

AWARD NUMBER: W81XWH-20-1-0081

TITLE: Cytoplasmic Suppression of Inflammatory Signaling

PRINCIPAL INVESTIGATOR: Brian Aneskievich, Ph.D.

CONTRACTING ORGANIZATION: University of Connecticut

REPORT DATE: JULY 2022

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				<i>Form Approved</i> OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE JULY 2022		2. REPORT TYPE Annual		3. DATES COVERED 01 July 2021-30 June 2022	
4. TITLE AND SUBTITLE Cytoplasmic Suppression of Inflammatory Signaling				5a. CONTRACT NUMBER W81XWH-20-1-0081	
				5b. GRANT NUMBER PR192438	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Brian Aneskievich, Ph.D. E-Mail: brian.aneskievich@uconn.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Connecticut 438 Whitney Rd Extension Office for Sponsored Programs Unit 1133 Storrs, CT 06269-1133				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Development Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES Published data is in journal article (Biomolecules. 2020 Nov 10;10(11):1531) included as appendix in previous year report.					
14. ABSTRACT The anti-inflammatory protein ABIN-1 (also referred to as TNIP1), independently and together with one of its binding partners, A20, has been linked to inflammatory bowel disease (IBD). Cells deficient in ABIN-1/TNIP1 mount an excessive inflammatory response because they are hypersensitive to debris shed from bacteria. Thus ABIN-1 suppresses cytoplasmic signaling steps leading to inflammation. Our data submitted with the original application and award-supported publication stemming provide new findings for to confirm and extend our hypothesis of that conformational flexibility for ABIN-1 is likely to affect its function. This concept of flexibility helps resolve i) how ABIN-1 interacts with its diverse protein partners, ii) the relatively short duration time of ABIN-1 protein during inflammation, and iii) what protein characteristics about it could be co-opted for future cell-based studies of its anti-inflammatory capacity.					
15. SUBJECT TERMS ABIN-1, A20-binding Inhibitor of NF-kappa B activation-1 (also known as TNIP1); IBD, inflammatory bowel disease; IEC, intestinal epithelial cell					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	10	19b. TELEPHONE NUMBER (include area code)

TABLE OF CONTENTS

	<u>Page</u>
1. Introduction	4
2. Keywords	4
3. Accomplishments	4
4. Impact	6
5. Changes/Problems	6
6. Products	7
7. Participants & Other Collaborating Organizations	7
8. Special Reporting Requirements	9
9. Appendices	follow page 8

1. INTRODUCTION:

Subject: Inflammatory bowel disease (IBD) at the cell biology level culminates from a “perfect storm” of pre-disposing genetics, local stress signals, and cell-cell interactions. Together these factors result in unrestrained inflammatory signaling within intestinal epithelial cells (IEC) resulting in their damage and loss. The most frequent clinical presentations of such injury are classified as ulcerative colitis and Crohn's disease. Current therapies include relatively non-specific immune system chemical intervention and more-targeted biologic intervention with antibodies.

Purpose: The above therapies have significantly restricted application due to adverse drug effects, infusion reactions, bone marrow failure/blood cell reduction, skin reactions, and increased risk of new or emergence of latent bacterial or viral infections and malignancy. There is significant and urgent need for cell biology level research to discover and validate new therapeutic targets that would allow for drug identification outside of current anti-inflammatory, anti-immune, and anti-receptor approaches. The core of this project is that the ABIN-1 (also known as TNIP1) protein is such a new target justified by its anti-inflammatory function, its genetic relevance to IBD, and unique protein shape properties suggested by preliminary data included with the proposal submission.

Scope: IBD diagnosis for a U.S. cohort of over 3 million adults is associated with appreciably reduced quality of life, medical treatments with significant likelihood of adverse reactions, and organ function decline often resulting in surgical intervention. Our novel approach to ABIN-1 /TNIP1 protein shape (conformation) is likely to reveal new functional and structural biology insights as to its ability to repress inflammatory signaling in IEC. Results from this project will guide our future research aims to further demonstrate the ABIN-1 /TNIP1 protein as a logical therapeutic target possibly enhancing its anti-inflammatory role. Such ultimate clinical translation of these results would have significant impact for numerous IBD patients.

