of CUNY o/b/o John Jay College	PD/PI

OMB Number: 4040-0001
Expiration Date: 06/30/2016

APPLICATION FOR FEDERAL ASSISTANCE
SF 424 (R&R)

3. DATE RECEIVED BY STATE	**State Application Identifier**

1. TYPE OF SUBMISSION*

❍ Pre-application ❍ Application ● Changed/Corrected Application

4.a. Federal Identifier [redacted]

b. Agency Routing Number

2. DATE SUBMITTED	**Application Identifier**	**c. Previous Grants.gov Tracking Number**
		GRANT12199564

5. APPLICANT INFORMATION **Organizational DUNS*:** 6201288630000

Legal Name*: Research Foundation of CUNY o/b/o John Jay College
Department:
Division:
Street1*: [redacted]
Street2:
City*: [redacted]
County:
State*: [redacted]
Province:
Country*: [redacted]
ZIP / Postal Code*: [redacted]

Person to be contacted on matters involving this application
Prefix: First Name*: Artem Middle Name: Last Name*: Domashevskiy Suffix: Ph.D
Position/Title: Assistant Professor
Street1*: [redacted]
Street2: [redacted]
City*: [redacted]
County:
State*: [redacted]
Province:
Country*: [redacted]
ZIP / Postal Code*: [redacted]
Phone Number*: [redacted] Fax Number: Email: [redacted]

6. EMPLOYER IDENTIFICATION NUMBER *(EIN) or (TIN)** [redacted]

7. TYPE OF APPLICANT* H: Public/State Controlled Institution of Higher Education

Other (Specify):
Small Business Organization Type ❍ Women Owned ❍ Socially and Economically Disadvantaged

8. TYPE OF APPLICATION*

❍ New ● Resubmission
❍ Renewal ❍ Continuation ❍ Revision

If Revision, mark appropriate box(es).
❍ A. Increase Award ❍ B. Decrease Award ❍ C. Increase Duration
❍ D. Decrease Duration ❍ E. Other *(specify)* :

Is this application being submitted to other agencies?* ❍Yes ●No What other Agencies?

9. NAME OF FEDERAL AGENCY*	**10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER**
National Institutes of Health	TITLE:

11. DESCRIPTIVE TITLE OF APPLICANT'S PROJECT*
Development of a Novel Inhibitor of Ricin: A Potential Therapeutic Lead against Deadly Shiga and Related Toxins

12. PROPOSED PROJECT		**13. CONGRESSIONAL DISTRICTS OF APPLICANT**
Start Date*	Ending Date*	NY-010
04/01/2017	03/31/2020	

SF 424 (R&R) APPLICATION FOR FEDERAL ASSISTANCE

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix: First Name*: Artem Middle Name: Last Name*: Domashevskiy Suffix: Ph.D
Position/Title: Assistant Professor
Organization Name*: Research Foundation of CUNY o/b/o John Jay College
Department: Sciences
Division:
Street1*: [redacted]
Street2: [redacted]
City*: [redacted]
County:
State*: [redacted]
Province:
Country*: [redacted]
ZIP / Postal Code*: [redacted]
Phone Number*: [redacted] Fax Number: Email*: [redacted]

15. ESTIMATED PROJECT FUNDING

a. Total Federal Funds Requested*	$[redacted]
b. Total Non-Federal Funds*	$0.00
c. Total Federal & Non-Federal Funds*	$[redacted]
d. Estimated Program Income*	$0.00

16.IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?*

a. YES ❍ THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:
DATE:

b. NO ● PROGRAM IS NOT COVERED BY E.O. 12372; OR
❍ PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

17. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. (U.S. Code, Title 18, Section 1001)

● I agree*

** The list of certifications and assurances, or an Internet site where you may obtain this list, is contained in the announcement or agency specific instructions.*

18. SFLLL or OTHER EXPLANATORY DOCUMENTATION File Name:

19. AUTHORIZED REPRESENTATIVE

Prefix: Ms. First Name*: Susy Middle Name: G. Last Name*: Mendes Suffix:
Position/Title*: Director
Organization Name*: Research Foundation of CUNY o/b/o John Jay College
Department: Sponsored Programs
Division:
Street1*: [redacted]
Street2: [redacted]
City*: [redacted]
County:
State*: [redacted]
Province:
Country*: [redacted]
ZIP / Postal Code*: [redacted]
Phone Number*: [redacted] Fax Number: Email*: [redacted]

Signature of Authorized Representative*
Susy Mendes

Date Signed*
06/22/2016

20. PRE-APPLICATION File Name:

21. COVER LETTER ATTACHMENT File Name:1241-Cover_Letter_ADomashevskiy.pdf

424 R&R and PHS-398 Specific Table Of Contents

OMB Number: 4040-0010
Expiration Date: 06/30/2016

Project/Performance Site Location(s)

Project/Performance Site Primary Location

❍ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name: John Jay College of Criminal Justice
Duns Number: [redacted]
Street1*: [redacted]
Street2:
City*: [redacted]
County:
State*: [redacted]
Province:
Country*: [redacted]
Zip / Postal Code*: [redacted]
Project/Performance Site Congressional District*: [redacted]

File Name

Additional Location(s)

Funding Opportunity Number: PA-16-200. Received Date: 2016-06-22T16:33:35.000-04:00

OMB Number: 4040-0001
Expiration Date: 06/30/2016

RESEARCH & RELATED Other Project Information

1. Are Human Subjects Involved?* ❍ **Yes** ● **No**

1.a. If YES to Human Subjects

Is the Project Exempt from Federal regulations? ❍ Yes ❍ No

If YES, check appropriate exemption number: _ 1 _ 2 _ 3 _ 4 _ 5 _ 6

If NO, is the IRB review Pending? ❍ **Yes** ❍ **No**

IRB Approval Date:

Human Subject Assurance Number

2. Are Vertebrate Animals Used?* ❍ **Yes** ● **No**

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? ❍ Yes ❍ No

IACUC Approval Date:

Animal Welfare Assurance Number

3. Is proprietary/privileged information included in the application?* ❍ **Yes** ● **No**

4.a. Does this project have an actual or potential impact - positive or negative - on the environment?* ❍ **Yes** ● **No**

4.b. If yes, please explain:

4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed? ❍ Yes ❍ No

4.d. If yes, please explain:

5. Is the research performance site designated, or eligible to be designated, as a historic place?* ❍ **Yes** ● **No**

5.a. If yes, please explain:

6. Does this project involve activities outside the United States or partnership with international collaborators?* ❍ **Yes** ● **No**

6.a. If yes, identify countries:

6.b. Optional Explanation:

	Filename
7. Project Summary/Abstract*	1236-Abstract_ADomashevskiy.pdf
8. Project Narrative*	1237-Public_Health_Relevance_Statement.pdf
9. Bibliography & References Cited	1238-Bibliography_ADomashevskiy.pdf
10.Facilities & Other Resources	1239-Facilities_and_Other_Resources.pdf
11.Equipment	1240-Equipment_ADomashevskiy.pdf

Funding Opportunity Number: PA-16-200. Received Date: 2016-06-22T16:33:35.000-04:00

Abstract

The PI proposes a high-impact collaborative research project to develop new inhibitors for ribosome inactivating proteins (RIPs), such as ricin and Shiga toxin. Ricin toxin, produced by the castor bean plant, has a nefarious past. Ricin is a well-known homicidal poison and has been used in several bioterrorist attacks. Shiga toxin is a deadly product of enterobacterial *Escherichia coli*. RIPs are RNA *N*-glycosidases that enzymatically remove specific purine residues from the universally conserved sarcin/ricin loop (S/R loop) of large ribosomal RNA, causing cellular death. There are no effective pharmaceuticals for either ricin or Shiga toxin poisoning. The PI proposes using a viral protein (VPg) from turnip mosaic virus that we have shown to inhibit ricin activity *in vitro*. The goals of this proposal are: 1) Establish conditions for VPg-ricin complex formation and the rates of VPg-ricin interactions; 2) Determine minimum VPg peptide that effectively inhibits ricin and Shiga toxins; and 3) Optimize conditions to enhance inhibition of these toxic proteins. This is an innovative activity. Based on the structural and mechanistic similarities of these toxins and previously acquired data showing that VPg peptides inhibit ricin activity *in vitro*, we believe the outcomes of this activity will provide leads for the synthesis of therapeutic peptides. In turn, this will serve as a catalyst for the development of constructively applied solutions for the inhibition of these deadly toxins. Furthermore, this proposal will enhance the infrastructure of research and education at John Jay College, introducing biochemical and biomedical research experiences to underrepresented minority and female students, who would otherwise lack such opportunities. This would allow them to experience a broad spectrum of techniques, and acquire skills such as data analysis used in modern scientific investigations, while developing a vast network of partnerships among scientists from national and international institutions.

Public Health Relevance Statement

Ricin and related Shiga toxins are potentially lethal poisons, which as of yet, have no effective pharmaceutical treatments or preventions. The global problem of these poisons is an increasing threat to public health, directly relating to the mission of the National Institutes of Allergy and Infectious Diseases. Ricin, astoundingly easily acquired, is an ideal toxin for bioterrorism, while the widespread transmission of *E. coli* poisoning is responsible for many severe human illnesses and several fatalities. In the future, it may be possible to utilize these toxins for their public health benefit, by repurposing them to attack specific human and animal diseases.

Facilities and Other Resources

Student Profile
In the last year, John Jay had a total enrollment of 12,969 undergraduates and 1,763 graduate students. Of the 14,732 enrolled students, 20% identify as African American/Black and nearly 41% identify as Hispanic. Nearly 57% of the student body is female. (John Jay College, Office of Institutional Research, Fall 2015).

Institutional Characteristics
John Jay College is designated as both a Minority and Hispanic-Serving Institution by the U.S. Department of Education and as a Primarily Undergraduate Institution by the National Science Foundation. Many of our students are eager to take advantage of opportunities in research and academia. The College offers baccalaureate and advanced degrees in the biomedical and behavioral sciences, e.g. forensic psychology, forensic science, cell and molecular biology, toxicology, etc.

Laboratory
Dr. Domashevskiy (the PI) directs a research laboratory (05.65 & 05.69 New Building) specifically tailored to his research interests. In addition to Dr. Domashevskiy access to a personal computer and printer, John Jay College is equipped with printers for producing large size posters for conference presentations. The College provides centralized computing services through the offices of the Department of Information Technology (DoIT). This Department supports a comprehensive range of computing, networking, file transfer and data communication services, as well as access to computer-related equipment, such as optical scanners, laser printers, etc. DoIT staff provides sophisticated programming and consultation. John Jay currently supports wireless access at several locations throughout its campus.

Office
The PI also has an office, located at 05.66.25 New Building of John Jay College, 524 West 59th St., New York, NY 10019. In 2012, the College completed construction on this new 620,000-square-foot building, 13-story vertical campus, that houses 56 state-of-the-art classrooms, new cyber lounges, computer labs, cutting edge science facilities, Emergency Management and High Rise Simulator labs, conference rooms, an exhibit gallery, a black box theater, great dining facilities and many other educational and administrative features in a 100 percent wireless environment. This office is hardwired to the CUNY system, including the library.

Other Assets
Resources typically found as part of a major liberal arts college are available at John Jay College. The College and CUNY provide state-of-the-art library resources. All faculties have onsite and remote online access to the CUNY+ library system which provides access to the millions of books and thousands of periodicals held by the 18 libraries of CUNY as well as New York City's public libraries. The Lloyd Sealy Library on the campus at John Jay itself holds more than 300,000 books, periodicals, and microforms. The CUNY+ library system provides online access to over 150 online databases including Medline, PsychInfo, Sociological Abstracts, and Anthropological Abstracts, and to thousands of online periodicals. CUNY+ maintains a membership in the Inter-University Consortium for Political and Social Research (ICPSR) and provides access to ICPSR data files without charge to CUNY faculty and advanced students engaged in social science research. CUNY+ maintains affiliations with many New York area libraries and provides an Inter-Library Loan service for books and periodicals that are not available within the CUNY system. In addition to library services, John Jay College also maintains excellent audiovisual facilities, a duplicating and print shop, and a telecommunications system that enables faculty and collaborators to communicate electronically and to retrieve bibliographic sources online.

Student Research Programs
The most developed student research program at John Jay College is the Program for Research Initiatives in Science and Math (PRISM). PRISM engages several hundred students annually in lectures, workshops, and outings centered on fostering interest in professional careers in science. PRISM also organizes a research

training course twice per year and funds more than 40 students per term (fall, spring, and summer) in mentored research relationships with faculty. The program provides travel funding for students and mentors to academic conferences, and supports the acquisition of equipment and supplies related to undergraduate research by PRISM faculty. The program organizes an annual research symposium at which some 40 students give oral or poster presentations to a college-wide audience. With almost two dozen faculty members participating, the program prides itself on *deep mentoring*, fostering multi-year relationships between students and faculty that not only improve retention and graduation, but result in significant scientific achievements for our students. The rate of presentation at national research conferences or co-authorship on peer-reviewed publications in our program far exceeds rates identified in the literature for other research-training programs (**Table 1**).

Table 1. Statistics for student success at John Jay College.

	Thiry at al. (2012) n = 73	Hunter et al. (2006) n = 76	Chaplin *et al.* (1998) n = 47	PRISM n = 101
% Students w/ Academic Presentations	10%	9.2%	17.2%	38.2%
% Students with Publication Authorship	10%	6.6%	NR	28.2%

Founded in the early 2000s, PRISM has been highly effective at improving the success and post-graduate careers of students in sciences. The program has resulted in a 5-fold increase in the number of students moving on to Ph.D./M.D. degrees in math and science fields. This led to recognition in 2011 by the National Science Foundation with a Presidential Award for Excellence in Science, Math, and Engineering Mentoring given by President Barack Obama.

The College also maintains other mentoring programs. The College participates in McNair and NSF-STEP programs for supporting research among STEM students, and a Vera Foundation funded program supports student scholars focused broadly on issues of justice or disparity. In addition, the College operates a central Office of Undergraduate Research to coordinate, disseminate, and strengthen efforts aimed at undergraduate research mentoring, which was also launched with grant funding from the U.S. Department of Education. The Office organizes a week-long Celebration of Student Research and Creativity Event which brings together all students at the College in a series of lectures, poster presentations, performances, and other events that further promote and showcase student research activities at the College.

Speaker Series/Seminars

The College maintains numerous seminar series for the professional development of our students. STEM lecture series are organized at the College both by our science masters programs and by a Ph.D. program housed at the Graduate Center of the City University of New York. Psychology maintains a separate research seminar series specifically for topics in that field. PRISM organizers monthly meetings with professionals who come speak to our students about entry into professional STEM graduate programs. And finally, multi College-wide seminars exist that include biomedical and behavioral health topics such as the changing landscape of drug abuse in the 21st century, the biological effects of lead exposure on transport system in the brain, and the effect of incarceration on major issues of public health to name but a few.

Institutional Scientific Collaborations and Partnerships

John Jay College actively fosters and promotes relationships with Community Colleges in the sciences through the CUNY Justice Academy. These unique partnerships between John Jay and York, Queensborough, Bronx, Borough of Manhattan, and Hostos Community Colleges go beyond traditional articulation programs, in that admitted students are considered to be enrolled both at John Jay and the two-year partner from the point of admission. The program thus engages students in the senior college community at the point of admission, and the College invests significant resources in fostering the growth and successful completion of these students from their first year.

Currently, some 600+ students are in the community college partnership program with John Jay in STEM fields. Given that all of our community college partners are MSIs, and both Hostos and Bronx Community are leading 2-year HSIs, a large portion of these students hail from traditionally underrepresented groups in the STEM fields.

John Jay faculty members also maintain extensive relationships with post-baccalaureate training institutions. Over half of our science faculty and more than two-thirds of our psychology faculty serve as active members of doctoral programs and doctoral dissertation committees at the CUNY Graduate Center. Many of our faculty members maintain active collaborations at doctoral institutions in the metropolitan area including Columbia University, Mount Sinai Medical School, Albert Einstein College of Medicine, New York University, and others. The STEM programs at John Jay have also negotiated formal relationships with the University of Lincoln (U.K.) and a burgeoning EU-funded Erasmus Mundus consortium in which students from these campuses come to our campus to complete their thesis projects.

