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TITLE: Rescue of TET2 Haploinsufficiency in Myelodysplastic Syndrome Patients Using Turbo Cosubstrate

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Rescue of TET2 Haploinsufficiency in Myelodysplastic Syndrome Patients Using Turbo Cosubstrate

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**6. AUTHOR(S)**
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**5d. PROJECT NUMBER**

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**14. ABSTRACT**
During this period, we have demonstrated that 2-oxoglutarate (2OG) analogs can dramatically enhance the activity of TET2 enzymes from MDS patient. Further, we published a manuscript on convenient expression, purification and quantitative liquid chromatography-tandem mass spectrometry-based analysis of TET2. Finally, we have identified novel substrate specificity of TET2 oxygenase.

**15. SUBJECT TERMS**
Nothing listed

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1. **INTRODUCTION:** TET2 is one of the most frequently mutated genes in myelodysplastic syndromes (MDS). The TET2 mutations are also prevalent in a number of myeloid malignancies such as MDS-myeloproliferative neoplasms (MDS-MPN) and acute myeloid leukemia derived from MDS and MDS-MPN (sAML). One of the fundamental causes of these diseases is the presence of 5-methylcytosine (5mC) marks, particularly in the CpG islands of promoters, leading to gene silencing. The wild-type (wt)-TET2 protein, a putative tumor suppressor, is a non-heme iron(II), 2-oxoglutarate (2OG)-dependent dioxygenase which initiates 5mC demethylation by hydroxylating it into 5-hydroxymethylcytosine (5hmC). TET2-knockout mice, which are viable and grossly normal initially, with age, develop diverse myeloid malignancies similar to humans. The objective in this project is to develop effective strategies using 2OG analogs to enhance the activity of the wt-TET2 enzyme both in vitro and in vivo in order to overcome TET2 haploinsufficiency. We have developed large scale expression of TET2 dioxygenase and a convenient assay for TET2 dioxygenase, which will be utilized to determine the kinetics of wild-type and various clinical mutants of TET2 dioxygenase. Further, we made a library of putative 2-oxoglutarate analogs which will be used to rescue the activity of TET2 mutants from MDS patient. Initial results shows that the activity of TET2 can be modified using 2-oxoglutarate analogs.

2. **KEYWORDS:** Myelodysplastic syndromes (MDS), MDS-myeloproliferative neoplasms (MDS-MPN), Acute myeloid leukemia (AML), 5-methylcytosine (5mC), Mutation, Haploinsufficiency, Small molecule activators, TET2, Dioxygenase, 2-oxoglutarate (2OG).

3. **ACCOMPLISHMENTS:**

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

**Task 1: Screen a library of 2OG analogs and identify cosubstrates with better $K_M$ values towards wt-TET2 under *in vitro* assay conditions (months 1-26).**

**1a.** Scale-up the purification of wt-TET2 from the insect cells (SF9) using an N-terminal his-tag by affinity chromatography (months 1-6). We already have a stock of P1 virus, which will be used to produce ≈10 mg of pure wt-TET2 dioxygenase.

100% completed. Along with expression in the insect cell lines, we have developed a convenience expression of active TET2 enzymes in bacterial system, which is described later.

**1b.** Determination of kinetic properties ($V_{max}$, $K_M$, and $k_{cat}$) of wt-TET2 with respect to 2OG using the standardized *in vitro* HPLC assay (months 4-6). Using our reported methods we will determine the kinetic properties of wt-TET2 dioxygenase.

50% completed. Delays in progress is due to development of a reliable assay. As describe below we have tried several assays and found mass spectrometry-based assay to be most reliable.

**1c.** Synthesize, purify and characterize a library of 2OG analogs using the scheme reported in the application (months 1-18).

50% completed. We have had several problems with the synthesis of 2OG analogs.

**1d.** Determination of kinetic properties ($V_{max}$, $K_M$, and $k_{cat}$) of wt-TET2 with respect to 2OG analogs using the *in vitro* HPLC assay (months 6-24). This will be followed by selection of ≈10 best 2OG analogs with an improved $K_M$ value.