2. KEYWORDS:

ABIN-1, A20-binding Inhibitor of NF-kappa B activation-1 (also known as TNIP1); IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; IDP, intrinsically disordered protein; NMR, nuclear magnetic resonance; TNFalpha, tumor necrosis factor alpha

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Major Task 1: Multiple analyses of ABIN-1 amino acid sequence via local computer-based programs and web-based servers. As planned, completed in project year 1.

Major Task 2: Generation of conformational variants and their in vitro assessment. As planned, first phase protein variants were generated and subjected to biophysical analysis. This Major Task was partially completed in project year 2 and carries forward into the no cost extension year.

Note: Overlapping much of the end of project year 1 and early in project 2, we were under COVID-19 restrictions which reduced access to some laboratory facilities along with supplies backordered because of supply chain issues. We continued active remote work on the project addressing data analysis and in interpretation along with computer-based protein analysis.

Major Task 3: Assessment of conformational variants in context of mammalian cell response to inflammatory triggers. This Major Task is will be addressed in the no cost extension year.

What was accomplished under these goals?

For the reporting period covering project year 2, **Major Task 2** was a **major activity** and was mostly completed. The **specific objectives** for this activity were to generate (Figure 1) and analyze with biochemical (protease sensitivity) and biophysical methods (Figure 2) protein sequence variants (disorder-to-order) of

AHD1-UBAN domain of ABIN-1. In brief, our **findings** confirmed and extended preliminary data contained in the submitted proposal and project year 1 results. Specifically, that the ABIN-1 / TNIP1 protein throughout its entire length and especially in specific subdomains (AHD1 and UBAN) are, using field-specific terminology, “intrinsically disordered”, meaning that there is no rigid, permanent shape to the protein. The **activity findings** illustrated in Figure 1 confirm our hypothesis that this disorder can be significantly altered with just one amino acid variation (see E444F on graph) as compared to the wild-type sequence (WT). Flexibility previously investigated by protease sensitivity is better addressed with more recently qualified biophysical means, such as light scattering. This does not change the goal of the task; it applies a more-quantifiable technique. The flexibility portion of task 2 will be addressed in the coming year.

For the reporting period covering project year 2, **Major Task 2** was a **major activity** and, as planned, several biophysical analyses of proteins' flexibility were addressed. The specific objectives were to use a laboratory-generated ABIN-1 / TNIP1 protein subregion and subject it to measurements of protein flexibility by NMR (Figure 2). In brief, new **major findings** establish for the first-time biophysical proof the flexibility present in the AHD1-UBAN region is significantly affected by even one amino acid replacement. Spectral peaks (see yellow arrows, Figure 2) not similarly detected with the wildtype protein were detected for the E444F variant. The peak patterning supports **gain of order** as expected from Figure 1. Because anti-inflammatory function is dependent on ubiquitin binding, we also tested conformation of the AHD1-UBAN region with addition of triubiquitin (triUB, blue peaks, yellow arrowheads). The results show gain of order (more stable shape conformation) is consistent with triubiquitin binding. Possible changes in strength of binding will be tested in future studies.

These findings partly cover the subtasks as in the approved SOW for Major Task 2, some of which are delayed because of COVID-19-related disruptions and will be addressed in the no cost extension.

These **findings** can be qualitatively **summarized** in that they prove subregions within ABIN-1 / TNIP1 protein are highly flexible, a characteristic governed by specific amino acid residues (demonstrated by E444F variant). Such single amino acid residue level resolution of the region's function is **wholly new** information and likely to improve our **understanding** of ABIN-1 / TNIP1 function in limiting inflammatory disease.