Early State Investigator Support

John Jay College provides myriad support opportunities for the success of its faculty members. Early stage investigators are of particular interest. The University Chancellor, the College President and the Provost have pledged to fund "The Decade of Science" at CUNY, and have supported the expansion of research programs by offering competitive lab start up packages and increasing the allocation of space to construct new labs. Also, grant holders are able to purchase release time at the less expensive replacement time rate, thus resulting in savings to the grant agencies. The College has provided an additional incentive to match grant purchased course release time. Official start-up packages were first presented in 2006, and offers have increased since then. The College also provides all the necessary support and resources for classes, and holds frequent teaching and training programs to enrich faculty pedagogical approaches, advancement in career, assistance and guidance in the mentorship and supervision of the personnel involved in the proposed activity.

The Office for Advancement of Research (OAR) at John Jay College is dedicated to working with faculty in the pursuit of their research and scholarly goals, and is available to assist faculty in grant-seeking and publication activities. Faculty members are encouraged to consult with OAR on any aspect of these processes. OAR promotes faculty success through several funding programs, including: The Emergency Fund program (support is available for research or other scholarly activities that specifically address a need that is urgent and/or time sensitive in nature), See Money Requests (support pilot or preliminary work necessary to pursue a major study to gather background data toward supporting a major proposal), Proposal Peer Review (funds are available for faculty to obtain external reviews of grant proposals prior to their submission to an agency or funding organization), Open-Access Publication Funding (in an effort to broaden the dissemination of research of John Jay scholars, funds are available to faculty who wish to publish articles in open-access format in major, peer-reviewed, scholarly journals), Book Publication Funding (support faculty authors who are in the process of publishing books), and Enhanced Travel Funding (provides additional travel opportunities to the Department's allocations for travel for faculty to present their research at scientific conferences.

The Program for Research Initiatives for Science and Math (PRISM) at the Department is committed to providing its research faculty, mentoring PRISM students, with annual supplies and equipment funds. Students participating in the PRISM program engage in mentored-supervised research, receive stipend support, and present their work at college, state and countrywide symposiums. PRISM increased student participation over 60%. Thus, John Jay has a firm commitment to increase research productivity.

The PI's collaborators have offered their full support and experience in this activity. Dr. Dixie Goss (Hunter College, CUNY) has offered her laboratory space, her time, and instrumentation to perform fluorescence titration experiments requiring high lamp intensity. Her lab will assist in recombinant protein purifications, as well as kinetic studies. Dr. Vern Schramm (Albert Einstein College of Medicine) has offered his full expertise in the proposed activity, and has supplied our laboratory with recombinant ricin and other proteins used in catalytic assays. Dr. John Latham (Denver University) will devote his time to conduct isothermal calorimetry experiments that will identify important thermodynamic parameters of ricin-inhibitor interactions. Dr. David Jeruzalmi (City College, CUNY) is an expert in X-ray crystallography, and will aid in protein crystallization, diffraction, and data analysis. Dr. Alison O'Brien (Uniformed Services University of

Health Sciences) has gladly agreed to perform *in vivo* experiments that will test our central hypothesis that VPg will inhibit RIP activity of RTA and Shiga toxin in Vero cells. Dr. Nancy Greenbaum (Hunter College, CUNY) has offered her aid in the development of experiments that involve NMR spectroscopy and data analysis. Dr. Kathi Hudak (York University, Ontario, Canada) will provide her expert advice and protocols for primer extension and determination of depurination sites. Drs. Shaneen Singh and Emilio Gallicchio (Brooklyn College) will provide their expertise and knowledge regarding molecular modeling, docking and scoring, and will aid in mapping the binding interactions between RTA and VPg.

The Department of Sciences at John Jay College provides its research faculty with safe facilities to conduct their research that may involve potentially toxic and biohazardous substances. Biosafety cabinets and fume hoods are available in the PI's laboratory. All faculty and students involved in experimental research participate in mandatory annual seminar/training on Hazardous Waste Disposal. Additional seminars on Biohazardous Substances, Radioactivity, etc. are also available to those researchers involved with them. Finally, all key personnel have satisfied RCR and COI training. Experiments involving radioactivity (primer extension assay) will be conducted at Dr. Goss laboratory at Hunter College. The Department of Sciences at John Jay is in the process of acquiring their radioactivity license, expected by the end of 2016. Dr. O'Brien's laboratory at the Uniformed Services University of Health Sciences is fully equipped to handle Shiga toxin and cytotoxic ricin, and will perform all the experiments involving these toxins in live cells.

Equipment at John Jay College

The Department of Sciences at John Jay College comprises three floors in a three-year old state of the art building on 59th Street. Spread across these three floors are 15,405 feet^2 of space dedicated to research laboratories and 29,501 feet^2 dedicated to teaching laboratories. There are 27 biosafety cabinets in the department, 77 fume hoods, three full-sized and two table-top autoclaves, three water purification systems, two glassware facilities, three microscopy suite, each for different types of microscopy (fluorescent, FT-IR, and a scanning electron microscopy facility), and a nuclear magnetic resonance (NMR) facility with a 300MHz magnet. There are three cell culture facilities dedicated as such with additional cell culture work taking place as well.

Major shared instrumentation existing in core shared research space (located 30 feet away PI's laboratory) include five -80°C freezers, one Sorvall floor centrifuge, one Beckman ultracentrifuge, and one X-series ICP-MS. The following equipment exists across three dual-use teaching/research facilities and are available for faculty to use for research: Three GC/FID instruments, one fluorescence spectrometer, one X-ray diffractometer, three HPLC units, four GC-MS units (including one triple quadripole unit), two atomic absorption spectrometers, five GC units, three microplate readers, one LC-MS instrument, three FT-IR spectrometers, one Luminex autoplex analyzer, three Agilent Bioanalyzers, two ABI 7500 real-time PCR units, one Life Technologies 3500 Genetic Analyzer, and six nano-drop 2000C spectrophotometers.

We include here the major equipment inventories of several teaching and research laboratories of individual faculty members:

Dept. of Sciences – Teaching Laboratory Equipment and Instrumentation		
Course	**Description**	**Quantity**
Chemistry	THERMO SCIENTIFIC GENESYS 20 UV SPECTROPHOTOMETER	18
Biology	MICROSCOPE OLYMPUS CHT	24
	MICROSCOPE LEICA STRATA LAB	10
	WALTER STEREO MICROSCOPE	16
	E-GEL IBASE	10
	INVITROGEN E-GEL SAFE IMAGER	4
	MONOLUXHD WIFI MICROSCOPE IPAD	16
Toxicology	TECAN MICROPLATE READER	2
	THERMO TRACE DSQ - MS	1
	THERMO TRACE GC ULTRA	1
	AGILENT HPLC 1100	2
	AGILENT 6890N GC SYSTEM	1
	SHIMADZU UV-2600 UV/VIS SPECTROPHOTOMETER	3
	BIOTAGE TURBOVAP LV EVAPORATION SYSTEM	1
	TRIPLE QUADRIPOLE LCMS SYSTEM - SHIMADZU LCMS-8030 SYSTEM	1
Molecular	HYBRIDIZATION INCUBATOR	1
Biology	REVCO ULTRA LOW FREEZER	1
	PERKIN ELMER/ LUMINEX CS 1000 AUTOPLEX ANALYZER + STAND	1
	AGILENT 2100 BIOANALYZER	2
	ABI GENE AMP PCR 9700	2
	ABI 7500 REAL TIME PCR SYSTEM	2
	LIFE TECHNOLOGIES 3500 GENETIC ANALYZER	1
	AMERSHAM ULTROSPEC 1100 PRO UV SPECTROPHOTOMETER	13

Faculty Mentor	Description of Equipment
Dr. Domashevskiy	AKTA PURE PROTEIN PURIFIER SYSTEM
	HORIBA JOBIN YVON FLUOROMAX-3
	BIORAD HELIOS GENE GUN & PREP STATION
	SHIMADZU UV-VIS SPECTROPHOTOMETER
	GENO GRINDER, TISSUE HOMOGENIZER
	PHAST SYSTEM ELECTROPHORESIS
	EPPENDORF REFRIGERATED MICROFUGE
	WATERS HPLC WITH ABSORBANCE DETECTOR, FLUORESCENCE DETECTOR, CONTROLLER AND AUTOSAMPLER
Dr. Rauceo	REAL TIME PCR DETECTION SYSTEM
	CARL ZEISS DISSECTING MICROSCOPE OR STEREO MICROSCOPE
	EPPENDORF MASTERCYCLER EPGRADIENT S
	EPPENDORF EPGRADIENT S MASTERCYCLER
	QIAGEN TISSUELYSER II
	NANODROP SPECTROPHOTOMETER
Dr. Carpi	TEKRAN 2537B MERCURY VAPOUR ANALYZER
	OCEANAIRE AIR BOSS AC
	MICROSCOPE NIKON ECLIPSE E600
	PERKIN ELMER THERMAL CYCLER 480
	MERCURY TEKRAN VAPUOR ANALYZER 2537A
Dr. Lents	PERKIN ELMER GELIANCE 200
	PERKIN ELMER GELIANCE 600
	EPPENDORF MASTERCYCLER EPGRADIENT
	AB APPLIED BIOSYSTEM 2720 THERMALCYCLER
	TURNER BIOSYSTEMS LUMINOMETER
	AGILENT 2100 BIOANALYZER
	APPLIED BIOSYSTEM 7500 REAL TIME PCR SYSTEM
	AIRCLEAN PCR WORKSTATION 600
	BIORAD CHEMIDOC MP IMAGING SYSTEM
	BIORAD TC20 AUTOMATED CELL COUNTER
	THERMOFISHER NANODROP SPECTROMETER
Dr. Kobilinsky	0700 XES CONTROL X-Ray Unit
	PANASONIC WV-BL200
	DELTASPHERE IN IM2720 CASE
	NIKON MICROSCOPE WITH CAMERA
	NIKON FX-35
	BIORAD BENCHMARK PLUS MICROPLATE SPECTROPHOTOMETER
Dr. Yaverbaum	TOBII TX300 EYETRACKER
Dr. Wallace	AB APPLIED BIOSYSTEM GENEAMP PCR SYSTEM 2400
	310 GENETIC ANALYZER
Dr. Cheng	APPLIED BIOSYSTEMS CYTOMETER - ATUNE
	INVERTED TISSUE CULTURE MICROSCOPE
	EPPENDORF REFRIGERATED MICROFUGE
	SANYO CO2 INCUBATOR

OMB Number: 4040-0001
Expiration Date: 06/30/2016

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Project Director/Principal Investigator

Prefix: First Name*: Artem Middle Name Last Name*: Domashevskiy Suffix: Ph.D

Position/Title*: Assistant Professor
Organization Name*: Research Foundation of CUNY o/b/o John Jay College
Department: Sciences
Division:
Street1*: [redacted]
Street2: [redacted]
City*: [redacted]
County:
State*: [redacted]
Province:
Country*:
Zip / Postal Code*: [redacted]

Phone Number*: [redacted] Fax Number: E-Mail*: [redacted]

Credential, e.g., agency login: [redacted]

Project Role*: PD/PI Other Project Role Category:

Degree Type: PhD Degree Year: 2011

	File Name
Attach Biographical Sketch*:	1235-Biosketch_ADomashevskiy.pdf
Attach Current & Pending Support:	

Tracking Number: GRANT12199590 Funding Opportunity Number: PA-16-200 . Received Date: 2016-06-22T16:33:35.000-04:00

OMB No. 0925-0001 and 0925-0002 (Rev. 10/15 Approved Through 10/31/2018)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Domashevskiy, Artem V.

eRA COMMONS USER NAME (credential, e.g., agency login): [redacted]

POSITION TITLE: Assistant Professor of Biochemistry

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

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
Hunter College, City University of New York	B.A.	01/2006	Chemistry
Graduate Center, City University of New York	M.A.	05/2009	Biochemistry
Graduate Center and Hunter College, CUNY	Ph.D.	06/2011	Biochemistry
John Jay College of Criminal Justice, CUNY	Postdoctoral	08/2012	Biochemistry/Biophysics

A. Personal Statement

I possess the knowledge and expertise required to execute the proposed research project with the motivation, devotion and leadership necessary to see it to a successful end. I have a broad background in biochemistry, molecular biology and biophysics, with specific training in eukaryotic and viral translation initiation, gene and protein expression, kinetic assay analysis and fluorescence. My research involves plant protein toxins known as ribosome inactivating proteins (RIPs), specifically pokeweed antiviral protein (PAP) isolated from common pokeweed plant (*Phytolacca americana*), with the goal of exploring and utilizing its antiviral properties. My current research entails the detailed study of related RIPs: ricin and Shiga toxin. As a PI, I have developed a mature research program involving undergraduate and graduate students. My research is highly collaborative; having laid the groundwork for the proposed research by developing vast networks of relationships with local, national and international researchers. I mentor and supervise the students, stress awareness of timelines for research, foster effective communication, and prepare them to perform research of their own after attaining graduate degrees in their chosen fields. The current application builds upon my previous research.

1. Domashevskiy, A.V., Miyoshi, H. and Goss, D.J. (2012) Inhibition of Pokeweed Antiviral Protein (PAP) by Turnip Mosaic Virus Genome-Linked Protein (VPg). *J. Biol. Chem.* 287, 29729-29738.
2. Domashevskiy, A.V. and Goss, D.J. (2015) Pokeweed Antiviral Protein (PAP), a Ribosome Inactivating Protein: Activity, Inhibition and Prospects. *Toxins (Basel)* 7, 274-298.
3. Domashevskiy, A.V. and Cheng, S.Y. (2015) Thermodynamic Analysis of Binding and Enzymatic Properties of Pokeweed Antiviral Protein (PAP) toward Tobacco Etch Virus (TEV) RNA. *J. Nat. Sci.* 1, e82. ISSN: 2377-2700.
4. Aitbakieva, V.R. and Domashevskiy, A.V. (2016) Insights into the Molecular Antiviral Mechanism of Pokeweed Protein from *Phytolacca americana*. *Biochem. Pharmacol (Los Angel)* 5, 210.

B. Positions and Honors

Positions and Employment

2006-2011 Adjunct Lecturer, Department of Chemistry, Hunter College, New York, NY
2008-2012 Lecturer, Department of Sciences, John Jay College, New York, NY
2011-2012 Adjunct Assistant Professor, Department of Chemistry, Hunter College, New York, NY
2011-2012 Adjunct Assistant Professor, Department of Sciences, John Jay College, New York, NY
2012 Adjunct Assistant Professor, New York Institute of Technology, New York, NY
2012-Present Assistant Professor, Department of Sciences, John Jay College, New York, NY

Other Experience and Professional Memberships

2006-Present Member, American Chemical Society (ACS)
2006-Present Member, Undergraduate Poster Judge, American Society of Biochemistry and Molecular Biology (ASBMB)
2006-Present Member, New York Academy of Sciences
2012-Present Research Poster Judge, New York City Science and Engineering Fair (NYCSEF)
2012-Present Member, Undergraduate Poster Judge, American Plant Biology Society (ASPB)
2013-Present Reviewer, Division of Biological and Earth Sciences, Professional Staff Congress (PSC), CUNY
2014-Present Member, American Phytopathological Society (APS)
2014-Present Member, Biophysical Society
2014-Present Member, The RNA Society
2014 Program Committee Member, Forensic Science Criminalistics Research (FSCR)
2015-Present Reviewer, Toxins
2015-Present Reviewer, Biophysica et Biochimica Acta
2015-Present Editorial Board, Jacobs Journal of Molecular and Translational Medicine
2015-Present Editorial Board, Journal of Pharmaceutical Sciences and Emerging Drugs (JPSEM)
2015-Present Editorial Board, Journal of Forensic Science and Legal Medicine
2015-Present Reviewer, Division of Chemistry and Biochemistry, Professional Staff Congress (PSC), CUNY

Honors

2002-2011 Julianne B. Fusco Scholarship, Dynamite Youth Foundation, Inc. Brooklyn, NY
2006 Helen S. Schectman Award, Hunter College, CUNY, New York, NY
2006-2007 Science Fellowship, Graduate Center, CUNY, New York, NY
2007-2011 CUNY Fellowship, Graduate Center, New York, NY
2015-2016 William Stewart Award, CUNY Academy for the Humanities and Sciences, New York, NY
2016 Henry Wasser Award, CUNY Academy for the Humanities and Sciences, New York, NY

C. Contribution to Science

1. My early research involved investigations into plant viral protein synthesis. Plant viral diseases affect a significant number of food crops world-wide, and can have severe impact on economic conditions and crop yield. Protein synthesis is a key step in viral infection, yet is not well understood. The research involved interactions of translation initiation factors with a set of viral RNAs of varying secondary structures. The findings (preference of the tobacco etch virus RNA to bind eIF4G rather than eIF*iso*4G; viral protein recruits cellular translational components and increases viral protein translation) increased our understanding of plant viral protein synthesis, and how this important biochemical process can be regulated. This information can potentially be used to shut off viral protein synthesis either through genetic engineering or biochemical agents. Furthermore, viral protein synthesis in general is simpler than host protein synthesis, so there is potential to use this research data to develop systems that produce desired proteins which are nutritionally beneficial, antibodies, or have other economic uses. I directed equilibrium and kinetic studies of the experiments, engaged

in recombinant protein purifications and analysis, designed and performed transcriptional and translational assays.

a. Ray, S., Yumak, H., Domashevskiy, A., Gallie, D.R. and Goss, D.J. (2006) Tobacco Etch Virus mRNA Preferentially Binds Wheat Germ Eukaryotic Initiation Factor (eIF)4G rather than (eIF)iso4G. *J. Biol. Chem.* 281, 35826-35834.
b. Goss, D.J., Khan, M.A., Domashevskiy, A.V. and Yumak, H. (2008) Biophysical Insights into the Mechanism of Viral Protein Synthesis. *MARM ACS J.* 40, 254.
c. Goss, D.J. and Domashevskiy, A.V. (2015) Messenger RNA (mRNA): The Link between DNA and Protein. *Encyclopedia of Cell Biology*. Vol. 1 pp. 341-345. Academic Press, Eds. Bradshaw, R.A. and Stahl, P.D.
d. Domashevskiy, A.V. (2016) Turnip Mosaic Virus Genome-Linked Protein May Confer Resistance to Plant Defense Ribosome Inactivating Protein from *Phytolacca americana*. *Virol Mycol.* Submitted; under reviews.