Ongoing. ***Our initial results shows for the first time that the activity of TET2 can be modulated, even enhanced in case of some MDS mutants, using 2-oxoglutarate analogs.*** We are continuing on this extremely exciting results and hope to publish some very significant papers from this grant.

**1e.** The shortlisted 2OG analogs (≈10) will be assayed with histone lysine demethylases, HIF prolyl hydroxylases and AlkB2 dioxygenases to identify 2OG analogs that show specificity towards wt-TET2 activation compared to other dioxygenases (months 12-24). From these experiments two 2OG analogs will be selected for the cell-based studies.

2-5 2OG analogs are found to enhance the activity of TET2 mutants found in MDS patients. No compounds are found that enhanced the activity of wt-TET2.

**Task 2: Develop strategies to improve wt-TET2 activity in haploinsufficient lymphoid cells from MDS patients (months 9-36).**

**2a.** Selection at least two TET2 mutated haploinsufficient patient cell line and one normal cell line with wt-TET2, used as a control (months 12-18).

100% completed.

**2b.** Chemical modification (esterification) of the two 2OG analog selected from task 1e (months 24-26) and 2OG, which will be used as a control in every experiment.

Ongoing with selected compounds.

**2c.** Co-culture of the two TET2 mutated haploinsufficient patient cell line from task 2a in the presence or absence of the two modified 2OG analogs at five times $K_M$ concentration. As a control, a normal cell line from healthy donor with wt-TET2 will be grown without any 2OG analogs (months 26-28).

Not started yet.

**2d.** Quantitation and analysis of 5mC/5hmC and gene expression levels in the three cell lines cultured in the presence or absence of modified 2OG analogs (months 28-36).

Not started yet.

- **What was accomplished under these goals?**

In Year 4 (June, 2016 – May, 2017) we continued with our focus on enhancing the activity of TET2 enzymes found in MDS patients. Please find below a brief description of our results:

1. During the last yr we published our first manuscript entitled "*Convenient expression, purification and quantitative liquid chromatography-tandem mass spectrometry-based*

*analysis of TET2 5-methylcytosine demethylase*" in Protein Expression and Purification. In this manuscript, we describe the cloning of untagged human TET2 demethylase using Gateway technology and its efficient expression in E. coli (Figure 1 and 2). The untagged TET2 enzyme was purified using cation exchange and heparin sepharose chromatography. In addition, a reliable quantitative liquid chromatography-tandem mass spectrometry-based assay was utilized to analyze the activity of TET2 oxygenase (Figure 3 and 4). This assay was further used to analyze the activity of a number of clinical TET2 variants with mutations in the 2OG binding sites. Our results demonstrate that the activity of one TET2 mutant, TET2-R1896S, can be restored using an excess of 2OG in the reaction mixture (Figure 5). These studies suggest that dietary 2OG supplements, which are commonly used for several other conditions, may be used to treat some patients with myeloid malignancies harboring TET2-R1896S mutation. Results described in this paper serve as a foundation for better characterization of wild type as well as mutant TET2 demethylases.

2. *Substrate specificity of Tet2 oxygenase*: As a side project we have for the first time demonstrated the detailed substrate specificity of Tet2 oxygenase. These remarkable results will be published within next few months (detailed results will be published soon).

3. *Rescue of TET2 activity using 2OG analogs*: This remains our main focus and as stated before that the activity of TET2 can be modulated, even enhanced in case of some MDS mutants, using 2-oxoglutarate analogs. We are in process of understanding these dramatic results based on crystallography (detailed results will be published).