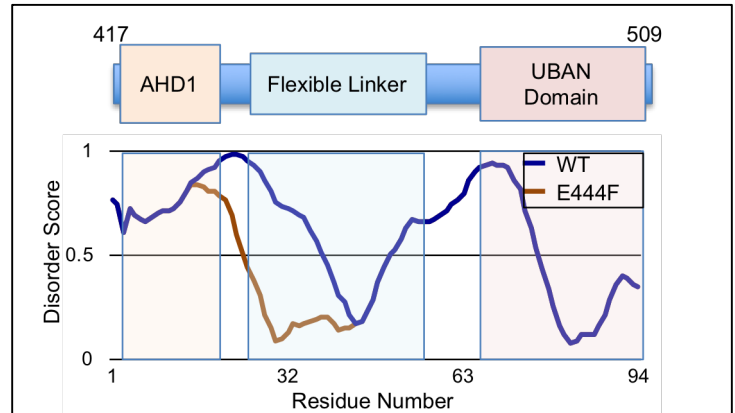


Figure 1. Example of Major Task 2 activity. The designed protein sequence variant (E444F) significantly alters wildtype (WT) protein's regional shape (conformation) as detected by computer-based analysis. Disorder (scored 0–1 on y-axis) along all of regions' amino acids (residue positions on x-axis) assessed by our bio-informatics studies.

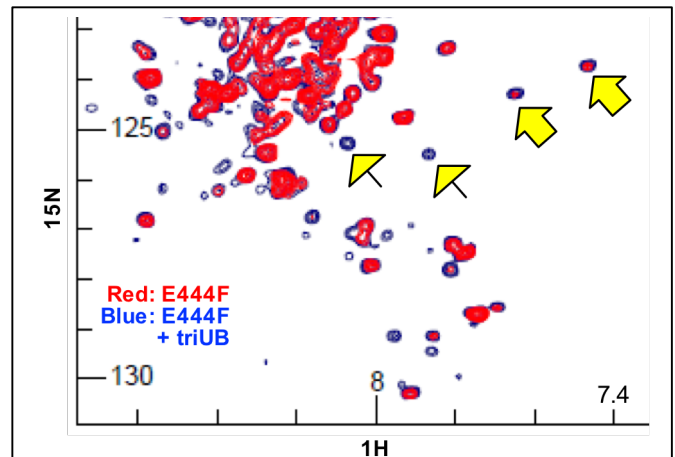


Figure 2. Example of Major Task 2 activity. NMR spectral analysis shows peak detection for the conformational variant E444F. Only partial areas of spectra are included in figure for space considerations. triUB, triubiquitin added as protein binding partner. For the E444F variant protein alone versus it binding to triubiquitin, contrast red versus blue patterns.

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

A training opportunity was available to the Graduate Assistant (R. Shamilov) who was partially supported by the project. He was able to assist in training a beginning PhD candidate (M. Samulevich). Mr. Shamilov was able to guide and inform Mr. Samulevich's entry into inflammatory disease research both via technological and educational interactions. Mr. Shamilov prepared and gave two department-level seminars on new project findings during the reporting year. He completed his PhD in 2021 with partial support from this project.

How were the results disseminated to communities of interest?

We had planned to present project findings at inflammation biology and cell signaling conferences. However, this were not possible because travel and in-person gathering were significantly restricted because of COVID mitigation practices. Two department-level seminars on the project were presented.

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

At this time, **Major Task 2** second phase is near completion with only a few biophysical tests remaining. These and other Major Task 2 results and findings reported the previous project year will be assembled for manuscript submission. We will also address **Major Task 3** covering assessments of ABIN-1 / TNIP1 protein in living cells grown in petri dishes. This connects computational and biophysical studies (Major Tasks 1 and 2) with the protein's biological function (Major Task 3).

4. IMPACT:

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

The impact from the reporting year is the discovery that flexibility of the anti-inflammatory protein ABIN-1 / TNIP1 is highly sensitive to even single amino acid changes, a trait not previously known for the protein. The biophysical assays, such as NMR studies (Figure 2) validated the biocomputational approach (Figure1). This finding provides a new understanding of ABIN-1 / TNIP1 interaction with its partner proteins as the "brakes" on runaway inflammation characterizing IBD. Additionally, both the investigational approach starting with computer-based assessments leading to biophysical assays worked not only for this particular project but also can provide a model for "structure – function" investigations of other important cell signal-controlling proteins.

What was the impact on other disciplines?

