

AWARD NUMBER: W81XWH-16-1-0322

TITLE: Novel Hypoxia-Directed Cancer Therapeutics

PRINCIPAL INVESTIGATOR: Fraydoon Rastinejad

CONTRACTING ORGANIZATION: Sanford Burnham Prebys Medical Discovery Institute
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REPORT DATE: July 2017

TYPE OF REPORT: Annual

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

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1. REPORT DATE July 2017		2. REPORT TYPE Annual		3. DATES COVERED 1 July 2016 – 30 June 2017	
4. TITLE AND SUBTITLE Novel Hypoxia-Directed Cancer Therapeutics				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-16-1-0322	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Fraydoon Rastinejad E-Mail: frastinejad@sbdpdiscovery.org				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Sanford Burnham Prebys Medical Discovery Institute 10901 North Torrey Pines Rd La Jolla, CA 92037-1005				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel 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					
14. ABSTRACT The hypoxia-inducible factors (HIFs) are proteins that have remained largely unexplored as possible targets for cancer therapeutics; yet there is widespread recognition these proteins directly contribute to the progression of many types of human solid tumors. Having previously discovered that the HIF proteins contain small-molecule binding pockets within their architectures, we sought to identify small-molecules that bind and act directly through the HIF proteins to block their functions. Over the first year of funding, we produced large quantities of highly pure HIF proteins (both HIF-1alpha/ARNT and HIF-2alpha/ARNT) and used these proteins for conducting high-throughput screens with 32,000 different small-molecules. The screen was efficient and a mass-spectrometry based assay, and the "hits" obtained were further confirmed by a counter-screen using another protein unrelated to HIFs. To understand the relative binding affinities of the hits, we carried out a biochemical assay to measure the equilibrium dissociation constants of the small molecules in binding to the HIF complexes. Over the coming year, we plan to examine these small-molecules in a number of cell-based studies to identify which ones are potent inhibitors that may be useful as possible use as anti-cancer therapies.					
15. SUBJECT TERMS Hypoxia-inducible factors, mass-spectrometry, drug discovery, kidney cancer					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES 12	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (include area code)

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1. INTRODUCTION:

The hypoxia-inducible factors (HIFs) are transcription factors that bind to DNA and control genetic programs required for driving solid tumor growth in cancers of kidney, pancreas, stomach, colon and skin. We seek the discovery of drug-like molecules that bind to and inhibit HIF proteins. We will use purified HIF proteins and a chemical library of 32,000 small molecules that have drug-like properties. We will employ a high-throughput screen to select small-molecules that bind tightly to the HIF proteins and further identify those compounds that can also inhibit the HIF functions required for tumor progression. Our goal is to identify inhibitors for HIF proteins that are effective in cell culture studies, and this goal will be met within the two-year funding period of this application.

2. KEYWORDS:

Hypoxia-inducible factors, mass-spectrometry, drug discovery, kidney cancer.

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

What were the major goals of the project?

All the original milestones listed for the first 12 months of the grant period were:

- 1) Produce 15 mg of HIF-1 α /ARNT and HIF-2 α /ARNT heterodimers.
- 2) Conduct separate AS-MS screens using 32,000 small-molecules for HIF-1 α /ARNT and HIF-2 α /ARNT
- 3) Counter-screen all hits from above and eliminate non-specific molecules.
- 4) Measure the K_d values for top hits with HIF-1/ARNT and HIF-2 /ARNT using SPR assay.

What was accomplished under these goals?

We essentially met the year-1 major goals of the project. We were able to successfully co-express in *E. coli*, each of HIF-1 α /ARNT and HIF-2 α /ARNT heterodimers, consisting of the bHLH-PAS-A-PAS-B domains of these proteins, as originally planned. Shown in Figure 1 are representative SDS-PAGE (commassie-blue stained) gels showing the level of purity obtained in each case. We used each of these purified proteins in AS-MS screens, in which a library of ~32,000 distinct compounds was used to detect those compounds that could bind best to each of these two heterodimers. Figure 2 shows a representative cross-section of the AS-MS data obtained when using each of HIF-1 α /ARNT and HIF-2 α /ARNT heterodimers. As planned, we also ran a similar AS-MS screen, with the same set of 32,000 compounds, on the distantly related heterodimer of Aryl hydrocarbon receptor (AHR) with ARNT. This counterscreen step allowed us to remove the "non-specific hits", i.e., those compounds that were not specific binding molecules for either HIF-1 α and HIF-2 α subunits. Finally, we established a biochemical binding assay (SPR) that allowed us to measure the direct binding of the small-molecule hits for the HIF-1 α /ARNT and HIF-2 α /ARNT heterodimers. Figure 3 shows a representative example of the SPR assay results. We also developed and use a more sensitive and rapid biochemical binding assay, based on microscale thermophoresis (MST). The MST assay uses less protein and is less expensive to run, therefore, we chose to more routinely use this assay in place of SPR assays, to detect the K_d for compound binding to each heterodimer.