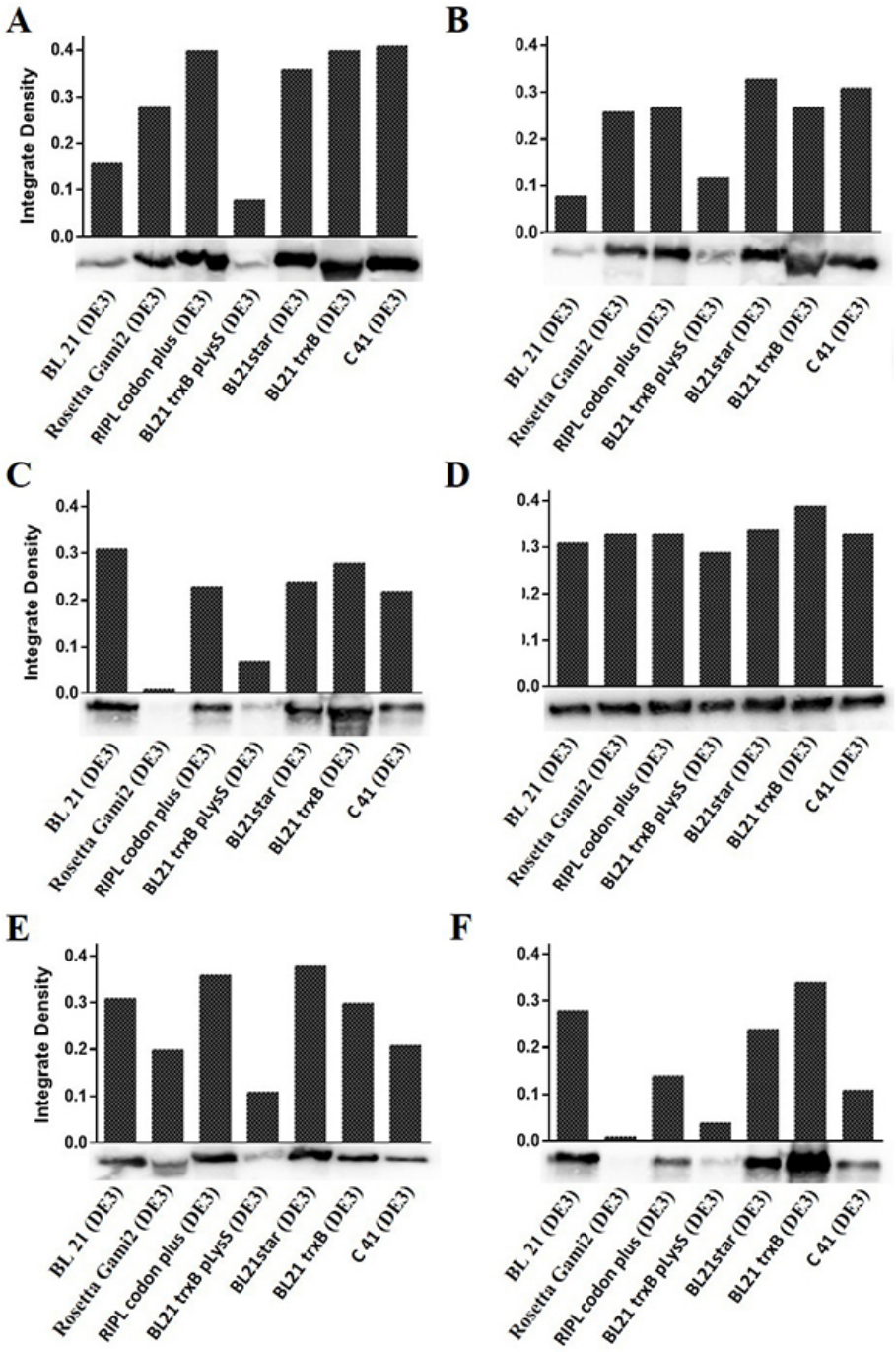


**Figure 1:** Western blot analysis of TET2 oxygenase expression in seven different strains of *E. coli* when induced at the indicated temperature and IPTG concentration: 16°C for 16 h with 0.5 mM IPTG (A), 27°C for 10 h with 0.5 mM IPTG (B), 37°C for 4 h with 0.5 mM IPTG (C), 16°C for 16 h with 1 mM IPTG (D), 27°C for 10 h with 1 mM IPTG (E), and 37°C for 4 h with 1 mM IPTG (F).

