Development of Biochemical Assay Tools to Enhance Drug Discovery Efforts Targeting RNA Modification Regulators.

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Introduction

Epitranscriptomics refers to biochemical modifications found in RNA that occurs post transcriptionally and provides another layer of gene expression regulation beyond genetic sequence and epigenetic regulations. N6-methyladenosine (m6A) is the most abundant epitranscriptomic marker found in eukaryotic mRNA and lncRNA. Expression of m6A is dynamically regulated in cells, m6A modifications added to RNA by m6A writers, removed by m6A erasers and processed by m6A readers. [1]

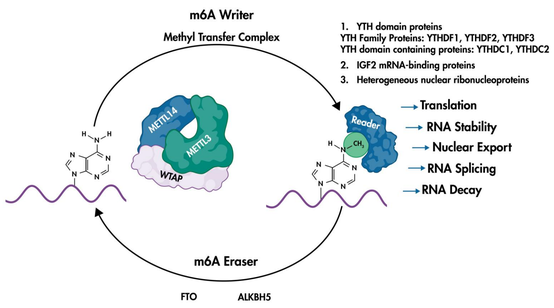


Fig. 1: m6A regulation machinery and signaling

Abnormal expression of m6A and proteins in its regulatory machinery is associated with tumorigenesis, cancer stemness and drug resistance of various cancers.[2,3] High METTL3 expression is found in several cancers and a METTL3 catalytic inhibitor is found to delay AML progression in mouse models. First METTL3 inhibitor STC15 enters phase1 clinical trial, in subjects with advanced malignancies.[4]

Several YTH family readers are shown to play oncogenic roles in cancers including AML, breast, lung, CRC and glioblastoma. Recent studies suggest inhibition of m6A binding of individual YTH family proteins is a promising therapeutic strategy however, potent inhibitors are yet to be identified.[5]

At Reaction Biology Corp., we have developed screening assays to facilitate drug discovery targeting oncogenes in the m6A machinery.

METTL3/METTL14 Screening Assay Development in HotSpot

RNA m6A modifications are deposited by writer known as Methyltransferase complex(MTC), which contains METTL3-METTL14-WTAP as core components. The catalytic component METTL3 is activated by heterodimer formation with METTL14. We have developed miniaturized radioisotope-based activity assay in HotSpot format, suitable for inhibitor screening applications for METTL3:METTL14 heterodimer protein.

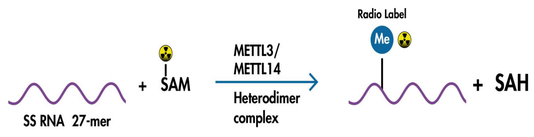


Fig. 2: HotSpot Assay Design METTL3:METTL14 complex transfers tritium-labeled methyl from S-adenosyl-L-methionine (SAM) to RNA substrate. Reaction mixtures are incubated and spotted onto filter papers, which are then washed to remove unreacted SAM, leaving the bound radiolabeled product for detection.