2. Additionally, I have developed synthesis of a novel fluorescent 5'-cap analog (Anthranioyl-m^{7}GpppG), and a novel method for labeling messenger RNA with this fluorescent probe. Nucleic acids have poor intrinsic fluorescence, and, other extrinsic fluorophores must be employed to study nucleic acid molecular dynamics and structure. The molecule, Ant-m^{7}GTP could be specifically incorporated into the cap site to yield Ant-m^{7}GpppG-capped mRNA or oligonucleotide. What makes this extrinsic fluorophore unique and valuable in research is a combination of high synthesis yield, efficiency in RNA capping and unique properties of the fluorophore itself (absorbance and emission maxima of this probe is far removed from native protein fluorescence). This fluorescent probe and the method of capping developed, has a great potential for studies of nucleic acid structure and function, their interactions with proteins, and possibly imaging. The Ant-m^{7}GTP-capped RNA provides an important tool for monitoring capping reactions, translation, and biophysical studies.

a. Gunawardana, D., Domashevskiy, A.V., Gayler, K.R. and Goss, D.J. (2015) Properties of mRNAs containing a novel fluorescent analog: Anthranioyl-m^{7}GpppG. *Translation* 3, e988538.
b. Rodriguez, D. and Domashevskiy, A.V. (2015) Synthesis of Fluorescently-Labeled Tobacco Etch Virus (TEV) RNA and Its Interactions with Pokeweed Antiviral Protein. *FASEB J.* 29:LB222.
c. Rodriguez, D. and Domashevskiy, A.V. (2016) Interactions of a Fluorescently-Labeled Tobacco Mosaic Virus (TMV) RNA with Pokeweed Antiviral Protein (PAP). *FASEB J.* 30:591.1.
d. Domashevskiy, A.V., Rodriguez, D.J., Gunawardana, D., and Goss, D.J. (2016) Preparation of functional, fluorescently labeled mRNA capped with Anthraniloyl-m^{7}GpppG. In Synthetic mRNA: Production, Introduction into Cells, and Physiological Consequences. Ed. Rhoads, R. *Methods Mol. Biol.* 1428, 61-75.

3. A unique aspect of my research experiences are plant protein toxins, known as RIPs. Ricin is a lethal poison that has no treatment or prevention. Other RIPs have been explored for their antiviral and antifungal properties. Pokeweed antiviral protein isolated from pokeweed plant, is a cap-binding protein and an RNA *N*-glycosidase that depurinates ribosomes. PAP exerts potent antiviral properties. The antiviral activity of PAP differs from its ribosome depurinating activity. PAP lowers infectivity of many plant and animal viruses by directly depurinating viral RNA. My research involved interactions of PAP with a small viral genome-linked peptide VPg from Turnip Mosaic Virus. VPg is important in many different viral functions. My findings showed that VPg binds to PAP with high affinity, and it inhibits PAP enzymatic activity. I believe that VPg may be an evolutionary adaptation of the virus against plant defense mechanism – PAP. An evolutionary arms race between plants, synthesizing RIPs, and their pathogens that have evolved to produce a small peptide that inhibits RIP activity, was characterized in these studies. Antiviral properties of PAP are investigated in my laboratory. I am the Principal Investigator on these projects, and have conducted fluorescence studies, produced depurination and catalytic activity assays, HPLC serration and quantification.

a. Aitbakieva, V. and Domashevskiy, A.V. (2015) Characterization of Pokeweed Antiviral Protein (PAP) Isoforms and Comparison of Their Enzymatic Activities toward Tobacco Etch Virus RNA. *FASEB J.* 29, LB130.

b. Williams, S. and Domashevskiy, A.V. (2014) Pokeweed Antiviral Protein Binds to Structures Present in the 3' Untranslated Regions of Viral mRNA. *Annual Biomedical Research Conference for Minority Students*. San Antonio, TX.
c. Cheng, S.Y., Domashevskiy, A.V. and Kobilinsky, L. (2014) The Effect of Eukaryotic Initiation Factors on the Activity of Pokeweed Antiviral Protein. *Society of Toxicology Annual Conference.* Phoenix, AZ.
d. Domashevskiy, A.V. and Goss, D.J. (2013) Overcoming Plant Defense Mechanisms. *American Society of Plant Biologists Annual Conference*. Providence, RI.

4. A novel avenue of my research involves investigation into how male routine infant circumcision (RIC) affects the length and degradation of chromosomal telomeres and development of autism as a direct consequence of RIC, as well as advocacy for rights of children. This is a highly contagious subject in the United States, with much convoluted information. While female circumcision is explicitly forbidden and punishable under the current laws of the United States, male circumcision is not. It is considered a non-therapeutic elective surgery legally performed by surrogate consent, violating the basic human rights of male children and in direct violation of the US Constitution granting equal protection under the law.
   a. Domashevskiy, A.V. and Domashevskiy, J.A. (2016) Ethics Pertaining to the Legalities of Male Routine Infant Circumcision and Surrogate Consent to Non-Therapeutic Surgery. *J. Clin. Res. Bioeth.* Submitted; under reviews.

Complete List of Published Work in My Bibliography:
http://www.ncbi.nlm.nih.gov/sites/myncbi/1p3WpcuErld5c/bibliography/40599058/public/?sort=date&direction=ascending

D. Research Support

Ongoing Research Support

Professional Staff Congress (PSC), Cycle 47 City University of New York internal grant Investigating effects of plant antiviral protein on HIV RNA.	Role: PI	2016-2017

Completed Research Support

Program for Research Initiatives for Science and Math (PRISM) Equipment Grant to purchase BioRad Helios gene Gun.	Role: PI	May 2016
Seed Grant, Office of Advancement of Research (OAR) John Jay College of Criminal Justice Synthesis of a functional, fluorescently-labeled RNA.	Role: PI	08/2015-06/2016
Seed Grant, Office of Advancement of Research (OAR) John Jay College of Criminal Justice Development of a novel inhibitor of ricin.	Role: PI	07/2015-05/2016
Program for Research Initiatives for Science and Math (PRISM) Equipment Grant to purchase AKTA Pure protein purifier system.	Role: PI	June 2015
Program for Research Initiatives for Science and Math (PRISM) Equipment Grant to purchase electroanalytic station (potentiostat).	Role: co-PI	June 2015

Program for Research Initiatives for Science and Math (PRISM) Role: co-PI June 2014
Equipment Grant to purchase Geno/Grinder, a high-throughput put tissue homogenizer.

Seed Grant, Office of Advancement of Research (OAR) Role: PI 02/2014-02/2015
John Jay College of Criminal Justice
Targeting HIV with Liposomal Pokeweed Antiviral Protein.

Professional Staff Congress (PSC), Cycle 44 Role: PI 03/2013 – 06/2014
City University of New York internal grant
Characterization of Enzymatic Mechanism of Pokeweed Antiviral Protein.

Program for Research Initiatives for Science and Math (PRISM) Role: co-PI June 2014
Equipment Grant to purchase a Circular Dichroism Spectrophotometer.

Startup Fund Role: PI 08/2012 – 07/2015
John Jay College, CUNY

OMB Number: 4040-0001
Expiration Date: 06/30/2016

RESEARCH & RELATED BUDGET - SECTION A & B, Budget Period 1

ORGANIZATIONAL DUNS*: [redacted]

Budget Type*: ● Project ❍ Subaward/Consortium

Enter name of Organization: Research Foundation of CUNY o/b/o John Jay College

Start Date*: 04-01-2017 **End Date*:** 03-31-2020 **Budget Period: 1**

A. Senior/Key Person

	Prefix	First Name*	Middle Name	Last Name*	Suffix	Project Role*	Base Salary ($)	Calendar Months	Academic Months	Summer Months	Requested Salary ($)*	Fringe Benefits ($)*	Funds Requested ($)*
1 .		Artem		Domashevskiy	Ph.D	PD/PI	[redacted]		[redacted]	[redacted]	[redacted]	[redacted]	[redacted]

Total Funds Requested for all Senior Key Persons in the attached file

Additional Senior Key Persons: File Name:

Total Senior/Key Person [redacted]

B. Other Personnel

Number of Personnel*	Project Role*	Calendar Months	Academic Months	Summer Months	Requested Salary ($)*	Fringe Benefits*	Funds Requested ($)*
	Post Doctoral Associates						
1	Graduate Students	4.50			[redacted]	[redacted]	[redacted]
3	Undergraduate Students	13.50			[redacted]	[redacted]	[redacted]
	Secretarial/Clerical						
1	Technician	11.82			[redacted]	[redacted]	[redacted]
5	**Total Number Other Personnel**					**Total Other Personnel**	[redacted]
						Total Salary, Wages and Fringe Benefits (A+B)	[redacted]

RESEARCH & RELATED Budget {A-B} (Funds Requested)

Funding Opportunity Number: PA-16-200 . Received Date: 2016-06-22T16:33:35.000-04:00

RESEARCH & RELATED BUDGET - SECTION C, D, & E, Budget Period 1

ORGANIZATIONAL DUNS*: [redacted]

Budget Type*: ● Project ❍ Subaward/Consortium

Organization: Research Foundation of CUNY o/b/o John Jay College

Start Date*: 04-01-2017 **End Date*:** 03-31-2020 **Budget Period: 1**

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

Equipment Item	**Funds Requested ($)***
Total funds requested for all equipment listed in the attached file	
Total Equipment	

Additional Equipment: File Name:

D. Travel	**Funds Requested ($)***
1. Domestic Travel Costs (Incl. Canada, Mexico, and U.S. Possessions)	[redacted]
2. Foreign Travel Costs	
Total Travel Cost	[redacted]

E. Participant/Trainee Support Costs		**Funds Requested ($)***
1. Tuition/Fees/Health Insurance		
2. Stipends		
3. Travel		
4. Subsistence		
5. Other:		
Number of Participants/Trainees	**Total Participant Trainee Support Costs**	

RESEARCH & RELATED Budget {C-E} (Funds Requested)

RESEARCH & RELATED BUDGET - SECTIONS F-K, Budget Period 1

ORGANIZATIONAL DUNS*: [redacted]

Budget Type*: ● Project ❍ Subaward/Consortium

Organization: Research Foundation of CUNY o/b/o John Jay College

Start Date*: 04-01-2017 **End Date*:** 03-31-2020 **Budget Period: 1**

F. Other Direct Costs	Funds Requested ($)*
1. Materials and Supplies	[redacted]
2. Publication Costs	
3. Consultant Services	
4. ADP/Computer Services	
5. Subawards/Consortium/Contractual Costs	
6. Equipment or Facility Rental/User Fees	
7. Alterations and Renovations	
Total Other Direct Costs	[redacted]

G. Direct Costs	Funds Requested ($)*
Total Direct Costs (A thru F)	[redacted]

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)*
1 . Salaries, wages and fringe benefits	[redacted]	[redacted]	[redacted]
		Total Indirect Costs	[redacted]

Cognizant Federal Agency (Agency Name, POC Name, and POC Phone Number) DHHS, Ryan Mccarthy, 212-264-2069

I. Total Direct and Indirect Costs	Funds Requested ($)*
Total Direct and Indirect Institutional Costs (G + H)	[redacted]

J. Fee	Funds Requested ($)*

K. Budget Justification*	File Name: 1234-Budget_Justification_ADomashevskiy rev.pdf (Only attach one file.)

RESEARCH & RELATED Budget {F-K} (Funds Requested)

Budget Justification

A. **Personnel:**

Principal Investigator *(1.28 Acad. Months Years 1-3; 2.0 Summer Months Years 1-3)*

The PI will devote himself to the experimental laboratory training of undergraduate and graduate students, demonstrate techniques, plan experiments, enforce protocols, and provide assistance with data fitting and analysis. He will also be responsible for manuscript preparations and in assisting students with poster presentations, by directing group meetings with his research team where practice presentations are performed. Additionally, these meetings will present training in basic topics of data search and analysis tools, fluorescence, spectroscopy, protein and RNA expression and purification techniques, and poster presentations. The group discussions will include current scientific literature and ethics. One course (3 hours) release and two (2) months of summer salary each year (2/9 of regular academic year salary) are requested for Dr. Domashevskiy. The full-time teaching load at John Jay College is 21 hours (7 courses teaching/year), thus this request equals 1.28 academic months in each year. He will continue to teach biochemistry undergraduate courses and the summer research training programs, but will have gained time adequate for the laboratory work, student training, manuscripts preparation, and other responsibilities that are directly related to this proposal.

Technician *($[redacted]/hr x 15 hrs/wk x 40 weeks) 3.94 Calendar Months each year*

Funds are requested for a part-time technician who will be responsible for assisting with undergraduate student training in techniques and protocols involving RTA, VPg, and other RIP purification, protein and RNA expressions, bacterial growth, and general assistance in overseeing the laboratory, sharing her expertise and knowledge, while continuing her own training in the PI's laboratory. The technician will greatly facilitate preparations, assisting in: ordering supplies, maintenance of chemical inventory, and compliance with safety regulations. Training of the undergraduate and graduate students is the cornerstone of the PI's research plan.

Graduate Student *($[redacted]/hr x 19 hrs/wk x 12 weeks) 1.5 Calendar Months each year*

Undergraduate Students *($[redacted]/hr x 19 hrs/wk x 12 weeks x 3 UG students) 1.5 Calendar Months per UG student each year*

Summer support is requested for four undergraduate students and one Masters student, who will learn protein and RNA expression and purification, basic biochemistry, perform most of the titration experiments, perform depurination and crystallography screening assays and molecular modeling, analyze data, and write preliminary drafts of reports, posters, and manuscripts. The MS student will perform fluorescence titrations, oversee depurination assay design and methods, and perform extrinsic fluorophore protein labeling. All undergraduate students will have a unique part of the proposed Specific Aims assigned to them for investigation.

B. **Fringe Benefits:** Fringe benefits are calculated by the City University of New York (CUNY) and the Research Foundation of CUNY, and are as follows:

- Release Time: 49%
- Summer Salary: 26%
- Part-time B (working 19 hours/week or less) Project Staff: 9.84%
  (A 0.34% MTA tax is included in the calculation for FT/PTA/PTB project staff.)

C. **Equipment:** N/A

D. **Travel:** The PI requests travel funds of $3,686 for the second and $3,470 for the third year of activity. Dr. Domashevskiy and one undergraduate student will attend relevant scientific conferences and will present their research as poster/oral presentations. The conferences planned for the team's attendance will include American Society if Plant Biology (ASPB) (July 14-18, 2018; Montreal, Canada), and Biophysical

Society (February 15-19, 2019; San Diego, CA). Estimated breakdown of the ASPB conference expense (US Department of State; Foreign Per Diem Rates): Plane ticket ($275 x 2 = $550), lodging ($179/night x 4 nights = $716 x 2 = $1,432), meals per Diem ($93 x 4 days = $372 x 2 = $744), local travel ($100 x 2 = $200), conference registration fees ($475 + $85 undergrad registration = $560), abstract submission fee ($60 x 2 = $120), other unexpected expenses ($200) = total $3,806. Estimated breakdown of the Biophysical Society conference expense (2016 Domestic Per Diem Rates): Plane ticket ($477 x 2 = $954), lodging ($153/night x 4 nights = $612 x 2 = $1,224), meals per Diem ($64 x 4 days = $256 x 2 = $512), local travel ($100 x 2 = $200), conference registration fees ($260 + $120 undergrad registration = $380), abstract submission fee ($60 x 2 = $120) other unexpected expenses ($200) = total $3,590.