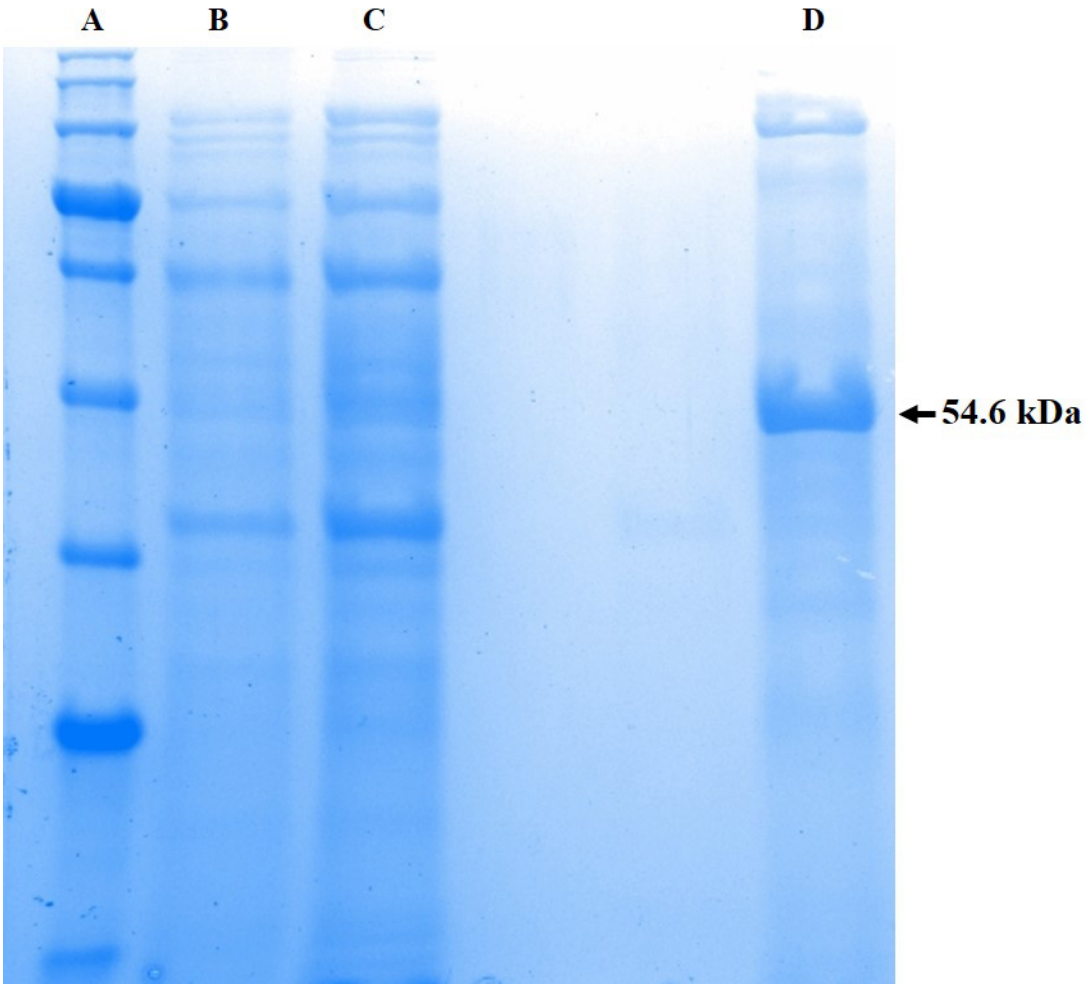


**Figure 2:** SDS-PAGE analysis of purified untagged TET2 demethylase from *E. coli* BL21 trxB (DE3) cells. Lane indicates: marker (A), unintroduced TET2 (B), induced TET2 protein (C), and TET2 protein purified using SP sepharose high-performance strong cation exchange resin (D). The total size of the untagged TET2 demethylase is ~54.6 kDa as indicated by the arrow.

