Identification of a Novel Small Molecule Inhibitor of the RNA Demethylase FTO using MST

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Introduction

- N-6-methyladenosine (m6A) is the most abundant RNA chemical modification found in the eukaryotic transcriptome [1,2].
- Fat mass and obesity associated protein (FTO) is a member of the non-heme dioxygenase superfamily and is one of two 'erasers' responsible for the demethylation of m6A (Figure 1)[3].
- Alteration in the m6A profile due to FTO dysregulation has been associated with the progression of various types of cancer and metabolic diseases [4,5].
- FTO is a high interest therapeutic target with a lack of potent and selective inhibitors currently available.
- In this study a robust microscale thermophoresis (MST) FTO assay was used to screen 100 compounds selected from the Library of Pharmaceutically Active Compounds (LOPAC) and identified five novel FTO binders.
- Inhibitor profiling via an FTO activity assay identified a novel, small molecule FTO inhibitor.

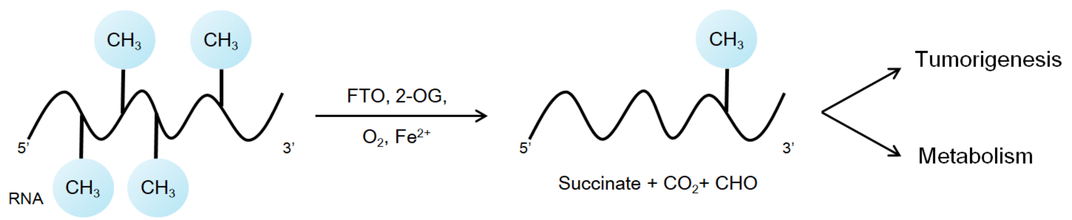


Figure 1: Schematic representing the role of FTO in the regulation of the transcriptome by removing the m6A modification from RNA. The downstream effects from this have been implicated in tumorigenesis and metabolic disease.

Microscale Thermophoresis Assay Development

- MST is a powerful solution-based technique for quantifying biomolecular interactions by detecting changes in fluorescence as a result of IR laser induced temperature change.
- MST enabled significantly lower enzyme consumption than traditional FTO enzyme activity based screens.
- MST data are represented as the change in Fnorm upon ligand binding . Fnorm is the ratio of the fluorescence measured before and during thermophoresis.

His-tag Labelling Approach

- Initially Red-tris NTA labelling of human recombinant FTO's His-tag was attempted.
- The dye should have a high affinity for the His-tag with a K_D <10 nM.
- Low affinity of the dye for the target can increase assay interference by free dye.
- The affinity of the dye for the N-terminal His-tag on FTO was lower than required as evidenced by the K_D of 270 nM (Figure 2).
- NTA His-tag labelling was not a viable approach for FTO.

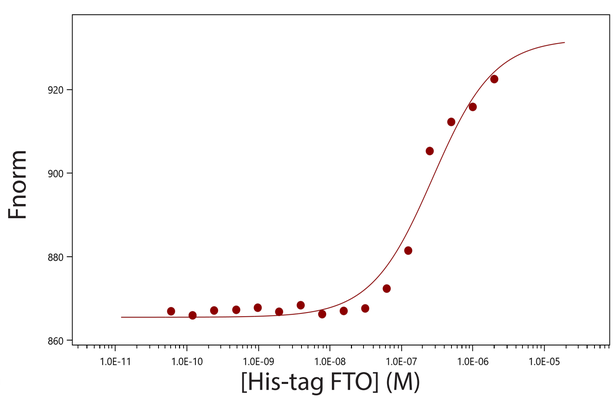
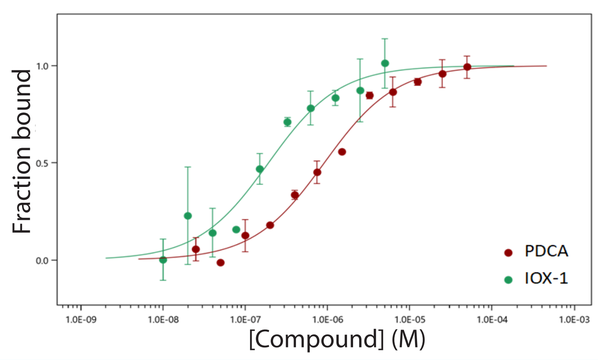


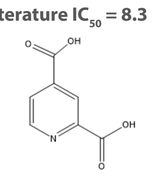
Figure 2: Titration of unlabelled FTO with 10 nM labelling dye revealed a lower affinity (K_D = 270 nM) than is required for the MST measurement.