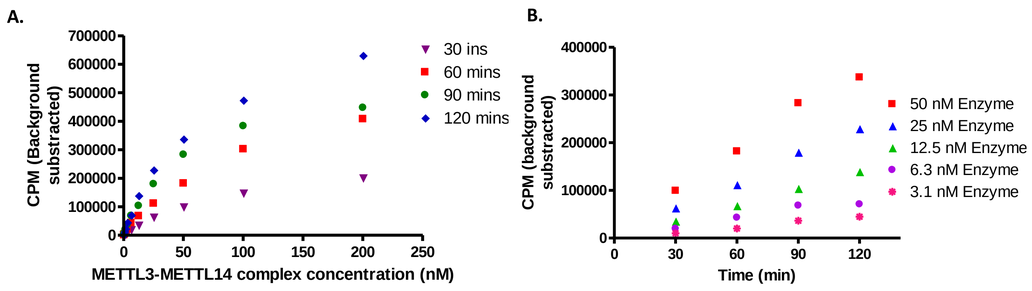
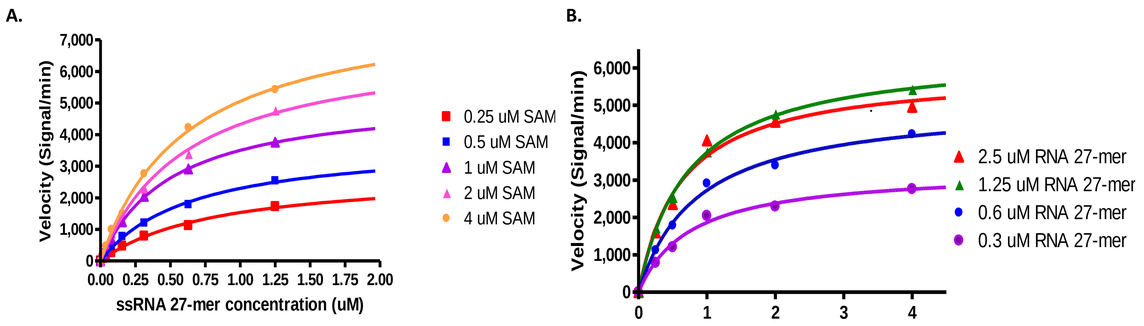


Fig. 3: Enzyme activity determination Enzymatic activity test for METTL3:METTL14 complex at 1mM SAM and 1mM ss RNA 27-mer substrate. 3A. Enzyme titration at each reaction time. 3B. Reaction linearity with time up to 120 minutes at constant enzyme concentrations.

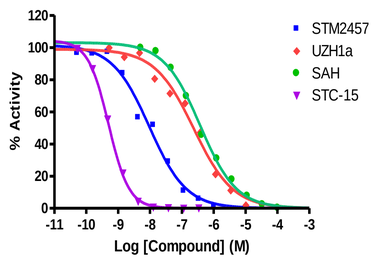


	0.25 uM SAM	0.5 uM SAM	1 uM SAM	2 uM SAM	4 uM SAM
VMAX	2810	3790	5268	6988	8186
KM	0.8335	0.6499	0.5042	0.6152	0.6191

	2.5 uM RNA 27-mer	1.25 uM RNA 27-mer	0.6 uM RNA 27-mer	0.3 uM RNA 27-mer
VMAX	5918	6435	5083	3280
KM	0.6231	0.7217	0.8653	0.7584

Fig. 4: Kmapp determination for each substrate at fixed enzyme concertation and reaction time 4A. Km of ss RNA 27-mer at varying SAM concentrations. 4B. Apparent Km of SAM at varying concentrations of ssRNA 27-mer.

Fig. 5: Control inhibitor validation



Compound	IC_{50}
STM2457	9.5nM
UZH1a	235nM
SAH	376nM
STC-15	0.5nM

YTH Inhibitor screening assay Development

All five proteins of YTH family m6A readers, YTHDF1–3 and YTHDC1–2 shares YT521-B homology (YTH) domain. These proteins recognize m6A sites in target mRNA and direct them to functionalities such as translation, stability, localization or splicing.

Targeting individual YTH family proteins for favorable therapeutic outcome in cancer treatment is gaining attention in recent literature.[5,6] To facilitate these efforts, we have developed screening assays suitable to identify YTH protein m6A interaction inhibitors in all five YTH family proteins using recombinant full-length proteins.