The PI also requests travel funds for the first and second year of activity for him and one undergraduate student to visit laboratory of a collaborating researcher. During the first year, the team will visit Dr. John Latham (Denver University, CO) to get acquainted with the techniques of isothermal titration calorimetry proposed in the activity, and to learn the protocols necessary to successfully accomplish the activity. Estimated breakdown for this travel expense: (2016 Domestic Per Diem Rates): Plane ticket ($400 x 2 = $800), lodging ($172/night x 2 nights = $314 x 2 = $688), meals per Diem ($69 x 3 days = $138 x 2 = $276), local travel ($200), other unexpected expenses ($200) = total $2,302. During the second year, the team will visit Dr. Allison O'Brien (Uniformed Services University, MD) to get acquainted with the techniques involved in Aim 1C of the activity, and to learn the protocols necessary to successfully accomplish the activity. Estimated breakdown for this travel expense: (2016 Domestic Per Diem Rates): Plane ticket ($200 x 2 = $400), lodging ($226/night x 2 nights = $452 x 2 = $904), meals per Diem ($69 x 3 days = $138 x 2 = $414), local travel ($200), other unexpected expenses ($200) = total $2,118.

There will be some local travel costs between John Jay College, Hunter College, City College, and Albert Einstein College of Medicine. The amount requested for local MTA travel is $300. It is anticipated that some funds will be available from the college. Undergraduate student travel will be supported in part by the Program for Research Initiatives for Science and Math (PRISM), Collegiate Science and Technology Entry Program (CSTEP), and the Louis Stokes Alliance for Minority Participation (LSAMP).

E. **Participant/Trainee Support Costs:** N/A

F. **Other Direct Costs**

Materials and Supplies: The cost estimate is based on the costs incurred during the PI's previous award experiences, taking price increases into consideration, and a small amount that will be available from the department. PI requests $24,000 per year in supplies, reagents, and small equipment below $5,000. The supplies will include media for bacterial growth (Fisher Sci.: Tryptone, #BP1421-2, $660.14; Pepton, #BP1420-2, $487.47; NaCl, #S271-1, $80.71; Agar, #A360-500, $353.93; Sigma-Aldrich: Agarose, A9539-250G, $608.00), plasmid, RNA, and protein purification media kits (Promega: DNA Clean-up wizard, #A9282, $450.00; RNA purification kits, #AS1050, $359.00; Quagen: Plasmid purification kits, #10043, $276.00; DNA extraction kits, #28704, $113.00) and supplies, competent cells (ThermoFisher, Sci.: DH5α, #1825012, $212.00; BL21, C600003, $288.00; Sigma-Aldrich: Vero cells, V0180000, $205.50), restriction enzymes (New England Biolabs: NdeI, #R0111L, $249.00; XmaI, #R0180M, $262.00; StuI, #R0187M, $241.00; DNA and RNA ladders, buffers: N3232L, $208.00; N3231L, $220.00; N0364S, $65.00; #N0362S, $65.00; mutagenesis kit, #E0554S, $185 x 3), antibiotics (Fisher Sci.: Ampicillin, #BP1760-25, $103.76; Chloramphenicol, #BP904-100, $202.45; Kanamycin, #BP2643-1, $101.97), RiboMax Transcription kits and Rabbit Reticulocyte and Wheat Germ Lysates (Promega: SP6, P1280, $328.00; T7, P1300, $328.00; #L4960, $188.00; #L4380, $188.00), protein-protein interaction (ThermoFisher: #82015, $100.00) and protein labeling and cross-linking kits (ThermoFisher Sci.: #A10239, $427.00; #P6305, $306.00; #53031, $298.00), general biochemicals and solvents (Promega: Nuclease-free water, #P1197, $77.00; Sigma-Aldrich: DEPC, #D5758-100ml, $437.00; Fisher Sci.: ethanol, #BP8202-4, $248.25; n-butanol, #BP505-500, $195.47; isopropanol, #A416-4, $208.89; methanol, #A456-4, $168.83; Across Organics: diethal ether, AC32686-0010, $78.28), reagents (Fisher Sci.: EDTA, #S312-500, $370.97 and #BP188-500, $105.95;

DTT, #BP172-25, $536.47; IPTG, #FERR0393, $540.00; protease inhibitor cocktails, PI-78415, $265.55; Sigma-Aldrich: chloroacetaldehyde, #317276-250ml, $41.60; aniline, 242284-500ml, $82.00; TEMED, T7024-50ml, $63.50; $(NH_4)_2S_2O_8$, A3678-100G, $42.40; MP Biomedicals: PMSF, #ICN19538105, $90.22), and buffers (Fisher Sci.: Tris base, T393-500, $209.89; Tris-HCl, PR-H5123, $140.20; HEPES, ICN10559383, $232.20; MOPS, AC172635000, $301.88; MES, AC327765000, $465.99), electrophoresis supplies (Sigma-Aldrich: Acrylamide, A9099-500G, $188.00; bis-Acrylamide, 146072-500G, $97.60; TAE 10x, #574797-1L, $40.20; TBE 10x, 574795-1L, $50.50; SDS, #L3771-500G, 229.00; BioRad buffers, gels, protein standards: #1610374, $125.00 x 2; #1610394S, $24.00; #1610373, $97.00; #1610375S, $135.00; #4561043EDU, $84.00 x 5; #4561045EDU, $84.00 x 5; #1610145EDU, $108.00;), fluorescence and UV-VIS cuvettes (Starna: #9/9-SOG-10, $98.00 x 4; #9B9-SOG-10, $129.00 x 4; #71-Q-10, $411.00; #9F-Q-10, $252.00 x 4; #46F-Q-10, $520.00), pH electrode and buffers (Fisher Sci.: SB115-500, $43.56; SB99-500, $123.34; SB107-500, $41.86; #13-620-133, $491.80; #13-AB1-50AP, $660.25), chromatographic supplies (GE Healthcare: HiTrap CM- #17-5155-01, $309.06; DEAE- #17-5154-01, $309.00, heparin- #17-0406-03, $801.00; and Ni-Sepharose, #17-3712-06, $728.00 Fast Flow columns, ThermoFisher Sci.: C_{18} HPLC columns, #079013, $109.66 x 2; HPLC solvents), crystallography screens (Hampton Research, # HR2-142, $116.00; #HR2-134, $169.95; #HR2-084, $340.00; #HR2-086, $189.00; #HR2-107, $340.00; #HR2-109, $ 340.00; #HR2-130, $189.00), Mettler Toledo balances (Fisher Sci. # 01-910-216, $4,690.00); UV Transilluminator (Fisher Sci. #11-993-16, $1,803.00); Microbiological incubator (Fisher Sci. #15-103-0515, $2,675.00); autoclaving tape and supplies, glassware, miscellaneous chemicals and crystallography reagents and supplies, NMR solvents, crystallography facility fees at City College, NMR facility fees at Hunter College, plasmid sequencing fees.

G. **Direct Costs** (Total): $297,254.27

H. **Indirect Costs:** The Research Foundation of CUNYo/b/o John Jay College of Criminal Justice has a federally-negotiated indirect cost rate agreement with the Department of Health and Human Services, at a rate of 69.5% of personnel including salaries and fringe benefits. Total requested is $148,131.10.

I. **Total Costs**: $445,385.37

RESEARCH & RELATED BUDGET - Cumulative Budget

	Totals ($)	
Section A, Senior/Key Person		[redacted]
Section B, Other Personnel		[redacted]
Total Number Other Personnel	5	
Total Salary, Wages and Fringe Benefits (A+B)		[redacted]
Section C, Equipment		
Section D, Travel		[redacted]
1. Domestic	[redacted]	
2. Foreign		
Section E, Participant/Trainee Support Costs		
1. Tuition/Fees/Health Insurance		
2. Stipends		
3. Travel		
4. Subsistence		
5. Other		
6. Number of Participants/Trainees		
Section F, Other Direct Costs		[redacted]
1. Materials and Supplies	[redacted]	
2. Publication Costs		
3. Consultant Services		
4. ADP/Computer Services		
5. Subawards/Consortium/Contractual Costs		
6. Equipment or Facility Rental/User Fees		
7. Alterations and Renovations		
8. Other 1		
9. Other 2		
10. Other 3		
Section G, Direct Costs (A thru F)		[redacted]
Section H, Indirect Costs		[redacted]
Section I, Total Direct and Indirect Costs (G + H)		[redacted]
Section J, Fee		

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OMB Number: 0925-0001

Expiration Date: 10/31/2018

1. Human Subjects Section

Clinical Trial? ○ Yes ● No

*Agency-Defined Phase III Clinical Trial? ○ Yes ○ No

2. Vertebrate Animals Section

Are vertebrate animals euthanized? ○ Yes ● No

If "Yes" to euthanasia

Is the method consistent with American Veterinary Medical Association (AVMA) guidelines?

○ Yes ○ No

If "No" to AVMA guidelines, describe method and proved scientific justification

3. *Program Income Section

*Is program income anticipated during the periods for which the grant support is requested?

○ Yes ● No

If you checked "yes" above (indicating that program income is anticipated), then use the format below to reflect the amount and source(s). Otherwise, leave this section blank.

*Budget Period	*Anticipated Amount ($)	*Source(s)

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4. Human Embryonic Stem Cells Section

*Does the proposed project involve human embryonic stem cells? ○ Yes ● No

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: http://grants.nih.gov/stem_cells/registry/current.htm. Or, if a specific stem cell line cannot be referenced at this time, please check the box indicating that one from the registry will be used:

Specific stem cell line cannot be referenced at this time. One from the registry will be used.

Cell Line(s) (Example: 0004):

5. Inventions and Patents Section (RENEWAL)

*Inventions and Patents: ○ Yes ○ No

If the answer is "Yes" then please answer the following:

*Previously Reported: ○ Yes ○ No

6. Change of Investigator / Change of Institution Section

❑ Change of Project Director / Principal Investigator

Name of former Project Director / Principal Investigator

Prefix:

*First Name:

Middle Name:

*Last Name:

Suffix:

❑ Change of Grantee Institution

*Name of former institution:

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This is a resubmission (A1) of application 1R15AI124044-01, "**Development of a Novel Inhibitor of Ricin: A Potential Therapeutic Lead against Deadly Shiga and Related Toxins**," which was reviewed in October 2015 at the MSFB study section. The initial submission received an impact score of 38. The initial submission was not funded. As a new investigator, I am grateful for the opportunity to present this revised application. I have made every effort to address the critique thoroughly, and I believe that the proposed studies have emerged considerably stronger and more focused on relevant aspects of structural quantification of RTA-VPg interactions. This resubmission has undergone substantial revision, both in response to the reviewers' comments and in order to meet the new guidelines for R15 application. For that reason, changes are not marked in the text.

From the summary statement for the A0 application, I understand that the reviewers thought "*the overall impact of the application was moderately high*." The significance of the proposed research was deemed as "*quite high*," and that the PI is "*fully capable of carrying out the project*." The proposed research was thought to be "*well-written, with clear rationale, and easy for undergraduate students to get involved*." The reviewers believed that "*inclusion of well-planned education program was another major strength of the proposal, which provides resources and personnel to support training and supervision of these students*." However, the A0 application lacked preliminary data for RTA-VPg interactions and required biophysical methods of analysis, such as X-ray crystallography, and/or NMR spectroscopy, to fully characterize and map-out RTA-VPg binding site. Similarly, the reviewers thought that molecular dynamics simulation was not thoroughly though out.

In this A1 application, we provide preliminary evidence for the RTA-VPg interactions (48.5 nanomolar affinity), as determined by fluorescence spectroscopy; this binding data were confirmed by performing multiples of titrations, yielding a standard deviation of 4.1. In addition, Scatchard analysis has revealed that there is one binding site on RTA for VPg. Furthermore, increasing concentrations of VPg inhibit the RTA *N*-glycosidase depurinating activity exerted on eukaryotic 28*S* rRNA *in vitro*, and we have quantified the amount of adenines cleaved by RTA in the absence and presence of VPg (appropriate controls were considered). The structure of RTA has been solved in the presence of tetranucleotide inhibitor (PDB ID 3HIO), and RTA has been crystallized previously under numerous conditions. Our preliminary data and the availability of RTA structure, provides us with a starting point for crystallizing RTA-VPg complex. First, we will characterize RTA-VPg interactions thermodynamically and kinetically by employing fluorescence spectroscopy and isothermal calorimetry, and determine the minimum of VPg peptide that inhibits RTA activity *in vitro* and *in vivo*. Then, we will use two complementary structural techniques (crystallography and NMR spectroscopy) to map-out RTA-VPg binding site. Creation of truncated VPg mutants *in silico* and application of docking algorithms will provide confirmation of our findings. To increase effectiveness, we will use tools of molecular modeling and site directed mutagenesis to create point mutations in VPg, further enhancing the binding and inhibition of RTA. We have initiated collaborations with Dr. David Jeruzalmi (X-ray crystallographer at Structural Biology Center, City College, CUNY), Dr. Nancy Greenbaum (NMR specialist at Hunter College, CUNY), and Drs. Shaneen Singh and Emillio Gallicchio (experts in molecular modeling, docking and scoring algorithms from Brooklyn College, CUNY), who have reviewed the corresponding aims for soundness, and have agreed to provide their expert advice.

I have addressed other concerns expressed by the reviewers as well. The team will visit collaborators' laboratories to increase student involvement and study experimental techniques and procedures involved in off-site research. A section regarding biohazards was included. Isothermal calorimetry will be used as a complementary technique, and the collaborators have similar types of equipment to conduct parallel studies off-site. Both X-ray crystallography and NMR spectroscopy will be used, in parallel to molecular docking, to decipher and map-out RTA-VPg binding site. Molecular modeling was advanced and modified. An argument justifying the effectiveness of ricin toxin as weaponry was included. The conditions for achieving a stable RTA-VPg complex were described and elaborated on. The physiological relevance of RTA-VPg complex and effectiveness of pharmacokinetic properties of peptide inhibitors were addressed.

Thank you for your consideration.

Development of a Novel Inhibitor of Ricin: A Potential Therapeutic Lead against Deadly Shiga and Related Toxins

Specific Aims

Ricin is a plant derived protein toxin produced by the castor bean plant. The plant is widely distributed throughout the world, and the toxin is well-known for its nefarious past. Global cultivation of the plant, and the ease of the toxin extraction from its beans, makes ricin a particularly appealing agent with a strong potential to be employed as a biological weapon. Ricin has been used as a homicidal poison since antiquity, having a long history of uses in espionage and warfare. In the past several decades, ricin has been associated with terrorist organizations in the US and other countries. The Centers for Disease Control (CDC) classifies ricin as a Category B biological agent. **Currently, there is neither an effective vaccine that can be used to protect against ricin exposure nor a therapeutic to reverse the effects once exposed.**

Ricin is a type 2 ribosome inactivating protein (RIP). RIPs are produced by many plants, effectively protecting plants against viruses, bacteria, and other parasites. RIPs are also dispersed among fungi, alga, and bacteria. The deadliness of RIPs has been explored by political and military organizations to design biological weaponry, scientists to generate transgenic species of plants resistant to viral and fungal infections, numerous cancer researchers in production of immune-conjugate therapeutics, as well as mystery writers to engage their readers. RIPs are RNA *N*-glycosidases that inhibit advanced stages of protein synthesis by selectively modifying large rRNA molecules and deactivating ribosomes.

While the majority of RIPs have thus far been isolated from plant species, the Shiga and Shiga-like toxins are produced by gram-negative bacteria species. Stx (Shiga toxins) are type 2 RIPs; they are produced by pathogenic STEC (Stx-producing *E. coli*) strains and are responsible for severe human illnesses, including hemorrhagic colitis, infantile diarrhea and hemolytic uraemic syndrome. RTA (ricin A-chain) and Stx1 bind to their target cell via a common receptor. X-ray crystal structures of RIPs, such as ricin and Shiga toxin, have been solved and reveal great similarities in their active sites.