Amine Labelling Approach

- Amine labelling was used to covalently attach a fluorophore *via* a Red-NHS ester group which is reactive with primary amines (lysine residues).
- The assay was validated with FTO inhibitors; 2,4 Pyridine Dicarboxylic acid (PDCA) and IOX-1 (Figure 3).
- Although affinities for PDCA and IOX-1 with FTO have not been reported, our affinities are in broad agreement with reported IC_{50} (Figure 3).



PDCA
Geomean K_D = 1.4 µM (N=2)
Literature IC_{50} = 8.3 µM [6]



IOX-1
Geomean K_D = 0.17 µM (N=2)
Literature IC_{50} = 3.3 µM [6]

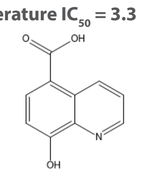


Figure 3: MST binding traces showing concentration responses of the FTO inhibitors IOX-1 and PDCA with the top concentrations of 5 µM and 50 µM respectively.

Pilot Screen of the LOPAC by MST

- A proof of concept pilot screen was performed with 100 compounds selected at random from the LOPAC.
- Initial binding was assessed by spot test at 100 µM and compared to a matched DMSO control for a shift in Fnorm.
- A significant shift in Fnorm was defined as greater than 3 standard deviations from the mean DMSO reference. at 5s MST time.
- Z' of the assay at 5s MST time = 0.6.
- Raw data was assessed for compound auto-fluorescence and aggregation.
- Sixteen compounds showed a significant shift in Fnorm (Figure 4) and were selected for K_D determination.
- 5 out of 16 compounds displayed concentration dependent binding with K_D values ranging from 0.78 - 190 µM (Figures 5 and 6).

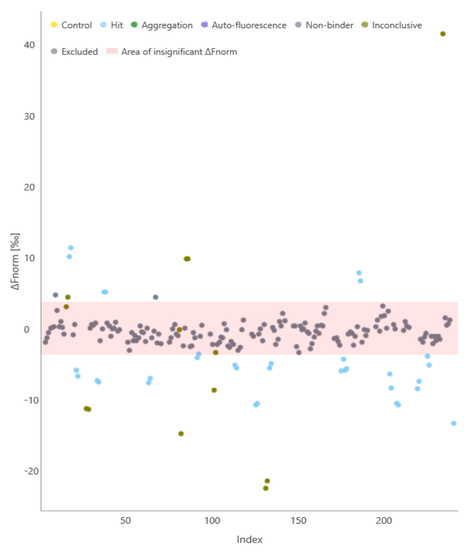
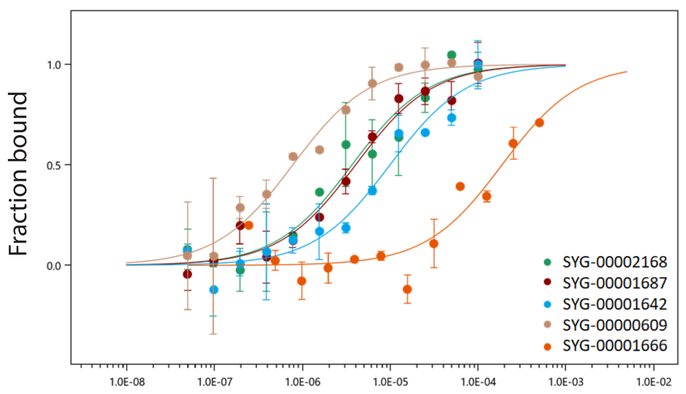
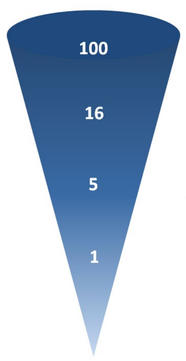


Figure 4 : Results of the initial spot tests carried out at 100 µM. Hits were defined as compounds with a shift in Fnorm greater than 3 SD from the mean DMSO reference.