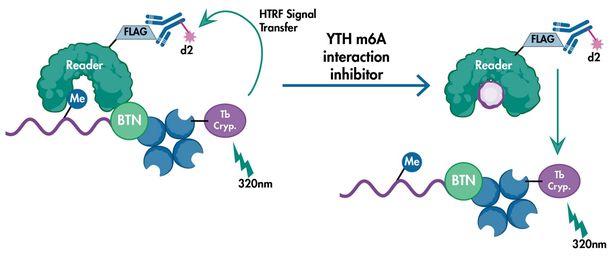
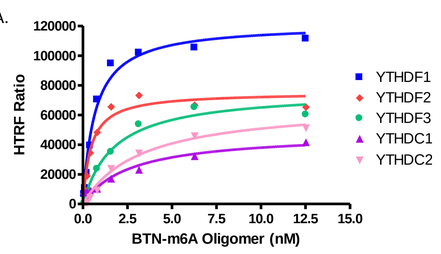


Fig. 7: HTRF based screening assay design Interaction between tagged YTH protein and Biotinylated m6A oligomer in presence of anti tagged HTRF reagents, brings the donor and acceptor to close proximity allowing HTRF signal transfer proportional to binding interaction. Presence of compounds inhibiting the interaction between YTH protein and m6A oligomer diminishes this association lowering HTRF signal.



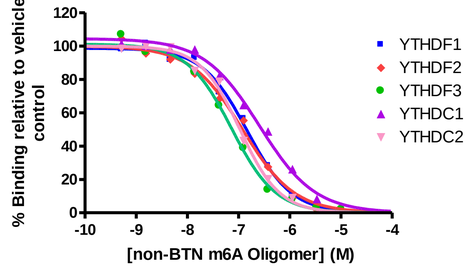
	YTHDF1	YTHDF2	YTHDF3	YTHDC1	YTHDC2
BMAX	121367	75249	76383	48008	67371
KD	0.6671	0.4419	1.744	2.644	3.262

B.

Target	Z'
YTHDF1	0.67
YTHDF2	0.77
YTHDF3	0.72
YTHDC1	0.67
YTHDC2	0.53

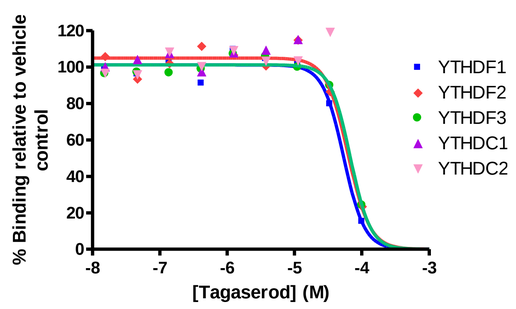
Fig. 8: KD and Z' Determination 8A. Binding curves for each protein at fixed protein concentration and varying oligomer concentration. 8B. Lists Z' values calculated for each target at finalized assay conditions to evaluate assay robustness.

A.



	YTHDF1	YTHDF2	YTHDF3	YTHDC1	YTHDC2
HILLSLOPE	-0.9959	-0.8562	-1.004	-0.7993	-1.120
EC50	1.467e-007	1.197e-007	7.317e-008	2.552e-007	1.068e-007

B.



	YTHDF1	YTHDF2	YTHDF3
HILLSLOPE	-2.815	-2.669	-2.928
EC50	5.378e-005	6.218e-005	6.749e-005

Fig. 9: Assay Validation
9A. Assay Validation using non-biotinylated m6A oligo that competes with labeled oligo to bind to YTH domain. 9B. Tagaserod, a compound identified as a YTHDF1 m6A interaction inhibitor in recent literature by structure based virtual screening,[6] was evaluated against the panel. Tagaserod shows selectivity towards YTH family proteins YTHDF1-3 compared to YTH domain containing proteins YTHDC1-2.

Summary

- Accumulating evidences suggest oncogenic roles of RNA m6A regulating machinery in various cancer types.
- We have developed activity assays METTL3:METTL14 heterodimer complex using our proprietary HotSpot assay technology. Here we show data for development and assay validation using previously reported METTL3 inhibitors.
- We show development of HTRF based screening assay for YTH domain family m6A readers using full length proteins. Using this assay panel, we evaluate the inhibition and selectivity Tegaserod, an FDA approved drug that was recently reported as YTHDF1 inhibitor.

References

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