(Please see Figures 1, 2 and 3 at end of report)

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

Two postdoctoral scientists were trained over the past year. A) Dalei Wu contributed to the project's goals over the past year in all aspects, and has recently taken a new position as Professor in China. B) Jingping Lu contributed to the project, and has received training in mass-spectrometry based high-throughput screening and biochemical binding studies involving proteins and small-molecules.

How were the results disseminated to communities of interest?

The PI presented lectures with data obtained over the past year, a) Conference on "Adaptations to Hypoxia in Physiology and Disease" in Whistler, British Columbia, Canada., b) ASBMB annual meeting in Chicago, USA, c) Dana-Farber Cancer Institute (Boston, USA), and Oxford University (Oxford, UK).

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

Over the coming year, we plan to a) carry out dose-response cell-based reporter transcription assays to assess which compounds may be inhibitors from those identified to bind to each of HIF-1alpha/ARNT and HIF-2alpha/ARNT; b) carry out cytotoxicity tests for inhibitors that are identified; c) carry out co-immunoprecipitation studies to determine if the inhibitors act by disrupting heterodimer stabilities of each heterodimer; d) carry out gene profiling studies, using RT-qPCR, to examine the effects of putative inhibitors on known target genes; and e) confirm if the compounds are impacting these target genes through HIF-1alpha/ARNT and HIF-2alpha/ARNT proteins in cells (using si-RNA).

- 4. IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

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

The findings show for the first time, that it is possible to inhibit both HIF-1alpha/ARNT and HIF-2alpha/ARNT proteins using small-molecules. The findings also show for the first time, that the mechanism of action for inhibiting these proteins is through disruption of their dimer interfaces. Both of these findings are viewed as proof-of-principle that should more widely (other laboratories and pharmaceutical industry) encourage drug-discovery against the HIF-1alpha/ARNT and HIF-2alpha/ARNT complexes, for a variety of unmet human diseases including cancers.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

- 5. CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not

previously reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:

Changes in approach and reasons for change

No changes.

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

None.

Changes that had a significant impact on expenditures

None.

Significant changes in use or care of human subjects

N/A.

Significant changes in use or care of vertebrate animals.

N/A.

Significant changes in use of biohazards and/or select agents

N/A.

6. PRODUCTS: Publications, conference papers, and presentations

Report only the major publication(s) resulting from the work under this award.

Journal publications.

1. Wu D, **Rastinejad F**. Structural characterization of mammalian bHLH-PAS transcription factors. *Curr Opin Struct Biol*. 2017 Apr;43:1-9. doi: 10.1016/j.sbi.2016.09.011. Epub 2016 Oct 6. PMID:27721191. Federal support acknowledged.
2. Wu D, Su X, Potluri N, Kim Y, **Rastinejad F**. NPAS1-ARNT and NPAS3-ARNT crystal structures implicate the bHLH-PAS family as multi-ligand binding transcription factors. *Elife*. 2016 Oct 26;5. pii: e18790. doi: 10.7554/eLife.18790. PMID: 27782878
Federal support acknowledged.

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

None.

Other publications, conference papers, and presentations.

None.

- **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**

None.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Fraydoon Rastinejad

Project Role: PI

Researcher Identifier: 0000-0002-0784-9352

Nearest person month worked: 3

Overall project management (together with co-I), including oversight, interpretation and quality control of all data, generated through all tasks and subtasks, and ensuring alignment of the team's daily activities with specific goals and timelines of the grant application. Contribution to Project: In SA1, Subtask 2: conduct separate AS-MS screens using 32,000 small-molecule library for each of HIF-1 α /ARNT and HIF-2 α /ARNT purified heterodimers. Subtask 3: Counter-Screen all hits from Subtask 2 against AHR/ARNT heterodimer and eliminate non-specific binding molecules (i.e. small molecules that also show binding to AHR/ARNT). In SA2, Subtask 4: RT-qPCR to evaluate target gene expression in response to inhibitors in cancer cell lines Subtask 4: Evaluate target engagement of compounds in cells using si-RNA.