Compound	Geomean K_D (µM) (N=2)
SYG-00002168	4.4
SYG-00001687	1.9
SYG-00001642	10.9
SYG-00000609	2.0
SYG-00001666	190

Figure 5: MST binding traces, SYG ID numbers and affinities of the five compounds which gave K_D values within the detectable range.

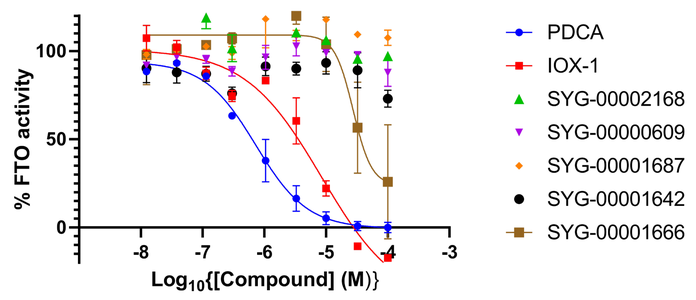


Initial spot test at 100 µM
↓
Significant change in Fnorm compared to the control
↓
K_D values within detected range
↓
Orthogonal assay (biochemical enzyme assay)

Figure 6 : Schematic of the screening cascade employed to identify novel binders of FTO via MST. The numbers are the successful compounds at each stage from the 100 compound pilot screen of the LOPAC.

Orthogonal Validation

- Commercially available Succinate-Glo ™ assay kit (Promega) was used to develop an FTO activity assay by measuring succinate production.
- Assay pharmacology was confirmed with the tool compounds PDCA and IOX-1.
- The five compounds active from the LOPAC pilot screen (Figure 5) were tested for their effect on FTO activity and revealed a novel FTO inhibitor (Figure 6).



Compound	Geomean IC_{50} (µM) (N=2)
PDCA	0.70
IOX-1	8.5
SYG-00002168	>100
SYG-00000609	>100
SYG-00001687	>100
SYG-00001642	>100
SYG-00001666	32

Figure 7: Concentration inhbition curves for IOX-1, PDCA and the 5 successful compounds from the MST pilot screen.

Summary

- A robust MST assay was developed, validated, and used to screen 100 compounds from the LOPAC for novel human FTO small molecule binders (Figures 4 and 8).
- Our proof of concept study identified a novel inhibitor, SYG-00001666 (K_D = 190 µM, IC_{50} = 32 µM, LE = 0.3).
- This fragment-like compound provides an attractive medicinal chemistry starting point for the generation of potent and selective FTO inhibitors.
- We are also interested in applying a FBDD approach to identify novel FTO inhibitors by screening our proprietary fragment library for FTO binders using the MST assay.

Compound	Geomean K_D (µM) (N=2)	Geomean IC_{50} (µM) (N=2)
PDCA	1.3	0.70
IOX-1	0.17	7.7
SYG-00002168	4.4	>100
SYG-00000609	2.0	>100
SYG-00001687	1.9	>100
SYG-00001642	10.9	>100
SYG-00001666	190	32

Figure 8: Table summarising the affinities and activities of PDCA, IOX-1 and the hits from the LOPAC pilot screen with FTO.

References

[1] Wei, C.M. et al. (1975) *Cell.* **4**: 379-386
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[4] Li, Z. et al. (2017). *Cancer Cell.* **31**(1): 127-141
[5] Fischer, J. et al. (2009). *Nature.* **458**: 894-898
[6] Aik, W. et al. (2013). *J. Med. Chem.* **56**: 3680-3688