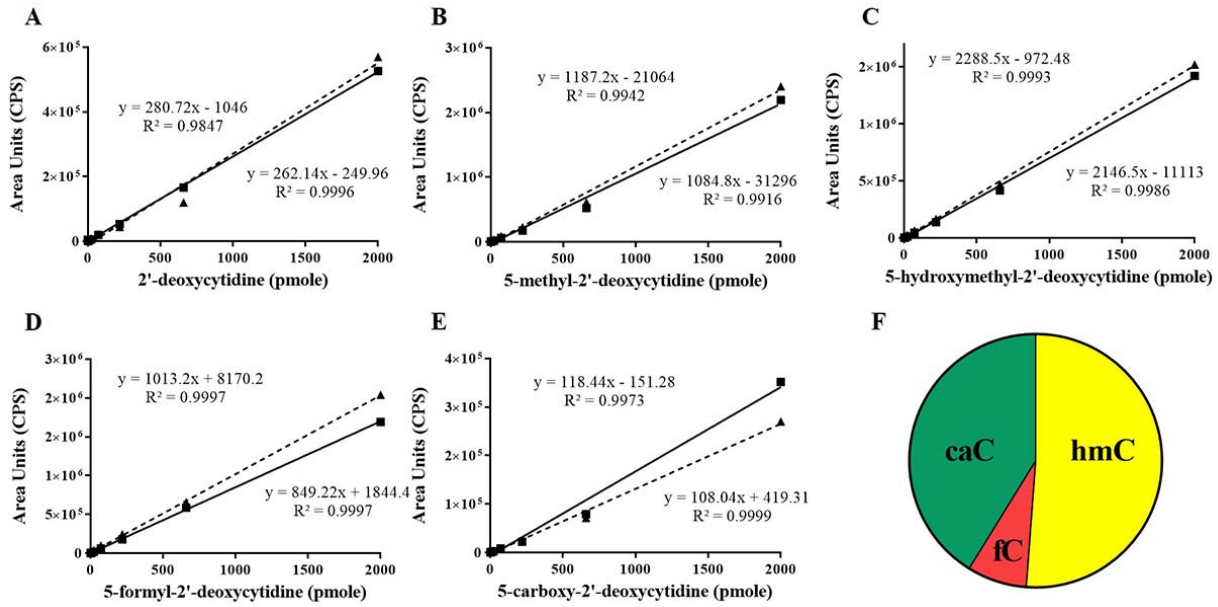


**Figure 3:** Standard curves of modified cytosine nucleosides dissolved in TET2 reaction conditions (straight line) or HPLC-grade water (doted line) showing insignificant effect on the quantification (A-E). A pie chart showing LC-MS/MS detection of 5hmC, 5fC, and 5caC formed after TET2 reaction (F).