Name: Sepideh Khorasanizadeh

Project Role: Co-Investigator

Researcher Identifier: 0000-0003-0372-0524

Nearest person month worked: 3

Co-management of team's daily activities. Training of scientists and review of data generated related to the biochemical, biophysical, and cell-based studies. Contribution to Project: In SA1, Measure the K_d values for top hits with HIF-1 α /ARNT and HIF-2 α /ARNT using biochemical binding assays. In SA2, Subtask 2: Cytotoxicity assay. Subtask 3: Co-IP studies to see if molecules disrupt heterodimer stability in cells. Subtask 4: RT-qPCR to evaluate target gene expression in response to inhibitors in cancer cell lines. Subtask 4: Evaluate target engagement of compounds in cells using si-RNA.

Name: Wu, Dalei
 Project Role: Postdoctoral Associate/Collaborator
 Researcher Identifier: 0000-0002-7880-1532
 Nearest person month worked: 5
 Contribution to Project: In SA1, Subtask 2: conduct separate AS-MS screens using 32,000 small-molecule library for each of HIF-1 α /ARNT and HIF-2 α /ARNT purified heterodimers. Subtask 3: Counter-Screen all hits from Subtask 2 against AHR/ARNT heterodimer and eliminate non-specific binding molecules (i.e. small molecules that also show binding to AHR/ARNT). In SA2, Subtask 1: Dose-response cell-based reporter transcription assays. Subtask 3: Co-IP studies to see if molecules disrupt heterodimer stability in cells.

Name: Chandra Vikas
 Project Role: Staff Scientist
 Researcher Identifier: 0000-0002-0768-0673
 Nearest person month worked: 4
 Contribution to Project: Participated in SA1 subtasks 1 and 2, by helping to develop the necessary purification strategy and quality control steps required to generate high-quality proteins for AS-MS screens. He additionally contributed to the SPR assay implementation in measuring the K_d values of ligands for HIF proteins (SA1).

Name: Jingping Lu
 Project Role: Postdoctoral Associate
 Researcher Identifier: 0000-0001-5620-0819
 Nearest person month worked: 2
 Contribution to Project: Assisted with SA1 subtasks 2 and 3, which involved producing the assay development and implementation related to mass-spectrometry based high-throughput screening and counter-screening.

Name: Nalini Potluri
 Project Role: Research Assistant II
 Researcher Identifier: 0000-0002-4921-4742
 Nearest person month worked: 4
 Contribution to Project: In SA1, Subtask 1: Produce 15 milligrams of highly purified (>99%) HIF-1 α /ARNT and HIF-2/ARNT heterodimers, as well as AHR/ARNT (for counter-screening) to be employed in Affinity-Selection Mass-Spectrometry (AS-MS) Screens and biochemical binding studies. Measure the K_d values for top hits with HIF-1 α /ARNT and HIF-2 α /ARNT using biochemical binding assays. In SA2, Subtask 1: Dose-response cell-based reporter transcription assays. Subtask 4: RT-qPCR to evaluate target gene expression in response to inhibitors in cancer cell lines. Subtask 4: Evaluate target engagement of compounds in cells using si-RNA.

Name: Xiaoyu Su
 Project Role: Research Assistant II
 Researcher Identifier: 0000-0002-0509-1007
 Nearest person month worked: 6
 Contribution to Project: Participated in preparing for meeting the goals of SA2 subtasks 1 and 3, by developing the necessary cell-based assay tools, reagents and techniques to initiate and carry out a) dose-reponse cell based reporter transcription assays, b) co-immunoprecipitation studies.

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

If there is nothing significant to report during this reporting period, state “Nothing to Report.” If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

New NIH R01 grant funded on June 1, 2017; 1 R01 GM120532-01. No overlap. PI and co-I have effort on this new grant. R01 DK094147 is complete/closed. R01 DK097475 is complete/closed. R01 DK101520 is complete/closed. CSRA 15-06FL is complete/closed.

What other organizations were involved as partners?