We have identified that RIP activity of RTA is inhibited by a viral peptide associated with the turnip potyviral genome (VPg). VPg has been implicated as a link to a variety of viral functions, including overcoming viral resistance in plants. *High affinity of VPg for RTA, its ability to inhibit RTA enzymatic activity, and structural similarities in RIP active sites provide a new direction in the search for a novel generation of RIP inhibitors.* Additionally, we have shown that a deletion mutant of VPg (VPg-71) is as effective an inhibitor as the full length VPg. ***The goal of the proposed research*** is to develop sufficient preliminary data to prove that VPg peptides can be used as RIP inhibitors. This knowledge will provide researchers with the tools to develop novel and more effective treatments against RIP toxicity, and advance strategies against the use of RIPs as agents of bioterrorism. In order to accomplish this goal, we propose the following two *Specific Aims*:

Specific Aim 1: **Establish conditions for RTA-VPg complex formation, and determine the minimum VPg peptide that binds and inhibits RTA.** Based on structural similarities in RTA and Shiga toxin active sites, and our preliminary studies showing that VPg binds RTA with high affinity and inhibits its activity *in vitro*, we will determine how RTA-VPg interactions are prompted by administering direct fluorescence titrations and using either intrinsic or extrinsic protein fluorescence. Employing quantitative methods of analysis, we ***expect*** to determine the extent of RTA inhibition by the VPg constructs, and identify thermodynamic and kinetic parameters of RTA-VPg interactions.

Specific Aim 2: **Mapping-out RTA-VPg interactions.** Mapping-out of RTA-VPg binding is an important step in understanding how these proteins interact. Initial studies will use deletion mutants of VPg. Once we have identified the minimum VPg that binds and inhibits RTA, we will co-crystallize RTA-VPg complex, and use two complementary high resolution structural biology techniques (X-ray crystallography and NMR spectroscopy) to map-out RTA-VPg binding site residues. Creation of the mutants *in silico* and application of docking algorithms will verify our findings. To further enhance effectiveness, we will use tools for molecular modeling and site directed mutagenesis to create point mutations in VPg, further enhancing the binding and inhibition of RTA.

Based on the outcomes of the *Specific Aims* above, we will test our inhibitor for the Shiga toxin *in vivo*.

Development of a Novel Inhibitor of Ricin:
A Potential Therapeutic Lead against Deadly Shiga and Related Toxins

Research Strategy

1. Significance

Ricin is a plant derived protein toxin produced by the castor bean plant (*Ricinus communis* L.). The toxin is well-known for its nefarious past. The plant is widely distributed throughout the world. Global cultivation of the plant and the ease of the toxin extraction from its beans made ricin particularly appealing agent with a strong potential to be employed as a biological weapon [1,2]. Ricin toxin (RT) has been used as a homicidal poison since antiquity [3]. The Centers for Disease Control (CDC) has classified ricin as a Category B biological agent [4]. **Currently, there is neither an effective vaccine that can be used to protect against ricin exposure nor a therapeutic to reverse the effects once exposed.**

Ricin is a ribosome inactivating protein (RIP). RIPs are produced by many varieties of plants, effectively protecting members of the *Plantae* kingdom against viruses, bacteria, and other parasites [5-7]. RIPs are also dispersed among fungi, alga, and bacteria [8-11]. Plant RIPs are phytotoxins and deactivate ribosomes [12]. Examples of plant-derived RIPs include ricin, abrin (from *Abrus precatorius*), saporin (from *Saponaria officinalis*) and pokeweed antiviral protein (PAP) (from *Phytolacca americana*) [13-15]. Since their discovery, RIPs have been of great scientific interest due to their importance in human heath (as both pathogenic agents and therapeutics), and their potential uses in biological warfare and bioterrorism [16-21].

RIPs are categorized into two major classes based on their physical properties. Type 1 RIPs consist exclusively of a single RNA *N*-glycosidase domain (e.g., PAP and saporin); they inhibit cell-free protein synthesis and are only mildly toxic to cells and animals. Type 2 RIPs (e.g., ricin, abrin, Shiga toxin, etc.), consist of an amino-terminal domain equivalent to type 1 RIPs (enzymatic domain with RIP activity; A-chain), joined by a disulfide bond to a different carboxyl-terminal domain with lectin properties (carbohydrate binding domain; B-chain) [22,23]. Type 2 RIPs are acutely toxic heterodimeric proteins. The lectin chain binds to galactosyl moieties of glycoproteins and/or glycolipids on the exterior of eukaryotic cells [24-26], promoting reverse transport of the A-chain to the cytosol [27-29]. The A-chain accesses translational machinery and depurinates ribosomes after it enters the cytosol. The extracellular location prevents contact between RIP and ribosomes of healthy cells, yet provides a direct source of toxin when the pathogen vector disrupts the cell.

Reports of the use of ricin as a weapon date back to ancient times; ricin has a long history of use in espionage and warfare. Nonetheless, usage of this toxin as customary weapons is quite a modern idea. The ease by which one may acquire large amounts of ricin makes this toxin a prime candidate for bioterrorism. In 1978, ricin was employed in the assassinations of Markov [3,30]; many more recent instances were identified where this assassination technique was used [31]. In the past decade ricin has been associated with terrorist organizations in several countries [32]. The potential use of ricin toxin as a biological weapon of mass destruction has been highlighted in a FBI report released in 2007, entitled Terrorism 2002-2005, stating that "Ricin and the bacterial agent anthrax are emerging as the most prevalent agents involved in WMD investigations" [33].

Recent economic surveys show that there is over 1 million tons of castor beans produced in the world annually, having the potential to yield approximately 50,000 tons of pure ricin. The fate of much of this ricin in countries outside of the US is unknown. Ricin toxin can contribute up to 5% of the total protein of the bean (consumption of 8 beans is considered to be dangerous; subcutaneous toxicity is 24 μg/100 hours); it can be easily extracted from the mash produced as a by-product of castor oil production by several simple enrichment steps, and is therefore easy to accrue. In 1952 the US Army filed a patent on how to prepare ricin for weaponry purposes [34]. Before the 1990 Gulf war, the Iraqi military attempted to devise ways to disseminate ricin as an explosive bomb, attempts which were stalled by the outbreak of war and ensuing events. While it is true that ricin, and similar toxins are often cited as major bioterrorism threats, it is also understood that they are not very effective with respect to mass dispersal. It should be stressed that in recent years, bioterrorist mass attacks have given way to a "lone wolf" strategy, where the assault is directed by a single individual or small group, increasing the chances of fatalities.

The majority of RIPs have thus far been isolated from plant species; although Shiga and Shiga-like toxins are produced by gram-negative bacteria species. Stx (Shiga toxins) [20,35] are produced by pathogenic STEC (Stx-producing *E. coli*) strains, which are responsible for severe human illnesses: including hemorrhagic colitis,

infantile diarrhea and HUS (hemolytic uraemic syndrome) [36]. HUS is the main cause of acute renal failure in children and is the consequence of intestinal infection by STEC.

In 2013 and 2014, the CDC reported a multistate outbreak of Shiga toxin-producing *E. coli* O121 (STEC O121), in which a total of 19 people were infected in the western United States; the infection was linked to raw clover sprouts and farm rich brand frozen food products [37,38]. Similarly, in 2014, the eastern US suffered another multistate outbreak of O157:H7 strain of *E. coli*; this specific outbreak was linked to ground beef contamination. Clearly, *E. coli* is still an important cause of human illness in the United States [39]. Among the multiple serotypes associated with disease, O157:H7 is responsible for more than 63,000 of the 175,000 total estimated STEC cases each year [40]. Ruminants, especially cattle, are the natural carriers of STEC, and these bacteria most commonly enter the food chain during beef processing [41-44]. Outbreaks and sporadic cases of STEC infection have also been attributed to contaminated fresh produce, person-to-person spread, and environmental sources [44,45]. Stx is the primary virulence factor associated with diseases caused by STEC. STEC produce two main types of bipartite toxins, Stx1 and Stx2, which are capable of binding to glycolipid receptors present on the surface of target cells through their B subunits [46]. After endocytosis, the A subunit damages ribosomes by releasing a specific adenine from 28S rRNA, and DNA, by releasing multiple adenines.

The cytotoxic effects of RIPs have long been attributed to translation inhibition [47]. RIPs specifically target and depurinate the conserved sarcin/ricin loop (SRL) of the large rRNA [48]. The SRL within the rRNA of different ribosomes is universally conserved across all species [49]. Consequently, both the specificity of different RIPs for their substrates, and the selectivity between ribosomes among species, are likely to come from deviations within RIPs themselves and the ribosomal proteins. Ricin, for instance, presents the highest activity toward yeast and mammalian ribosomes, but exhibits low activity on plant and *E. coli* ribosomes, whereas PAP depurinates ribosomes from plants, bacteria, yeast, lower and higher animals, as well as a variety of viral RNAs [5]. RIP substrate specificity may be influenced by the deviations in ribosomal proteins, which dictate RIPs activity and sensitivity toward different ribosomes. The L9 and L10e rat liver ribosomal proteins are targeted by the ricin A-chain (RTA) [50], however PAP has been shown to bind to the L3 yeast ribosomal protein [51]. The structures of ricin, PAP and other RIPs have been solved, and the presence of various substrate analogs interacting with RTA side chain amino acids has been mapped out [52,53]. *RTA and PAP reveal a high degree of similarity in their active site residues* (***Figure 1A***) [54-56]. Similarly, *RTA and Stx1 bind to their target cell via a common receptor, in addition to having analogous amino acid residue distribution within their active sites* (***Figures 1B and 1C***) [7,57,58].

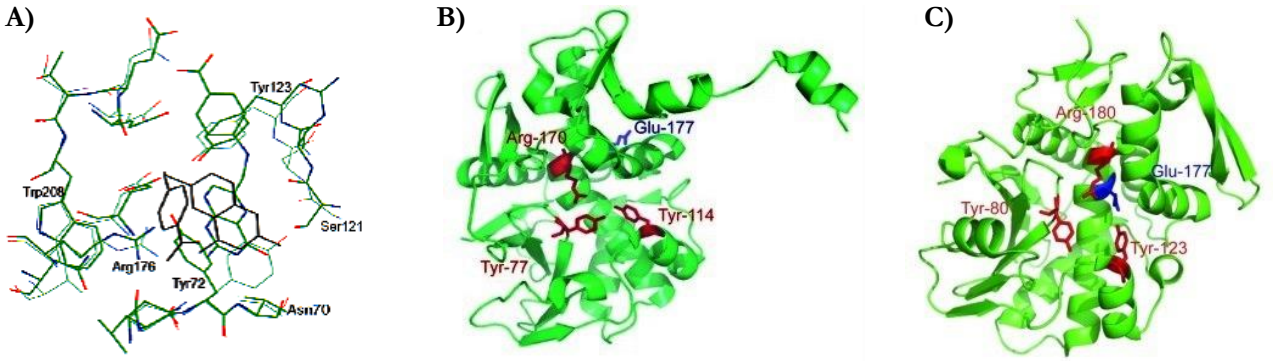

Figure 1. (**A**) Superimposition of PAP and RTA [54] active site residues. RTA atoms are in thin lines; PAP is in thick lines [55]. In RTA, the substrate is sandwiched between Tyr123 and Tyr80, making six H-bonds to the Val81, Gly121 and Arg180 within its active site [56]. Structures of RTA and Siga toxin, Stx2 [7]: (**B**) Structure of RTA in complex with the cyclic tetranucleotide inhibitor; PDB ID 3HIO; (**C**) Structure of Shiga-like Stx2 A-chain from *Escherichia coli* in complex with adenine; PDB ID 2GA4.

Preliminary Data:

Viral protein (VPg) from turnip mosaic virus (TuMV) binds to and inhibits RTA activity *in vitro*. We have shown that PAP is inhibited by a viral peptide associated with the turnip potyviral genome (VPg) [59]. By employing fluorescence spectroscopy, we have determined that VPg is also able to bind to RTA with great affinity (K_d = 48.5 ± 4.1 nM) in a 1 to 1 ratio, and inhibit RTA depurination of eukaryotic 28*S* ribosomal RNA *in vitro*. ***Figure 2*** presents the binding curve for RTA-VPg interactions, and the inhibitory effect of VPg on ribosomal depurination.

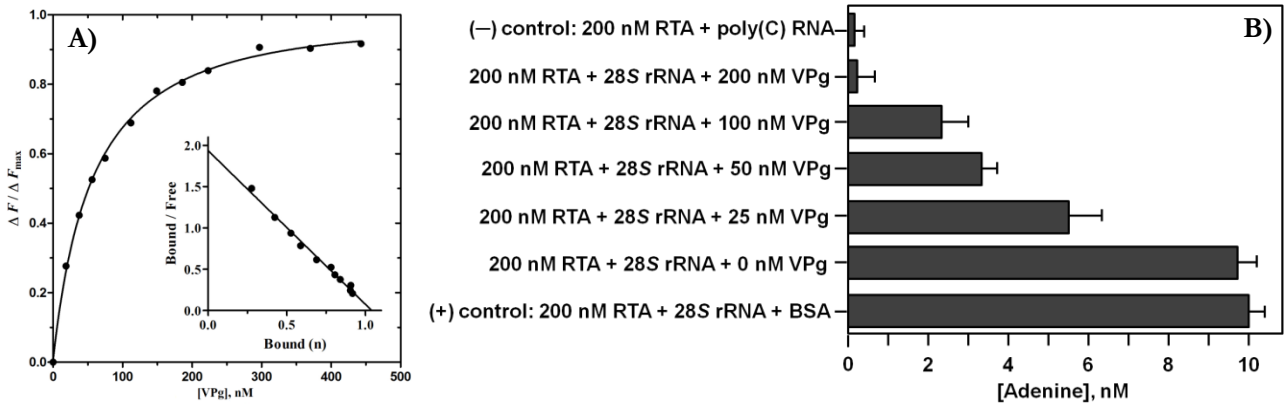

Figure 2. (**A**) Normalized fluorescence binding curve for a binary complex of RTA with VPg from TuMV. The *inset* depicts the Scatchard analysis at 25 °C of the titration data, indicating that there is one binding site on RTA for VPg. (**B**) Increasing concentrations of VPg inhibit RTA depurination of 28*S* rRNA *in vitro*, as determined by conversion of adenine released from rRNA to N^6-ethenoadenine, and its quantification on the HPLC.

We have also shown that a deletion mutant of VPg (VPg-71; lacks 70 *N*-terminal amino acids) is as effective an inhibitor of PAP activity as full length VPg [59]. *Our long-term goal* is to understand how viral infections affect protein synthesis and cellular antiviral strategies. Turnip mosaic virus (TuMV) is a member of the genus *Potyvirus* that infects cruciferous plants (e.g., horseradish, cabbage, broccoli, cauliflower) throughout the world [60,61]. Several functions have been attributed to potyviral VPg, including viral replication, infection, and cell-to-cell movement; it has been shown to play an important role in the viral resistance of plants [15,59,62-69]. We have proposed that VPg may provide an evolutionary advantage to the virus in the arms race with the plant defense mechanism – the RIP: VPg binds to PAP and inhibits its antiviral activity [15,59].

Ricin A chain (RTA) X-ray crystal structure has been solved. The structure of RTA has been solved and RTA has been crystallized under numerous conditions [56,70]. The 267 amino acid RTA consists of three domains: residues 1-117 contain a 6 stranded β-sheet that form the core of the protein and 2 α-helices; residues 118-210 are comprised of 5 α-helices and contain active site residues, E177 and R180; and the region from residue 211 to residue 267 forms the interface with RTB and has a two stranded anti-parallel β-sheet and one α-helix [71]. Schramm laboratory have published the 2.0 Å X-ray crystal structure of RTA (***Figure 1B***), and this offers a starting point for crystallizing RTA-VPg complex, in that it was done with a tetranucleotide inhibitor.

Scientific Premise:
The high affinity of VPg for RTA, its ability to inhibit RTA enzymatic activity, and structural similarities in RIP active sites, provides a new direction in the search for a novel generation of RIP inhibitors and is the essence of the scientific premise of the proposed research. We seek to characterize RTA-VPg interactions, determine the minimum VPg necessary to efficiently inhibit ricin activity, and show that VPg peptides can be used as RIP inhibitors. This knowledge will provide researchers with the tools to develop novel and more effective treatments against RIP toxicity, allowing for strategies against the use of RIPs as agents of bioterrorism. Fluorescence spectroscopy and isothermal calorimetry (ITC) employed in this activity, are two complementary and commonly employed techniques that will allow our team to study protein-protein interactions [72,73]. *To establish the stable conditions for RTA-VPg complex formations, we will vary pH, ionic strength, and buffer settings, leading to interactions that are enthalpically favorable and entropically driven, yielding the overall lowest energy.* Transient protein complexes form and break rapidly, whereas permanent complexes have a relatively long half-life. Kinetic characterization of RTA-VPg will yield a full profile for these interactions; individual kinetics of RTA-VPg interactions (k_{on} and k_{off}) are very significant, and will dictate the biological activity of these proteins [74,75]. We have previously employed physiologically relevant translational assays and showed that PAP's activity is inhibited in the presence of VPg [59]. Likewise, a rabbit reticulocyte lysate translational assay will provide *physiological relevance* to our experiments (in addition to *in vivo* experiments performed by Dr. O'Brian). The RTA structure reveals that it is an alpha-beta protein with ABA sandwich architecture (PDB ID 3HIO) [76], and there is enough beta-strand character, yielding fine resolution spectra with sufficient dispersion to be assignable. First, however, a battery of 3D experiments needs to be

performed on a ^{13}C and ^{15}N-enriched protein [77-80]. High yield of RTA expression is easily attainable from *E. coli* and/or yeast cells [81-84], and the protein remains clear in solution at concentrations suitable for NMR.