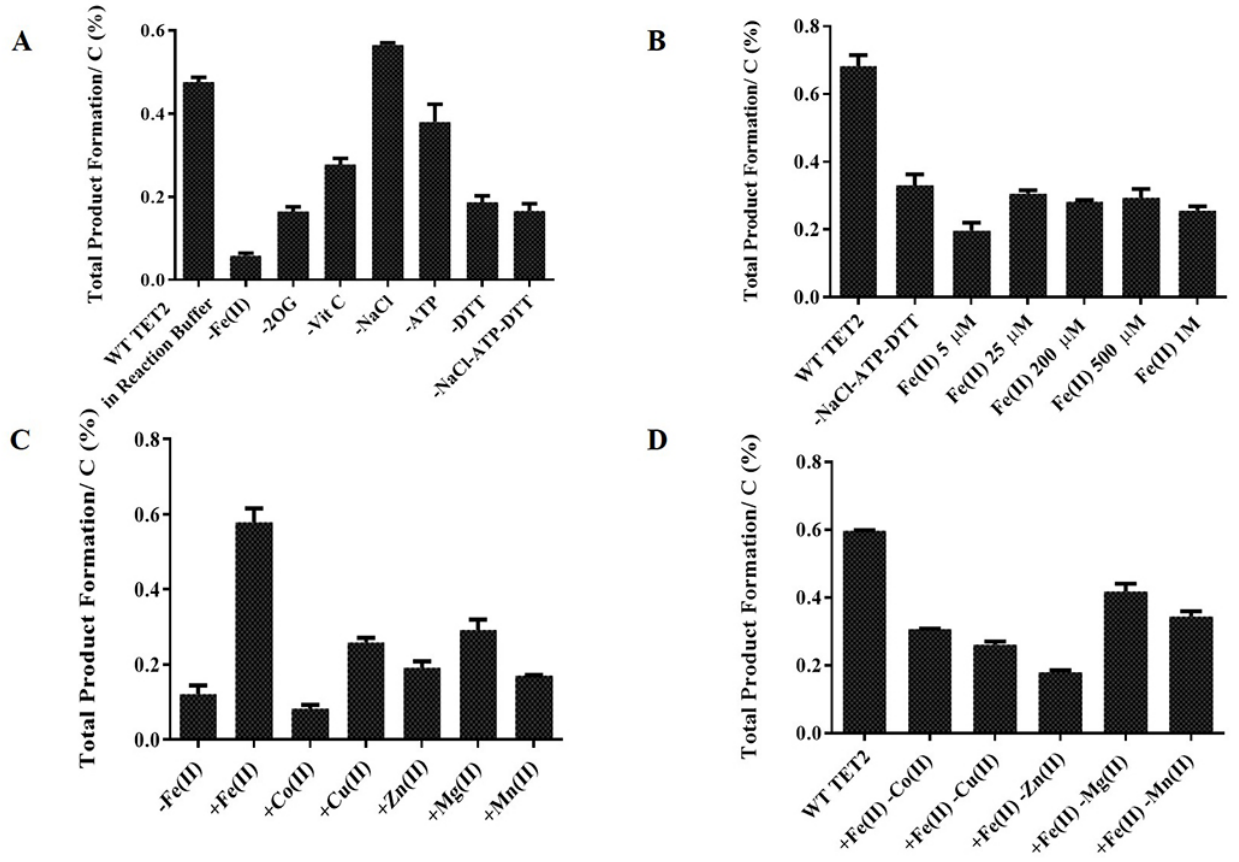


**Figure 4:** Characterization of the WT-TET2 demethylase. Effect of co-factors on the TET2-mediated demethylation (A). An increase in Fe(II) concentration from 75-1000 µM in the reaction buffer in the absence of ATP and DTT did not increase TET2 activity (B). Fe(II) could not be replaced by any of the alternative metal ions for TET2 activity (C). Several metal ions inhibited TET2 activity in the presence of Fe(II) (D).