While proper functioning of the ABIN-1 / TNIP1 protein is integral to blocking inflammatory signaling in pathologies such as IBD, our establishment of it as an "**intrinsically disordered**" protein informs the study in **other disciplines** of such flexible proteins in a variety of normal physiology and diseases.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report.

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

Due to restricted in-person lab time from COVID policies, there were delays and reduced time in accessing campus core equipment facilities for some protein measurements. There was less expenditure on supply and facility fees as we focused on computer-based remote-capable assessments, but we expect to use the funds as approved with the no cost extension. We have identified alternative resources for the back-ordered supplies.

Changes that had a significant impact on expenditures

As above, due to reduced in-person lab time from COVID restrictions, laboratory supply and core facility equipment costs had lesser expenditures as we focused on computer-based, remote-capable assessments. Those funds will be used in the coming year as we anticipate occupancy and pandemic measures will continue to be lifted or paused.

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

Nothing to report.

6. PRODUCTS:**Publications, conference papers, and presentations**

We had planned to present project findings at relevant conferences covering inflammation biology and cell signaling. These were not possible because travel and in-person gathering were significantly restricted during the reporting period because of COVID mitigation practices.

Website(s) or other Internet site(s)

Nothing to report.

Technologies or techniques

Nothing to report.

Inventions, patent applications, and/or licenses

Nothing to report.

Other Products

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:**What individuals have worked on the project?**

Name:	Brian J. Aneskievich
Project Role:	Principal Investigator
ORCID ID:	https://orcid.org/0000-0001-7440-1948
Effort:	No change from project submission
Name:	Rambon Shamilov
Project Role:	Graduate Assistant
ORCID ID:	https://orcid.org/0000-0003-0375-7482
Effort:	No change from project submission

Name:	Olga Vinogradova
Project Role:	Co-Investigator
ORCID ID:	https://orcid.org/0000-0001-5101-7361
Effort:	No change from project submission
Name:	Victoria Robinson
Project Role:	Co-Investigator
ORCID ID:	https://orcid.org/0000-0003-3838-6394
Effort:	No change from project submission

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

No change in support.

What other organizations were involved as partners?

Academic Institution

Organization Name: University of Connecticut, Department of Pharmaceutical Sciences

Location of Organization: Storrs, CT

Partner's contribution to the project:

Financial support;

A cost-share for the support of the Graduate Assistant on the project is in place and has not changed.

8. SPECIAL REPORTING REQUIREMENTS

Collaborative awards

There is no collaborative award component to this project.

Quad chart

A quad chart for the project report is included in the appendix.

Award chart

An award chart for the project report is included in the appendix.

9. APPENDICES

Quad Chart: attached

Award Chart: attached

Cytoplasmic Suppression of Inflammatory Signaling

PR192438

W81XWH2010081



PI: Brian Aneskievich

Org: University of Connecticut

Award Amount: \$322,000 (total costs, direct + indirect)

Study/Product Aim(s)

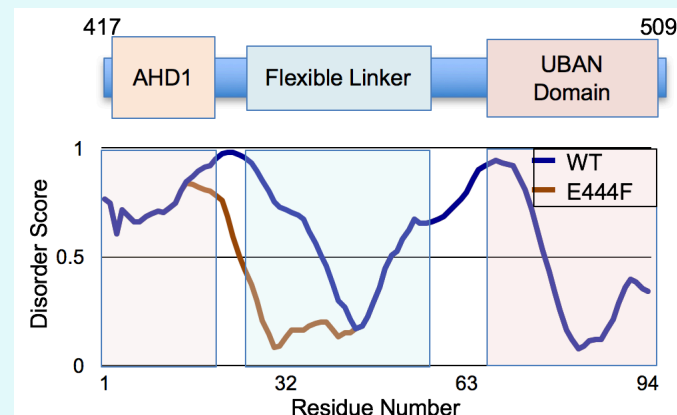
- 1)Complete bioinformatic analysis of ABIN-1/TNIP1
- 2)Extend biochemical and biophysical testing of laboratory-generated ABIN-1/TNIP1 protein regions
- 3)Conduct biological evaluation of ABIN-1 /TNIP1 conformational variants versus standard protein to test effect on inflammatory signaling

Approach

Synergistic protein conformation algorithms used for design of protein sequence variant – see bar graphic and plot, top right.