Currently, there is no inhibitor or therapeutic to counteract ricin poisoning, and those that have been investigated induce sever immune responses in animal models. This activity will provide the groundwork foundation and understanding into the mechanism of ricin inhibition by VPg peptide, providing models for designing molecules with greater inhibitory potency. In the early years of the pharmaceutical industry, and drug development pipelines, has been dominated by small molecules. For a drug to be marketed it has many obstacles to overcome; next to efficacy and tolerability, new drug candidates have to meet several requirements, including favorable pharmacodynamics (effects of a product related to its clinical activity), pharmacokinetics absorption, distribution, metabolism, and excretion), toxicity and safety issues [85]. Peptide attributes originally considered troublesome with respect to drug development may now turn out to be more convenient rather than unfavorable [85]. During the past decade, peptides have gained a wide range of applications in medicine and biotechnology, and therapeutic peptide research is also currently experiencing a renaissance for commercial reasons. Peptides are recognized for being highly selective and efficacious and, at the same time, relatively safe and well tolerated. Consequently, there is an increased interest in peptides in pharmaceutical research and development, and over 140 peptides are currently being evaluated in clinical trials as therapeutics [86]. Rational design of peptide therapeutics can start with a known crystal structure of the peptide providing the secondary and tertiary structure. Then, based on the structure-activity relation, it is important to identify essential amino acids that are chemically prone to events such as isomerization, glycosylation, or oxidation, which should be avoided or substituted. Additionally, it is important to improve physicochemical properties of peptides, which often have the tendency to aggregate, sometimes having poor solubility. Strategies to avoid aggregation include the corruption of hydrophobic patches, which is achievable by means of substitution or *N*-methylation of particular amino acids. Changing the charge distribution and the isoelectric point of the peptide leads to increased solubility. The physicochemical properties of peptides can also be improved by the introduction of stabilizing α-helices, salt bridge formulations, or other chemical modifications, such as lactam bridges [86,87].

2. Innovation

The PI proposes a high-impact experimental investigation of the effect exerted by a small genome-linked turnip mosaic virus protein (VPg) on the RIP depurinating activities of RTA and Shiga toxin. Ricin has been used in sporadic incidents of espionage and bioterrorism; therefore, there has been a great deal of interest in developing a safe vaccine or antidote to protect people from ricin poisoning. Although multiple types of vaccines have been tested and numerous ricin inhibitors or inactivators have been patented, none possess the ideal properties and standards of an effective vaccine. In terms of passive post-exposure protection, monoclonal neutralizing antibodies to passively protect animals are also under development.

Many of the inhibitors developed and tested concentrate on structure-based designing of the inhibition of ricin's *N*-glycosidase activity [56]. Recently, an inhibitor of the cellular trafficking of ricin has been reported [88]. Schramm *et al.* have reported oligonucleotide-based inhibitors of ricin [89]. The RiVax™ (Soligenix) ricin toxin vaccine developed to protect against exposure to ricin toxin is the most advanced vaccine available to date [90,91]. The immunogen in RiVax™ consists of a genetically inactivated subunit ricin A-chain that is enzymatically inactive and lacks residual toxicity of the holotoxin; this induces a protective immune response in animal models of ricin exposure, and functionally active antibodies in humans.

Immunization after ricin exposure is impractical on a large scale; four hours after exposure to lethal doses the effects are irreversible, taking three to five days to kill an individual. Ricin is cleansed rapidly from the blood and the symptoms of ricin intoxication mimic those of other diseases. Furthermore, the current expectation behind vaccine development is that ricin would most likely be distributed as an aerosol.

The research proposed by the PI and his team is innovative in nature; it involves investigating a novel peptide inhibitor of RIP activity of deadly ricin and Shiga toxin. Based on our experimental plan and preliminary data, we are confident that VPg constructs will serve as a new direction in the search for a novel generation of RIP inhibitors. Our research is an important milestone in an attempt to develop an effective vaccine for the inhibition of a wide variety of toxins with similar activities. A better ability to identify toxin in the body and improved vaccines [92] should mitigate the risk of its use in bioterrorism [3,93], in addition to providing more effective treatment for *E. coli* poisoning.

3. Approach

The PI and his research team (four undergraduate and two graduate students) propose a study that will investigate interactions between ricin A-chain (and Shiga toxin) and a viral peptide (VPg) from TuMV. We will determine the minimum VPg peptide required to efficiently inhibit RIP activity. The thermodynamic driving forces of these macro-molecular interactions are the critical parameters for the design of effective biomedical and pharmaceutical treatments. Once the minimum VPg peptide is established, we will crystallize the RTA-VPg complex and employ X-ray diffraction to map out the RTA-VPg binding site. Using bioinformatics tools, we will identify residue substitution in VPg, which may lead to more profound RIP inhibition. Our laboratory uses *in vitro* and *in vivo* analysis methods, and will investigate biochemical, molecular biological and biophysical aspects of this activity. This research is strongly collaborative in nature, combining the efforts of national and international institutions. **Goals** *of this proposal will be accomplished by integrating scientific research knowledge, classroom experiences, collaborative efforts with other researchers, and an interdisciplinary research team involving underrepresented minority students.*

Materials and Methods

VPg clones (VPg, VPg-71, VPg-81, VPg-91, etc.) are a gift from Dr. Miyoshi from St. Mariana University (Japan) and will be expressed in *E. coli*. We have a number of deletion mutants to test and since we have clones, other mutants can be readily created, both site specific and deletion. RTA clone is a gift from Dr. Schramm (Albert Einstein College of Medicine, NY); it is expressed in *E. coli* and purified in the PI's laboratory at John Jay College. All protein purifications will be accomplished using an AKTAPure system (GE Healthcare). We have requested assistance from Dr. O'Brien (Uniformed Services University, MD), who is equipped to safely handle ricin and Shiga toxins. The PI and accompanying students will travel to the collaborators' laboratories to learn the techniques used in this activity and assist in testing our inhibitor for that toxin as well.

Bio- and Radio-Hazardous Materials and Safety: All personnel are required to undergo training in chemical waste disposal, and biochemical and radiological safety. A primer extension assay will involve the usage of a P-32 radioisotope, an emitter of β radiation. All radiological samples and reactions will be performed in acrylic containers and tubes, with shields aiding in protection from radiation. Biohazardous and radiological chemical waste disposal will be carried out in accordance with the OSHA (Occupational Safety and Health Administration) regulations [94]. *It should be noted* that RTA cannot cross cell membranes and is not a bioterrorism agent in and of itself. The research requires no additional precautions for those experienced in working with PAP or RTA. Ricin and Shiga toxins are category B pathogens, and research involving these toxins will be performed in accordance with guidelines outlined by the OSHA by Dr. O'Brian's laboratory.

Rigorous Experimental Design

Fluorescence Data Acquisition and Analysis: Steady state fluorescence has been used extensively to study protein-protein and protein-nucleic acid interactions [95]. Fluorescence measurements will be taken at John Jay College in the PI's laboratory using a Horiba Jobin Yvon FluoroMax®-3 (equipped with a 150 W xenon lamp, photodiode array detectors and FluorEssence™ software). In experiments entailing high excitation intensity, a SPEX-Fluorolog-τ spectrophotometer (450 W) will be employed; the instrument is located in the Goss laboratory (Hunter College, CUNY). Fluorescence changes (quenching or enhancement) will be monitored using excitation and emission wavelengths appropriate to the experimental design. Data will be fit as previously described [96]. Briefly, data are normalized and subjected to statistical analysis using the following equation:

$$F_b = (F_{obs} - F_0)/(((F_\infty - F_{obs})*(F_\infty/F_0))+(F_{obs} - F_0)) \quad \text{Eq.1}$$

where F_b is the fraction bound, F_{obs} is the observed fluorescence, F_0 is the initial fluorescence value and F_∞ is the final fluorescence value. Normalized values are plotted and analyzed by a nonlinear least-squares fitting for K_d determinations. Data are fit and analyzed for both one- and two-site binding models. The uncertainty of fitting parameters, dilution corrections, and inner filter effects of titrant absorbance are considered in data analysis and interpretation. The software used is Graph Pad Prism®, version 5.

Analysis of Stopped-Flow Kinetic Data: Kinetic experiments will be performed on an OLIS RSM 1000 stopped-flow system (Dr. Dixie Goss, Hunter College) with a 1-ms dead time. Stopped-flow data for the binding of RTA to VPg will be analyzed using Global analysis software [97]. Data from the anisotropy experiments will be fitted to the single-exponential function:

$$r_t = \Delta r \exp(- k_{obs} * t) + r_f \quad \text{Eq. 2}$$

where r_t is the observed anisotropy at any time t, and r_f is the final value of anisotropy, Δr is the amplitude and k_{obs} is the observed first order rate constant; or the double-exponential function:

$$r_t = \Delta r_1 \exp(- k_{obs1} * t) + \Delta r_2 \exp(- k_{obs2} * t) + r_f \quad \text{Eq. 3}$$

where Δr_1 and Δr_2 are the amplitudes for the first and second components of the reaction with observed rate constants k_{obs1} and k_{obs2}, respectively. Data will be subjected to statistical analysis.

NMR Spectroscopy: Acquisition of 3D HNCO, HN(CA)CO, HNCA, HN(CO)CA, HNCACB, and CBCA(CO)NH spectra to assist in assignment of backbone resonances Cα, Cβ, CO, and amide N and ^{1}H of the RTA protein [98], and 2D [^{1}H, ^{15}N]-HSQC spectra to monitor chemical shift changes upon binding of VPg, will be performed at 298 K on a 600 MHz Bruker Avance spectrometer (equipped with a three-channel TXI-cryoprobe) in the NMR Facility at Hunter College with the aid of Dr. Nancy Greenbaum. NMR experiments will be acquired and processed using Bruker TopSpin 3.2 software in triplicates and statistically analyzed. Spectral visualization and chemical shift assignments will be performed with SPARKY [99]. For the experiments, samples of RTA will be labeled with ^{15}N and/or ^{13}C (as described in *Aim 2*) and will be carried out in in 90% H_2O/10% D_2O phosphate buffer in 5 mm micro volume NMR tubes (Shigemi, Inc.). Changes in ^{15}N and ^{1}H chemical shifts of the protein observed in a [^{1}H-^{15}N]-HSQC spectrum will be calculated using an adjusted chemical shift:

$$\Delta\delta = [(\Delta\delta_H)^2 + (0.2 * \Delta\delta_N)^2]^{1/2} \quad \text{Eq. 4}$$

where $\Delta\delta_H$ represents the chemical shifts change observed for amide ^{1}H and $\Delta\delta_N$ for amide ^{15}N. The mean and standard deviation σ of the shifts will be calculated and most perturbed residues will be identified as those whose shift changes are greater than σ. The fit will be performed using the SOLVER function of Graph Pad Prism®, version 5, by minimizing the sum of squared errors for Δδ.

We plan to accomplish our goal by directing the following *Specific Aims and Rigorous Experimental Design*:

Specific Aim 1*: Establish conditions for RTA-VPg complex formation, and determine the minimum VPg peptide that binds and inhibits RTA.** Based on structural similarities in PAP, RTA, and Shiga toxin active sites, and our preliminary studies showing that RTA binds to VPg with high affinity, and that increasing concentrations of VPg inhibit RTA activity *in vitro*, we will determine how RTA-VPg interactions are prompted by administering direct fluorescence titrations and using either intrinsic or extrinsic protein fluorescence. Employing quantitative methods of analysis, we ***expect to determine the extent of RTA inhibition by the VPg constructs, and identify thermodynamic and kinetic parameters of RTA-VPg interactions.

***Aim 1A:* Characterize RTA-VPg interactions and identify minimum VPg peptide that binds to RTA.** We will purify recombinant RTA, wild type VPg, and VPg mutants, and measure the equilibrium constants for RTA-VPg protein-protein interactions in a range of temperatures (e.g., 10, 20, 30 °C) using direct fluorescence titrations to determine pivotal thermodynamic binding parameters (e.g., ΔH, ΔS and ΔG) for RTA-VPg complex formation, and identify whether the binding is entropically favored or enthalpically driven. All titration experiments will be performed in triplicates and statistically analyzed to extract standard deviations. Once the basic binding parameters for wild type VPg-RTA complex are established, and the binding has been quantitatively characterized, we will determine the minimum VPg peptide required to bind to the RTA. All VPg constructs (VPg-71 through VPg-121 deletions and wild type VPg) are His-tagged, easing their purification by Ni-Sepharose affinity chromatography. We will measure binding constants for RTA-VPg constructs using direct fluorescence titrations and establish the affinity between the proteins. Those VPg mutants that do not bind RTA will not exhibit saturation binding behavior, nor will we observe the decrease in adenines released from the depurinated substrate RNA. The changes in fluorescence upon binding are directly related to the interaction of the two proteins. For intrinsic protein fluorescence (λ_{ex} = 280nm/λ_{em} = 332nm), the difference between identical concentrations of RTA and VPg, taken separately and when mixed together, will give the difference in fluorescence for the complex. The fluorescence intensity of RTA will be measured, then blank buffer will be titrated in, adding to the corrected intensities (F_s). Once RTA is titrated with VPg, the corrected fluorescence for the complex will be obtained (F_c). The difference in fluorescence intensity related to the complex is defined as $\Delta F = F_c - F_s$. The inner filter corrections will be applied, whenever necessary, using the equation:

$$F_{corr} = F_{obs} \cdot \text{antilog}\,[(A_{ex}+A_{em}) / 2] \quad \text{Eq. 5}$$

where F_{corr} and F_{obs} are the corrected and observed fluorescence intensities, respectively. The absorbance of the samples will be measured using an UV-VIS spectrophotometer. The normalized fluorescence difference ($\Delta F/\Delta F_{max}$), between the protein-protein complexes and the sum of the individual fluorescence spectra, will be used to determine the equilibrium dissociation constants (K_d) for the interactions at different temperatures, allowing for the construction of a van't Hoff plot. We will vary pH, ionic strength, and buffer settings.

Expected Outcomes and Alternative Approaches. At the conclusion of these experiments, we will quantitatively determine equilibrium binding constants for the interactions of RTA with VPg peptides, and will have detailed quantitative measurements of RTA-VPg protein-protein interactions. The energy profile of the RTA-VPg binding reaction is the final result of the combined fluorescence and ITC approach, and a cornerstone in the elucidation of these protein-protein interactions. The current deficiency in physical measurements will be addressed and our description of these interactions will lead to an understanding of these novel RTA-VPg interactions. Furthermore, the data will allow for comparisons of binding and VPg effects with other RIPs (e.g., PAP), providing comparative information of these interactions. Being the initial studies, they are particularly important. *Several complementary and alternative approaches to the above Aim are described below.*

Isothermal Titration Calorimetry (ITC): As a complementary approach to fluorescence, and to confirm the veracity of RTA-VPg thermodynamics, isothermal titration calorimetry (ITC) [73,100] will be employed. The PI and his students will travel to Denver University, and assist Dr. John Latham (Department of Chemistry and Biochemistry, Denver University, CO), in using the Nano ITC (TA Instruments) to determine the thermodynamics of RTA-VPg interactions in solution. Calorimetry is an excellent technique, allowing for characterization of the thermodynamic driving forces and defining molecular interactions. All binding events are accompanied by the evolution or absorption of heat (ΔH). With the appropriate experimental design, fundamental information regarding the molecular interactions driving the process, as well as stoichiometry of binding (n) and the binding constant (K_a), can be generated. The ITC experiment will be performed at a constant temperature by titrating one binding partner (VPg, the titrant) into a solution containing the other binding partner (RTA; the titrand) in the sample cell of the calorimeter. All experiments will be performed in triplicates. After each addition the heat released or absorbed in the sample cell is measured with respect to a reference cell filled with a reference buffer sample. Information obtained from the ITC experiment will include free energy, enthalpy, entropy, heat capacity changes, and any protonation effects that accompany RTA-VPg binding interactions. ITC will provide the global thermodynamic parameters of the RTA-VPg binding reaction. Data from non-calorimetric methods will complement this characterization [101].