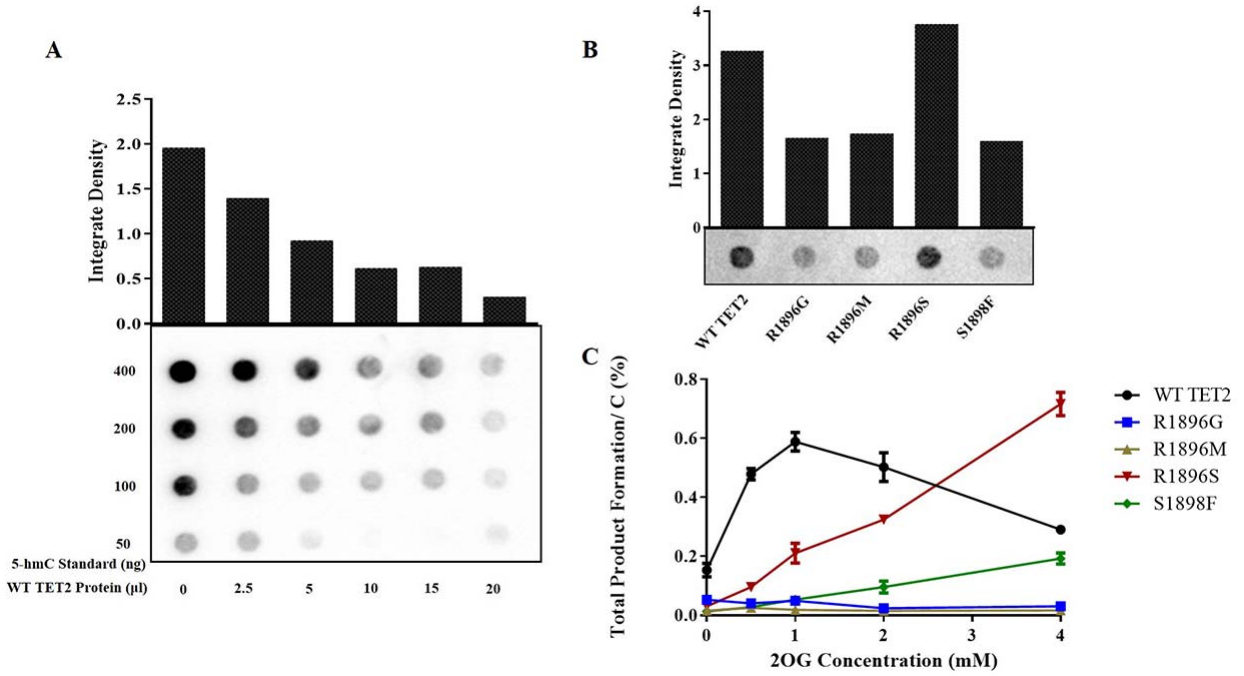


**Figure 5:** A comparison of the activities of wild type and some TET2 clinical mutants by dot-blot and our quantitative LC-MS/MS-based assay. The presence of the TET2 protein inhibited the detection of 5hmC (A), DNA substrate was purified using a Zymo Oligo purification columns before the blotting and detection by dot-blot (B), Catalytic activities of the wild type and clinical TET2 mutants (-R1896M/S/G, -S1898F) were characterized in the presence of different 2OG concentrations using the quantitative LC-MS/MS-based assay (C).

| Cytosine modification | Q1 | Q3 | $t_r$ (min) | DP (V) | EP (V) | CEP (V) | CE (V) | LOD (pmol) | LLOQ (pmol) |
|---|---|---|---|---|---|---|---|---|---|
| 2′-deoxycytidine | 228.1 | 112.1 | 11.50 | 11 | 6 | 16 | 21 | 1.00 | 3.30 |
| 5-methyl-2′-deoxycytidine | 242.2 | 126.2 | 12.57 | 30 | 6 | 14 | 23 | 0.10 | 0.33 |
| 5-hydroxymethyl-2′-deoxycytidine | 258.2 | 142.2 | 11.68 | 36 | 3.5 | 14 | 27 | 0.30 | 1.00 |
| 5-formyl-2′-deoxycytidine | 256.2 | 140.2 | 14.49 | 26 | 4 | 14 | 17 | 0.30 | 1.00 |
| 5-carboxy-2′-deoxycytidine | 272.2 | 156.2 | 11.84 | 56 | 6 | 24 | 15 | 3.00 | 9.90 |

**Table 1:** Summary of optimized positive mode IP-LC-MS/MS parameters and characteristics of different cytosine derivatives. For each parent ion nucleoside (Q1), the most intense product ion (Q3) was detected.

- **What opportunities for training and professional development has the project provided?**
  - This project has provided important opportunities to Dr Mridul Mukherji to attain conferences.
- **How were the results disseminated to communities of interest?**
  - Preliminary results were disseminated in the 34th Midwest Enzyme Chemistry Conference (MECC), University of Illinois at Chicago, Chicago, IL, 2016. We have published 1 manuscript in 2017 and at least 2 more manuscripts will be published soon in peer reviewed national/ international journals.
- **What do you plan to do during the next reporting period to accomplish the goals?**
  - We would like published our exciting results on substrate specificity of Tet2 oxygenase and try to solve the crystal structure of Tet2 enzyme (wild-type and mutants) to understand the mechanism of 2OG analog mediated modulation of activity.