Laboratory-generated ABIN-1/TNIP1 regions were subjected to nuclear magnetic resonance (NMR) spectral analysis yielding new information on interaction with partner proteins – described in report.

Wildtype and variant ABIN-1/TNIP1 proteins, including those plotted top right, will be tested in cell-based assays for signal repression.



Accomplishment: Successful design, production, and analysis of wildtype (WT) and conformational i.e., shape variant (E444W) of key ABIN-1 / TNIP1 region (417 – 509). Disorder (flexibility) scored 0 – 1 (y-axis) along amino acid residue positions (x-axis).

Timeline and Cost

Activities	CY	20	21	22	23
Bioinformatic analysis (completed)					
Biochemical & biophysical testing (1 st phase done; 2 nd ongoing)					
Biological evaluation (initiated, continuing in no cost ext. year)					
Estimated Budget (\$K) <u>direct</u>		\$49	\$25	\$48	

Updated: (July 25, 2022)

Goals/Milestones (for the reporting period)

CY 2021/2022 Goal – Generation and biophysical evaluation of conformational variants (project 2nd year)

- ☒ Variant design and biocomputational comparison against wildtype (see above diagram)
- ☒ First phase biophysical testing, completed (see report text)
- ☐ Ongoing – results confirmation in progress along with data assembly for second publication

Comments/Challenges/Issues/Concerns

- Restricted in-person lab time from COVID mitigation practices led to reduction in supply and facility fees expenditures as we focused on computer-based remote-capable assessments. Those funds will be used in the coming no cost extension year.

Budget Expenditure to Date

Projected Expenditure: direct costs, \$100K

Actual Expenditure: direct costs, ~\$72K

Award Log Number PR192438: Cytoplasmic Suppression of Inflammatory Signaling

PI: Brian Aneskievich, University of Connecticut, CT

Budget: Total Award Cost, \$322,000
(2 year plus approved no cost ext. year)



Topic Area: Inflammatory Bowel Diseases

Mechanism: Discovery Award

Research Area(s): SCS Coding 0100 Cell Biology
0107 Signal Transduction

Award Status: 01-JUL-2020 TO 30-JUN-2022 (initial)
TO 30-JUN-2023 (approved no cost extension)

Study Goals:

Goal: Study goals remain those as in approved project and reported previously. In brief, to test our central hypothesis that ABIN-1 (also known as TNIP1) protein has atypical and distinct shape (conformational) characteristics required for its function as an inflammatory signal repressor. This goal is a clear departure from typical lock-and-key thinking of protein-protein interactions regulating responses in inflammatory bowel disease. The ABIN-1/ TNIP1 protein qualities we newly predict may move the protein to candidacy as a novel therapeutic target. Testing ABIN-1/TNIP1 innate protein characteristics will significantly inform understanding of how it limits inflammation by recognizing signal-repressing partners (this project) and guide additional research to possible anti-inflammatory clinical interventions (future studies).

Specific Aims (SA):

SA1. Complete in silico analysis of ABIN-1 / TNIP1 protein. Several validated algorithms independently assessing intrinsically disordered proteins were used in an integrated manner, advantageous to corroborating shape (conformational) analysis.

SA2. Extend in vitro biochemical and biophysical testing. Full length wildtype and variant ABIN-1 / TNIP1 proteins or subdomains as required by analytical approach, engineered according to partner-binding and disorder-to-order design, are being tested (e.g., via protease sensitivity, NMR "fingerprint" experiments).

SA3. Conduct biological evaluation of ABIN-1 / TNIP1 variants versus proteins of standard (wildtype) conformation in repressing inflammation signaling in cultured intestinal cells by examining effects on inflammation-associated gene expression.

Key Accomplishments and Outcomes:

Protein conformation results: We found key amino acid positions along ABIN-1 / TNIP1 extensively control regional protein shape, along further distances than had been previously known providing significant support for the project hypothesis.

Patents: None to date.

Funding Obtained: No new funding obtained during this reporting period.