Kinetic characterization of RTA-VPg interactions: Protein motions, particularly their conformational dynamics, regulate and often constitute protein function. Probing the structural and internal dynamics of RTA-VPg complexes requires complementary experiments, allowing energetic and dynamic aspects in the formation and stability of the complex to be revealed. Fluorescence anisotropy studies will provide information on the binding mechanisms, and may lead to mechanistic models of RTA inhibition on the molecular level. To gain insight into the kinetic mechanism of RTA-VPg binding, and understand how this binding is affected, we will perform stopped-flow anisotropy measurements for RTA binding to VPg [72]. All experiments will be performed in triplicates. We will label RTA (or VPg) with fluorescein (λ_{ex} = 490 nm, λ_{em} = 520 nm) to aid in the sensitivity of measurements. The temperature of the flow-cell and solution reservoir will be maintained constant at 25 °C. After rapid mixing of RTA with various concentrations of VPg, the time course of the anisotropy change will be recorded by computer data acquisition. Statistical analysis of the acquired will be performed. In each experiment, 1000 pairs of data will be recorded and sets of data from 5 to 7 shots will be averaged to improve the signal-to-noise ratio. Data will be evaluated by fitting to the single- and double-exponential functions [102,103]. Each averaged set of stopped-flow anisotropy data will then be fitted into nonlinear analytical equations using Global analysis software provided by OLIS. For the stop-flow fluorescence measurements, we will excite RTA and will measure the fluorescence (voltage) after passing a 324 nm cut-on filter. A reference photomultiplier will be used to monitor fluctuations in the lamp intensity. The temperature of the flow cell and solution reservoir will be maintained by using a temperature controlled circulating bath. Corrections will be made to the anisotropy changes when titrating a buffer blank into RTA.

Extrinsic Fluorophore Labeling: Should the fluorescence difference spectra prove too small to give accurate results, as an alternative approach, we will label one, or both, of the proteins with an extrinsic fluorophore. There are wide varieties of probes to choose from (e.g., NHS-fluorescein from Pierce has λ_{ex} = 493 nm and λ_{em} = 516 nm that are far removed from native protein fluorescence). Typically, labeling is done at neutral pH so that only uncharged amines are labeled. Using a limited amount of probe, one or two sites are labeled on the protein. Determination of the amino labeled site will be confirmed by digestion of a control, unlabeled protein sample, and HPLC analysis. Binding of NHS-fluorescein-labeled RTA to VPg will serve as a functional comparison for the labeled and unlabeled proteins. We can label either protein, and should this method be necessary, labeling of each protein separately and titrating with the other will provide a control that the probe has not perturbed the equilibrium [59]. Simultaneous measurements of the individual protein spectra and of all buffer solutions will also be used as controls for the changing fluorescence signal. Data will be analyzed as described in *Fluorescence Data Acquisition and Analysis*. Averages of at least three titrations and use of at least two different protein preparations will be used as controls to verify the data. As an additional control, using catalytically inactive RTA, measurements will be made to determine whether it still binds to VPg, and if so, how it compares to native RTA.

Aim 1B: **Determine the extent of rRNA depurination by RTA in the presence and absence of VPg peptides, and identify shortest VPg necessary to inhibit RTA.** After establishing favorable conditions for the minimum VPg peptide binding to RTA, we will assay the inhibition of ricin activity, and correlate binding to depurination. Analysis of ricin activity will be determined as described previously [59]. Briefly, the amount of adenine released during depurination of eukaryotic rRNA will be determined by conversion of the purine to its fluorescent derivative, N^6-ethenoadenine, using chloroacetaldehyde. Samples will be analyzed with the Waters HPLC located in the PI's laboratory. The HPLC is equipped with Waters 2487 dual λ absorbance detector (set at 254 nm), Waters 2475 multi λ fluorescence detector (λ_{ex} = 315 nm and λ_{em} = 415 nm), controller, and an autosampler. The column to be used is a reverse-phase XBridge™ C_{18} column, and the elution solvent is ammonium acetate/methanol system. The areas under the elution peaks will be analyzed by Waters Empower™ chromatography software, and related to the amount of adenines released from rRNA by RTA activity. The retention time of the elution peak and the internal standard, N^6-ethenoadenine, will be used for the quantification of the adenines released by RTA. Eukaryotic 28S rRNA will provide a positive control for the experiments, whereas poly(C) will serve as a negative control for the depurination. Standard curve will be created and data will be statistically analyzed. We will then perform a primer extension assay on the RTA-depurinated rRNA substrate, determining and confirming the location of the depurination site. All of the above experiments and assays will be performed at least three times.

Expected Outcomes and Alternative Approaches. At the conclusion of these experiments, we will determine to what extent rRNA is depurinated by RTA, in the absence and presence of VPg peptides, and correlate this depurination with the physical binding measurements (see *Aim1A*). We expect to have detailed quantitative measurements of the effects exerted by VPg on the activity of RTA. *Several alternative and complementary approaches to the above methods are described below.*

Aniline Cleavage Assay of *N*-glycosidic Activity: Alternatively, the depurination can be determined by an aniline cleavage assay of *N*-glycosidic activity. After the adenine is removed the depurinated site becomes unstable, and is subjected to a β-elimination reaction when RNA is treated with acidic aniline. This cleaves the 3'-end of the RNA and the depurination product is detected by electrophoresis [104,105]. As a control, aniline treatment of RNA in the absence of RTA will confirm that the target RNA sample is free of ribonuclease activity. The extent of depurination is determined based upon the amount of nucleic acid chain cleavage [104,106]. The assays will be performed in triplicates.

Luminescent Assay for RTA Activity: In addition to the above methods to assay RTA activity, we will employ a sensitive luminescent coupled assay [107], for the measurement of adenine released from rabbit reticulocyte 80*S* rRNA by RTA in the presence or absence of VPg protein. Adenine phosphoribosyl transferase (APRTase) and pyruvate orthophosphate dikinase (PPDK) convert adenine released by RTA to ATP for quantification by firefly luciferase. The resulting AMP is cycled to ATP, so it produces sustained luminescence proportional to the adenine concentration [107]. Continuous measurements are accomplished in 96-well plate format by combining the luciferase reagent with APRTase/PPDK (adenine to ATP) coupling enzymes. Both, APRTase and PPDK

clone from *Clostridium symbiosum* are gifts from Dr. Schramm (Albert Einstein College of Medicine). Assay samples lacking VPg will constitute a positive control of the depurination, whereas RTA depurination assay samples lacking RIP will generate a negative control for this experiment.

Aim 1C: **Identify the effect exerted by VPg peptide on Shiga toxin activity.** Depending on the outcomes of the *Aims 1A and 1B*, the PI has arranged to send out VPg peptides to his collaborator at the Uniformed Services University of the Health Sciences, Dr. O'Brien, to test the effect of the VPg inhibitor on Shiga toxin (Stx) toxicity on Vero cells (CCL-81) [46]. The cells will be transfected with cDNA encoding for VPg peptides, and a cytotoxicity assay will be performed by Dr. O'Brien, revealing any inhibitory effects of VPg on cytotoxic ricin and Shiga toxin. The PI will visit Dr. O'Brien's laboratory to be fully involved in the techniques performed. As a negative control, a different His-tagged protein will be used; a positive control of ricin or Shiga toxin will lack VPg peptides. All experiments will be performed in triplicates.

Ricin and Shiga toxin (Stx) Cytotoxicity Assays: The Vero cytotoxicity assay [108,109]: Vero cells will be seeded in 96-well plates under appropriate conditions, and incubated in the presence and absence of Shiga toxin or intact ricin toxin. Consecutively, the inhibitory effect of VPg on the toxin will be tested by successive addition of the viral peptide to the toxin treated Vero cells. The cells will then be fixed with formalin and stained with crystal violet. The absorbance of the wells is then measured at 630 nm using a spectrophotometer.

Expected Outcomes and Alternative Approaches. We expect to have a detailed quantitative data on the inhibitory effect of VPg on the cytotoxicity of Shiga toxin and ricin toxin. These expectations stem from our preliminary data, and a central hypothesis stating that VPg will successfully bind to and inhibit the RIP activity of deadly Shiga toxin. This is essential in developing novel vaccines for the prevention of *E. coli* poisoning. As an alternative approach to the Vero cell cytotoxicity assay, HCT-8 cell line will be used in the same manner as Vero cells, except that the cells must be incubated prior to the toxin application. Controls will be similar to the Vero cell assay. The CD_{50} will be determined by the reciprocal of the toxin dilution that caused 50% of the cells in a monolayer, compared to control cells. Specific toxin activity is calculated as CD_{50}/mL divided by the toxin concentration in mg/mL [46].

Specific Aim 2**: Mapping-out RTA-VPg interactions.** A comprehensive understanding of RTA-VPg binding is an important step in understanding how these two proteins interact. Initial studies will use deletion mutants of VPg. Once we have identified the minimum VPg that binds and inhibits RTA, we will express these VPg mutants in either *E. coli* or purchase custom made truncated VPg peptides. We will co-crystallize RTA-VPg complex, and use two complementary high resolution structural biology techniques in parallel (X-ray crystallography and NMR spectroscopy) [110] to map-out RTA-VPg interacting residues. Creation of the mutants *in silico* and application of docking algorithms will confirm our findings. To further enhance effectiveness, we will use tools for molecular modeling and site directed mutagenesis to create point mutations in VPg, further enhancing the binding and inhibition of RTA.

Aim 2A: **X-ray crystallography to define sequence requirements of VPg binding to RTA.** The structure of RTA has been solved and RTA has been crystallized under numerous conditions [70,76]; however, no structure exists of VPg or RTA-VPg complex. We will seek crystallization advice, X-ray diffraction, and data analysis from the PI's collaborator Dr. David Jeruzalmi (City College, CUNY), who is an expert in this technique. To prepare crystals for structure determination using X-ray crystallography, we will initially follow a novel procedure for RTA-inhibitor crystallization established by the Schramm laboratory [76]. As necessary, crystallization trials for the entities under investigation here will also be carried out using the index, crystal and detergent screens (Hampton Research). These commercially available screens will allow us to sample 230 crystallization conditions; other screens available in the group of Dr. Jeruzalmi will also be tried. Once the ideal conditions for RTA crystallization are developed, we will soak RTA crystals with various concentrations of VPg (2-3-fold excess of RTA), and concentrate the drops by exposing them to dry air before mounting and freezing the crystals in liquid nitrogen. If the X-ray diffraction data acquired from these crystals yield weak electron density, we will vary crystallizing conditions to obtain crystals with higher VPg occupancy. X-ray diffraction data will be collected using Beamline X29A (CCNY) and the data will be processed with the HKL2000 program suite [111] and statistical software. The PI will incorporate the screening of VPg crystallization into the undergraduate biochemistry course offered by the Department of Sciences. Hampton Research provides crystallization screens

that are optimally designed to provide rapid screening of the crystallization of macromolecules. This is a highly effective approach to overcome the exhaustive search for suitable crystallization conditions; the formulation utilized evaluates 96 unique mixtures of pH, salts, polymers and organics, and their ability to promote crystal growth. In the event of unsuccessful crystallization, students will still gain valuable experiences in protein crystallography. X-ray diffraction data will be collected from several acquired crystals and compared for accuracy and consistency.

Expected Outcomes and Alternative Approaches. At the conclusion of these experiments, we *expect* to have a detailed description of interacting amino acid residues in RTA-VPg complex by probing the molecular basis of recognition between the proteins and employing X-ray crystallography. The technique requires fine crystals, and if we encounter difficulty in generating nicely diffractable crystals, we will send protein samples to Hauptman-Woodward Medical Research Institute (University of Buffalo, NY), which provides crystallization services.

NMR Spectroscopy: As an alternative to crystallography, we will employ NMR spectroscopy, a complementary technique used to map-out macromolecular interactions. NMR will require an isotopic enrichment with ^{13}C and ^{15}N of one of the proteins, and then the other; this will aid with NMR chemical shift assignments. We expect to identify chemical shift changes observed upon titration of RTA with VPg peptide that inhibits RIP activity. The PI and his students will employ NMR studies to map out interactions between RTA and VPg, gaining insight into the amino acid interactions between the proteins and realizing the sequence requirements for binding. To identify interacting amino acid residues and map out the interface of the binding site, we will analyze perturbations in resonances corresponding to amide N and H of uniformly ^{15}N-labeled RTA (or VPg as a control). The ^{1}H-^{15}N HSQC spectrum of RTA will allow us to assign sequence specific chemical shifts to the protein. Addition of stoichiometric amounts of VPg will result in the quantifiable perturbation of a number of backbone amide cross-peaks. Mapping the residues with the greatest chemical shift perturbations, in the presence and absence of VPg, will allow for the identification of these interacting residues. We will then fit the chemical shift perturbation data for each perturbed residue to an isotherm, calculated by the hyperbolic equation upon titration of ^{15}N-enriched RTA, with increasing amounts of VPg to derive the apparent K_d for each perturbed residue. This constant will be compared to the one acquired in the *Aim 1*. For isotopic enrichment with ^{13}C and ^{15}N, uniformly $^{13}C/^{15}N$ labeled recombinant RTA will be prepared to assist with NMR chemical shift assignments. The isotopically enriched protein will be obtained using a method developed by Bracken *et al.,* adapted to RTA [112].

Aim 2B: **Employ tools of molecular modeling/docking to map out RTA-VPg interactions.** After we identify the RTA-VPg interface residues, the PI and his students will employ bioinformatics tools; molecular modeling, docking and scoring algorithms will map out molecular interactions between RTA and VPg proteins, gaining insight into the dynamics of amino acid interactions. We will seek advice of Dr. Shaneen Singh and Emilio Gallicchio (Brooklyn College, CUNY), experts in molecular modeling. Molecular docking allows prediction of the orientation of two molecules relative to each other. Approaches include: (i) using complementary in shapes, and (ii) simulation of binding. Small molecule structures will be obtained from DrugBank 3.0. We are able to perform these structure-based virtual screening exercises because of readily available X-ray crystallographic structures for RTA (PDB ID 3HIO) and Shiga toxin (PDB ID 2GA4) proteins. The search for the correct binding mode (pose prediction) of the VPg constructs will be carried out by performing a number of trials and keeping those energetically best. This involves finding the correct orientation, and as most protein molecules are flexible, the correct conformation of the docked molecule. In order to explore a large search space, we will explore algorithms, specifically developed to keep track of previously discovered minima and guide the search into new regions [113,114]. The team will then apply affinity scoring functions to the energetically best pose, or *n* best poses for each of the RTA-VPg complexes, comparing the affinity scores for different complexes, thus providing their relative ranking-order. The assumption is that for a given molecule the best pose according to the affinity score is among the *n* saved poses identified with the dock score [115,116]. A large number of docking programs and search algorithms have been published. One criterion for classifying the underlying algorithms is how the proteins are threaded during docking. To start, we will use a program that builds the protein incrementally, starting from a docked 'base fragment' – FlexX [117]. Similarly, we will use a program, such as Ligand Docking (GOLD) [118], to thread the protein sequence in its entirety.

Expected Outcomes and Alternative Approaches. After the completion of this *Aim*, we will have a detailed description and identification of the amino acid residues that participate in the RTA-VPg binding interactions *in silico*. This will allow us to virtually substitute individual amino acids in the constructed models, and identify those substitutions that yield the lowest binding energy while retaining the overall structure and inhibitory characteristics. This will provide a future direction in the search for a novel peptide sequences that inhibit RIP activity of RTA and Shiga toxin RIPs. For those VPg deletion constructs whose structures are not available, we will resort to other techniques of protein structure prediction. As an alternative, we will apply 'threading' and homology modeling to determine these structures [119,120]. Hammerhead [121], AutoDock, and DOCK [122,123] programs may be used alternatively as docking algorithms. We will employ rational design of peptide therapeutics that will improve binding affinity of VPg for RTA or Shiga toxin and inhibition of their toxicities. Based on structure-activity relation, we will identify amino acids that promote isomerization, glycosylation, aggregation, and/or oxidation (Met, Cys, His, Trp, Tyr, etc.) and substitute them via site-directed mutagenesis (*Aim 2C*) to avoid these events [86,87,124].