4. **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**
  - For the very first time we have found that the activity of TET2 dioxygenase can be modulated using 2-oxoglutarate analogs. In addition, our results demonstrate that the activity of one TET2 mutant, TET2-R1896S, can be restored using an excess of 2OG in the reaction mixture. These studies suggest that dietary 2OG supplements, which are commonly used for several other conditions, may be used to treat some patients with myeloid malignancies harboring TET2-R1896S mutation. In addition, we have established novel substrate specificity of Tet2 oxygenase.
  -

- **What was the impact on other disciplines?**
  - There are many 2-oxoglutarate-depent dioxygenases like histone demethylases that regulate critical biological processes like HIF signaling, epigenetics etc. Our methods would make it possible to regulate the activity of these dioxygenases using 2-oxoglutarate analogs.

- **What was the impact on technology transfer?**

  A patent application will be filed soon.

- **What was the impact on society beyond science and technology?**
  - Nothing to Report.

5. **CHANGES/PROBLEMS:**

- **Changes in approach and reasons for change**
  - 1. We have had issues with development of a reliable TET2 dioxygenase assay. Progress of the project has been hampered by delays in permission to use the mass spectrometry instrument with ammonium salt (please note that our LCMS method requires use of ammonium salt in the buffer for proper separation of nucleosides). However, now we are able to use ammonium salt in the LCMS methods.

    While we were waiting for the permission to use LCMS system with ammonium salt, which took over one year, we spent significant time on developing alternative TET2 assays using (i) colorimetric TET Activity Assay from Epigentek, and (ii) Dot blot. However, both these assays detect 5mhC, using an antibody, and therefore these two assays were not very reliable because they don't detect 2$^{nd}$ (5fmC) and 3$^{rd}$ (5cmC) TET2 products. Only the LCMS assay detects all three products, namely 5hmC, 5fmC, and 5cmC, of TET2 enzyme.

2. We also had delays due to graduate students from my lab transferring to other pharmacy programs/ schools.

Due to delays in the progress of project a 12 month no cost extension was requested which was granted by DOD.

- **Actual or anticipated problems or delays and actions or plans to resolve them**
  - After one year we were able to use our LCMS system with ammonium salt. This has allowed us to progress with the project as proposed in the application. Further, I have admitted two new graduate students and they are making good progress.
- **Changes that had a significant impact on expenditures**
  - Since the alternative colorimetric assay from Epigentek requires a plate reader, we received a written permission from DOD to buy a demo molecular devices plate reader costing over $19,000. Along with the purchase of the plate reader, which was not allocated in the original approved budget by DOD, I had to pay students and buy reagents in order to develop alternative assays.
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**: Nothing to Report.
- **Significant changes in use or care of human subjects:** Nothing to Report.
- **Significant changes in use or care of vertebrate animals:** Nothing to Report.
- **Significant changes in use of biohazards and/or select agents:** Nothing to Report.

6. **PRODUCTS:** Nothing to Report.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

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

| | |
|---|---|
| Name: | Mridul Mukherji |
| Project Role: | PI |
| Researcher | |

| | |
|---|---|
| Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 0.36 |
| Contribution to Project: | *Dr Mukherji supervises the project on day-to-day basis and ensure that the participating graduate students learn appropriate skills to conduct their daily research independently and timely manner. He participate in writing progress reports and research publications.* |
| Funding Support: | |

| | |
|---|---|
| Name: | Chayan Bhattacharya |
| Project Role: | Graduate Student |
| Researcher Identifier (e.g. ORCID ID): | 16236402 (EMPLID #) |
| Nearest person month worked: | 3 |
| Contribution to Project: | He is working on purification and assay of TET2. |
| Funding Support: | |

| | |
|---|---|
| Name: | Aninda Sundar Dey |
| Project Role: | Graduate Student |
| Researcher Identifier (e.g. ORCID ID): | 16245682 (EMPLID #) |
| Nearest person month worked: | 3 |
| Contribution to Project: | He is responsible for LCMS-based TET2 assays. |
| Funding Support: | |

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

  Nothing to Report.

- **What other organizations were involved as partners?**

  - Nothing to Report.

    - **Organization Name:**
    - **Location of Organization:** *(if foreign location list country)*
    - **Partner's contribution to the projec**t *(identify one or more)*

8. **SPECIAL REPORTING REQUIREMENTS**: Nothing to Report.

9. **APPENDICES:** Nothing to Report.