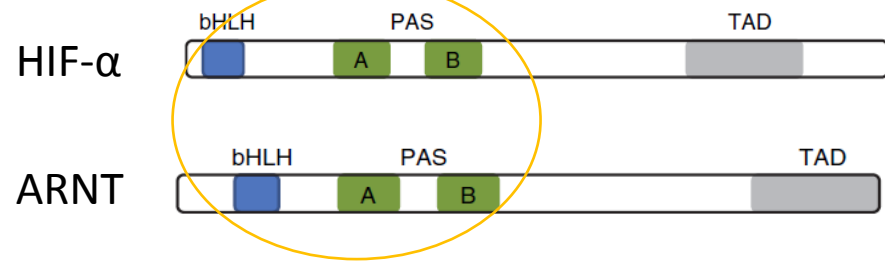
None

8. SPECIAL REPORTING REQUIREMENTS

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

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

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



bHLH, PAS-A&PAS-B domains expressed and purified

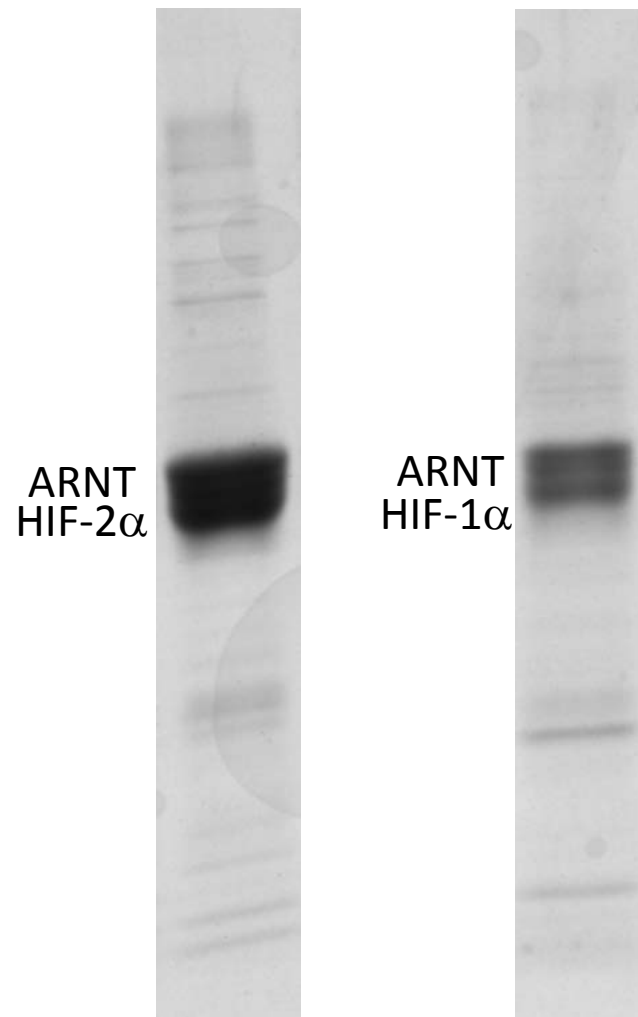


Figure 1

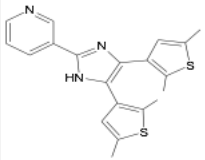
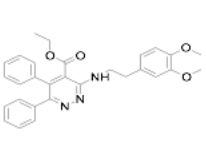
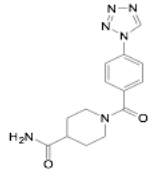
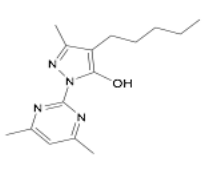
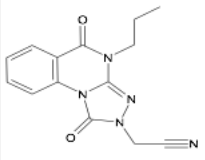
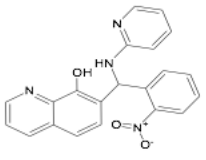
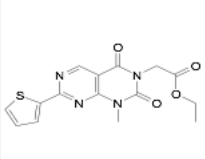
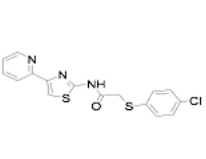
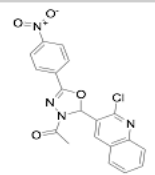
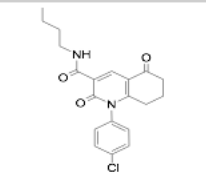
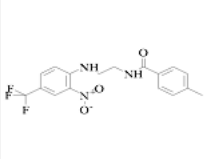
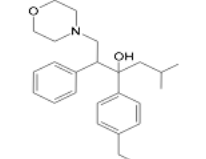
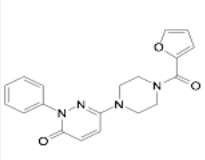
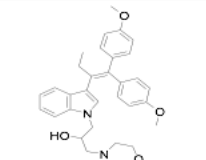
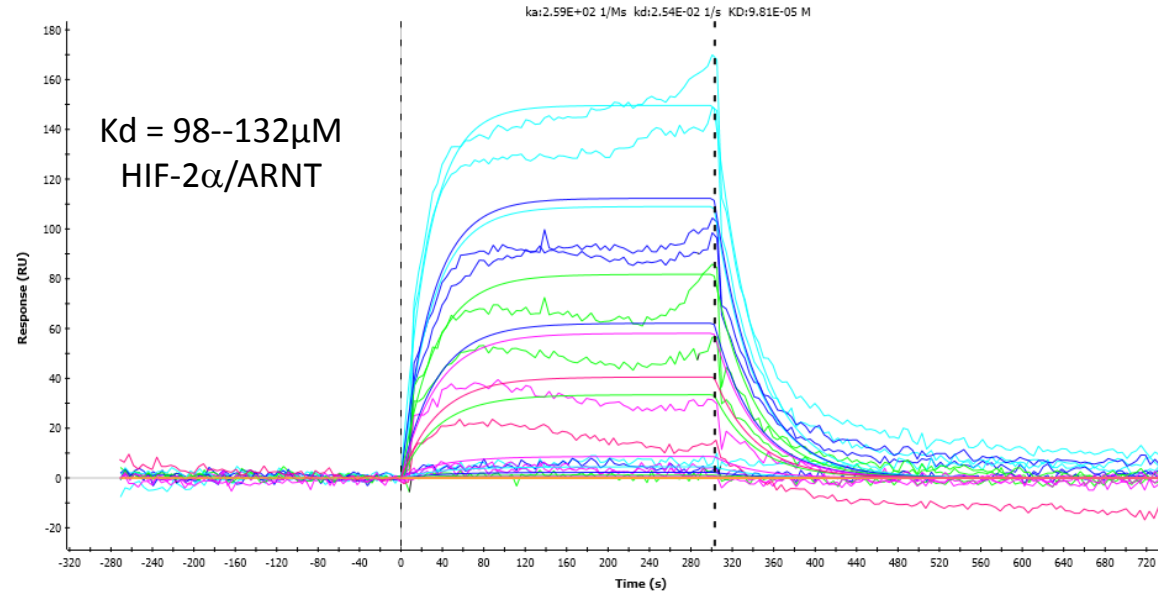
HIF1alpha:ARNT			HIF2:ARNT		
Compound ID	Structure	Abundance	Compound ID	Structure	Abundance
SBI-0177406		466,610	SBI-0311394		514960
SBI-0333234		460,120	SBI-0317522		484020
SBI-0391417		458,310	SBI-0171820		440800
SBI-0381624		348,460	SBI-0201312		437550
SBI-0339934		337,370	SBI-0171656		437300
SBI-0006774		329,990	SBI-0186731		429760
SBI-0355145		277,820	SBI-0307925		413730