***Aim 2C:* Site-directed mutagenesis of VPg to increase its inhibitory effect on RTA.** *In vitro* site-directed mutagenesis is an invaluable technique for studying protein structure-function relationships and gene expression, and for carrying out vector modifications [125]. Several approaches to this technique have been described [126-129]. These methods generally require single-stranded DNA (ssDNA) as the template and are labor intensive or technically difficult. We will use Stratagene's QuickChange® Site-Directed Mutagenesis Kit that will allow site-specific mutations to be performed in virtually any double-stranded plasmid, thus eliminating the need for subcloning into M13-based bacteriophage vectors. The system requires no specialized vectors, unique restriction sites, or multiple transformations. The process is rapid, generating mutants with greater than 80% efficiency. The positive control reaction for the mutagenesis will include pWhitescript 4.5-kb plasmid, which produces known mutations under the conditions of the reaction.

Expected Outcomes and Alternative Approaches. At the conclusion, we will successfully create a series of VPg constructs (e.g., constructs with point mutations, amino acid switching, and/or deletion/insertion of single or multiple amino acids) which, as predicted by the tools of bioinformatics, would have a stronger binding affinity for RTA and more pronounced inhibitory effect on its RIP activity. We will then express these constructs, and purify to homogeneity for further binding and enzymatic studies and testing.

Summary

Ribosome inactivating proteins (e.g., ricin and Shiga toxin) are highly toxic agents produced by the castor bean plant and pathogenic enteric gram-negative bacteria, respectively. Presently, there are no effective inhibitors of RIP activity. This proposal seeks to develop a novel peptide inhibitor of RIP toxicity, examine its interactions with RTA and Shiga toxin, and improve the potential to provide a therapeutic lead against these deadly toxins. This research is strongly collaborative in nature; it will provide excellent training opportunities for undergraduate and graduate students, in multiple biomedical and biophysical techniques, as well as data analysis. Additionally, by developing a vast network of partnerships among scientists from local, national and international institutions, this activity will enhance the infrastructure for research and education at John Jay College.

Tentative Timeline of the Proposed Activity: We expect to begin our proposed experiments in the fall of 2016, and anticipate for all studies outlined in this proposal, to be completed within 3 years, by the end of 2019 to early spring of 2020. Year 1 (2016-2017) will be devoted to purification of recombinant RTA and VPg proteins, and execution of fluorescence and ITC experiments that will characterize thermodynamics of RTA-VPg binding interactions (*Aim 1A*). We will also initiate crystallization studies during the first year (*Aim 2A*). During year 2 (2018), RTA-VPg interactions will be kinetically characterized by stop-flow anisotropy and fluorescence (*Aim 1A*). We will also probe catalytic assay for RIP activity and investigate VPg inhibitory effects, employing depurination assays (*Aim 1B*). VPg peptides will be sent to Dr. O'Brien's laboratory, who will examine inhibitory effects of VPg on ricin and Shiga toxins *in vivo* (*Aim 1C*). Crystallography of RTA-VPg complex, X-ray diffraction and NMR will begin during year 2 (*Aim 2A*) and continue into year 3 (2019). During year 3 (2019-2020), RTA-VPg binding studies we will performed by implementing extrinsic fluorophore labeling. Molecular modeling, docking and scoring algorithms, as well as site-directed mutagenesis, will be performed during year 3. The team will visit collaborators' laboratories, and get familiar with techniques and protocols described in this

activity. The laboratory technician will be responsible for preparation of routine biological media, plasmid preparation, protein purification and assays, equipment maintenance, etc. All research assistants will participate extensively in this project. The PI's central career goal is to establish a productive research program at John Jay College that engages students in modern experimental biomedical research techniques. In addition to enhancing the infrastructure of the College, this funding will be critical toward his career advancement, and will contribute to the maintenance of an on-going research program in an important area of plant defense mechanisms. We expect to generate enough data for at least one publication per each funded year and present our findings at the annual American Plant Biology Society and the Biophysical Society meetings. The funding will also foster networking and establish collaborations between the PI's and fellow scientists. This research is the collaborative effort of the PI, his students, and professional colleagues, and is an important contribution to his on-going research. It will provide opportunities for guest lecture invitations at other research universities, serving on grant review panels or journal editorial boards, further advancing the PI's career. The above activity will ensure the PI's competition for other funding mechanisms, such as collaborative grants. As an NIH supported laboratory in the Department, AREA support will foster the introduction of biomedical research to students not currently exposed; this is an essential resource for minority students interested in pursuing a career in biomedical research.

Mentoring Statement: The PI has developed his research career mentoring undergraduate and Master's students in a vibrant, diverse urban university system. All of the proposed projects are available for undergraduate student participation. Student researchers working in the PI's laboratory will have completed a minimum of their sophomore year and will co-enroll for the Undergraduate Research Internship course (FOS 402); this course is a capstone experience for the Forensic Science Major with hands-on teaching and laboratory training. The course is intended for students interested in graduate school and research careers. Since fall of 2015, the Department offers new majors in Cell & Molecular Biology and Toxicology; students in these majors are able to participate in this experimental course as an elective. The PI's proposed plan of research will fully support the objectives for this internship. Many students will have completed the Biochemistry course (CHE 315) concurrently with their lab research. CHE 315 course is coordinated and taught by the PI, preparing the students to participate meaningfully in the PI's research. The biochemistry lab course is a series of skill-building experiments, culminating in a high-level final experiment; competency in performance, knowledge, and written communication skills must be demonstrated by students at this point. This is a solid preliminary training for laboratory research. All of the students are participants of the PRISM (Program for Research Initiatives for Science and Math) Program, established at John Jay College and endorsed by President Obama. Launched in 2006, PRISM aims to provide science, technology, engineering, and math (STEM) students with faculty-led research experiences throughout their undergraduate career. It was created to improve student retention and graduation rates, increase student exposure and access to careers in STEM disciplines, and improve their competitiveness when applying to graduate schools. As a Hispanic-serving institution, John Jay is contributing to increased diversity in STEM fields. Students attend seminars and training programs to better prepare them for research. At the beginning of their junior year, students are matched with a faculty mentor. In addition to direct research experience, students are eligible for stipends for their research, conference travel grants, GRE preparatory help, advisement on graduate and professional school applications, and career planning. They can also look forward to guest lectures and informal seminars with research faculty, scientists, and other professionals. PRISM research experiences go beyond the traditional training received in the classroom; they include instruction on literature searches, project design, implementation, experimental sampling, data analysis, and scientific writing/presentation, as well as providing a community in which students are able to flourish. These experiences help to demonstrate that science is not exact, but an iterative process of questioning the world around us, providing students with the skills necessary to succeed in science beyond the classroom, and prepare them to join the global research community. All techniques and data analyses described in this proposal are an extension of laboratory classroom skills; the experiments lend themselves to a high degree of manageable compartmentalization and subsequent integration into the whole project. The research relies heavily on peers as co-researchers in its approach: experienced senior students are paired with incoming students. Since the experiments require a great deal of teaching/learning, and honing of technical skills, the PI is always accessible, working hands-on with individuals and the entire group. With this approach, students are immersed in science and incrementally learn techniques, quickly carrying high levels of responsibility.

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Anthony Carpi, PhD
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June 7, 2016

Proposal Review Committee
Academic Research Enhancement Award
National Institutes of Allergy and Infectious Diseases
National Institute of Health

Dear Review Committee,

I am writing this letter of recommendation in support of Dr. Artem Domashevskiy and his application to the National Institutes of Health Academic Research Enhancement Award. I am happy to support him in the project entitled "Development of a novel inhibitor of Ricin: A potential therapeutic lead against deadly Shiga and related toxins." The proposal focuses on the inhibition of the poison ricin and a related Shiga toxin produced by an enteric pathogenic bacteria by a viral peptide. Dr. Domashevskiy directs a research laboratory in the Department of Sciences at John Jay College with undergraduate and graduate students assisting him in his investigations of plants defense mechanisms. His proposed is a collaborative effort of his students and professional colleagues, and is an important contribution to his on-going success at John Jay College. Dr. Domashevskiy's laboratory is fully equipped with the facilities necessary for the successful completion of the project. I am confident that the results of this study will provide important new insights into the design of novel peptide inhibitors of plant phytotoxins. This research has excellent potential for success, and will enhance the environment of research and education at John Jay College.

I enthusiastically support his application the Award. Please feel free to contact me should you have any additional questions.

Sincerely,

[redacted]

Anthony Carpi, PhD

Department of Chemistry
Phone (212) 772-5330
Fax (212) 772-5332

June 15th, 2016

Proposal Review Committee
Academic Research Enhancement Award
National Institutes of Allergy and Infectious Diseases
National Institute of Health

Dear Committee,

It is a great pleasure to write this letter of collaboration for Dr. Artem Domashevskiy, in support of his Academic Research Enhancement Award. The project entitled "Development of a Novel Inhibitor of Ricin: A Potential Therapeutic Lead against Deadly Shiga and Related Toxins," encompasses a novel and interesting approach that may lead to new therapies and vaccines against deadly ricin and Shiga toxin poisoning. My laboratory is using biophysical methods and molecular biology to study protein-nucleic acid interactions and macromolecular assembly. We are particularly interested in mechanisms of assembly and how kinetics and equilibria influence the final composition of assembly complexes and ultimately biological function. Pertaining to this project, my laboratory will perform experiments requiring high lamp fluorescence intensity measurements, provide VPg constructs, investigate kinetics of the RTA-VPg interacts, and perform primer extension assays.

Dr. Domashevskiy has extensive experience in biophysical techniques including fluorescence spectroscopy and a solid molecular biology background. Recently, Dr. Domashevskiy and I have co-authored a manuscript describing a unique method for fluorescently labeling mRNA for use in biophysical studies a manuscript reviewing current knowledge on Pokeweed Antiviral Protein (PAP) and its inhibition by a viral peptide VPg. Dr. Domashevskiy's broad range of research experiences and productivity demonstrates his creativity and exceptional work ethic. He is well appropriate to receive the award in support of these studies.

Sincerely,

[redacted]

Dixie J. Goss, Ph.D.
Professor and Elion Endowed Scholar
Department of Chemistry
Hunter College, City University of New York

Albert Einstein College of Medicine
OF YESHIVA UNIVERSITY

Science at the heart of medicine

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June 8, 2016

Proposal Review Committee
Academic Research Enhancement Award
National Institutes of Allergy and Infectious Diseases
National Institute of Health

Dear Review Committee,

I am writing in support of the application submitted by Dr. Artem Domashevskiy who is applying for a National Institutes of Health Academic Research Enhancement Award.

I am pleased to be able to collaborate with Artem on his project entitled “Development of a novel inhibitor of ricin: A potential therapeutic lead against deadly Shiga and related toxins.” The research deals with the studies of a viral peptide and its inhibition of toxic ribosome inactivating proteins.

My laboratory has worked on ricin A-chain isolated from castor beans, expressed in *E. coli* and saporin L3 protein from soapwort leaves, expressed in excretion yeast vectors. We have developed exquisite assay systems for these difficult enzymes.

We are pleased to share these reagents with Dr. Domashevskiy for use in his research program. My laboratory is pleased to provide plasmids, reagents, and research advice to support Dr. Domashevskiy’s project. I am confident that the results of his study will provide interesting and important new insights into inhibition of ricin and Shiga toxin by a viral genome protein.

I enthusiastically support Dr. Domashevskiy’s application for the Academic Research Enhancement Award and look forward to our collaboration.

Please feel free to contact me should you have any additional questions.

Sincerely,
Vern L. Schramm

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4712

June 22, 2016

Proposal Review Committee
Academic Research Enhancement Award
National Institutes of Allergy and Infectious Diseases
National Institute of Health

Dear Review Committee,

I am pleased to collaborate with Dr. Artem Domashevskiy on his project entitled "Development of a Novel Inhibitor of Ricin: A Potential Therapeutic Lead against Deadly Shiga and Related Toxins." The proposal focuses on the inhibition of ribosome inactivating proteins, specifically ricin from castor bean plant and Shiga toxin from enteric bacteria by a viral peptide. Our laboratory is interested in defining the molecular mechanisms by which enterohemorrhagic *E. coli* cause hemorrhagic colitis and the hemolytic uremic syndrome. We have developed novel methods of Shiga toxin detection and investigate genetics and regulation of toxin synthesis. Our laboratory will test inhibitory effect of VPg from Dr. Domashevskiy laboratory on Shiga toxin (Stx) and ricin RIP activity in Vero cells. I am confident that the results of this study will provide important new insights into the design of novel peptide inhibitors of cytotoxic RIPs.

Sincerely,

[redacted]

Alison O'Brien, Ph.D.
Professor and Chair,
Department of Microbiology and Immunology
Uniformed Services University
of the Health Sciences
Bethesda, MD 20814

Learning to Care for Those in Harm's Way

DEPARTMENT of CHEMISTRY & BIOCHEMISTRY
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June 22, 2016

Proposal Review Committee
Academic Research Enhancement Award
National Institutes of Allergy and Infectious Diseases
National Institute of Health

Dear Review Committee,

I am pleased to collaborate with Dr. Artem Domashevskiy on his project entitled "Development of a Novel Inhibitor of Ricin: A Potential Therapeutic Lead against Deadly Shiga and Related Toxins." The proposal focuses on the inhibition of ribosome inactivating proteins, specifically ricin from castor bean plant and Shiga toxin from enteric bacteria by a viral peptide from turnip mosaic virus.

My research group laboratory is interested in the molecular mechanisms that underlie the faithful transmission of genetic information. We are currently focused on two areas, the machinery associated with DNA replication and 2) nucleotide excision repair. The goal of research in my laboratory is to provide a structural view of these "machines" and, by concomitant application of biochemical approaches, to provide a fundamental understanding of the underlying mechanisms. Our approaches are primarily structural, X-ray crystallography or cryo-electron microscopy.

My laboratory will aid Dr. Domashevskiy in the crystallization of the RTA-VPg complex, recording of X-ray diffraction, and data analysis. My research group has extensive experience in macromolecular structure determination using X-ray crystallography. The proposed crystallization trials will be greatly facilitated by availability of a complete automated crystallization facility at the CUNY-Advanced Science Research Center, just steps away from my research laboratory. The available instruments include: 2 Gryphon protein crystallization robots (at 25°C and 4°C), 2 Minstrel crystallization tray imagers (at 25°C and 4°C), and an Alchemist crystallization screen-maker. Lastly, my research group is actively measuring X-ray ray diffraction and determining crystal structures. As such, when diffraction data for the targeted entities becomes available, structure determination will proceed smoothly.

I am confident that the results of this study will provide important new insights into the design of novel peptide inhibitors of cytotoxic RIPs.

Sincerely,

[redacted]

David Jeruzalmi, Ph.D.

UNIVERSITY of DENVER
NATURAL SCIENCES & MATHEMATICS
Chemistry & Biochemistry

Artem V. Domashevskiy, Ph.D.
Department of Sciences
John Jay College of Criminal Justice
City University of New York
New York, NY 10019

14 June 2016

Dear Dr. Domashevskiy,

Your proposal to study the thermodynamic and binding properties of the RTA-VPg interaction has my enthusiastic support.

We have been using isothermal titration calorimetry as a primary technique to measure protein-peptide interactions in our research at the University of Denver for our work on radical-S-adenosylmethionine dependent peptide modification pathways. We currently operate a Nano ITC from TA Instruments, which is located in our lab. I have published results using ITC in the Journal of Biological Chemistry and, most recently, in FEBS Letters. We are happy to expand our knowledge of protein-peptide interactions by facilitating your research goals.

Best wishes for success on your grant application.

Kind regards,

[redacted]

John A. Latham, Ph.D.
Department of Chemistry and Biochemistry
Molecular and Cellular Biophysics Program
University of Denver
Denver, CO 80232

AUTHENTIFICATION OF KEY BIOLOGICAL AND CHEMICAL RESOURCES

All key resources for this proposal will be authenticated to enhance the reproducibility of our results, as appropriate and according to NIH policy.

Key Biological Resources that will be utilized in this proposal include:

Cell lines: DH5α (*E. coli* competent cells), BL21(DE3) (*E. coli* competent cells), Vero cells (from kidney epithelial cells extracted from African green monkey).

Cell lines MAX Efficiency® DH5α™ Competent Cells will be purchased from ThermoFisher Scientific (Invitrogen™ distributer; catalog number 18258012), and come with the company's authentication certificate.

Cell lines One Shot® BL21(DE3) Chemically Competent *E. coli* will be purchased from ThermoFisher Scientific (Invitrogen™ distributer; catalog number C600003), and come with the company's authentication certificate.

Cell lines Vero Cell Line will be purchased from Sigma-Aldrich® (84113001-1VL) and come with the company's authentication certificate.