Figure 2

SPR binding assay



MST binding assay

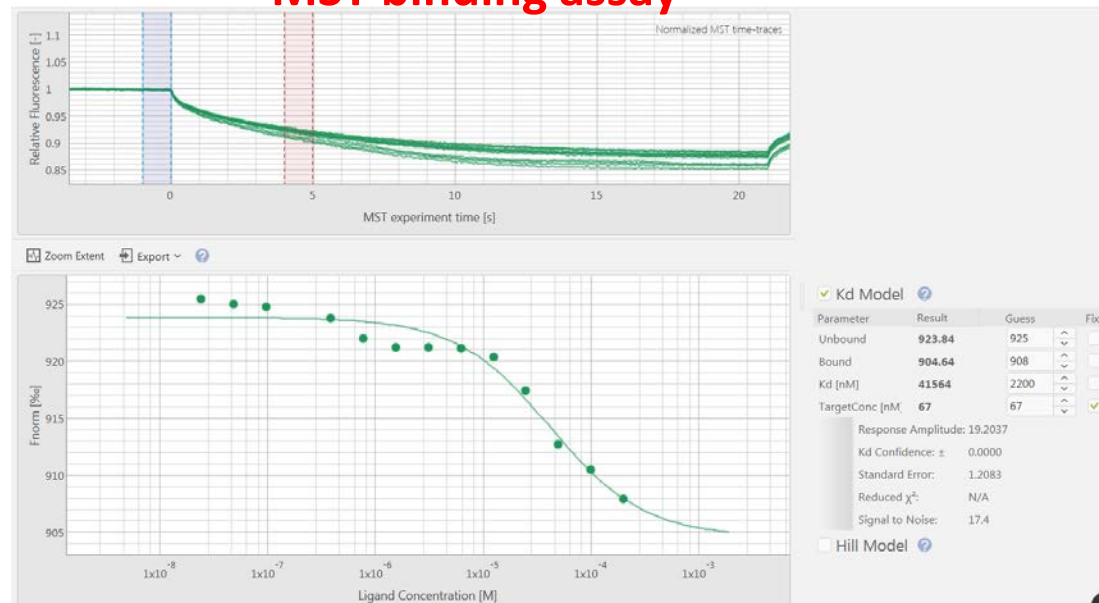


Figure 3