The Double Tudor Domain: a Novel Therapeutic Target within the Histone Lysine-Specific Demethylase Subfamily (KDM4) as a Means to Target Oncogenic Super Enhancer Function

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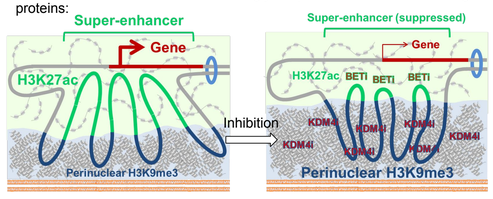
BACKGROUND

Background

Super-enhancers (SE) are elements of the human genome that contain clusters of enhancers, regions of DNA that facilitate the initiation of gene transcription and expression [2,3]. These enhancers are subject to extensive post-translational modifications, such as methylation and acetylation [3]. In cancer cells, super-enhancers activate a unique assortment of genes that produce mutative phenotypic effects that have been characterized as the hallmarks of cancer, such as metastasis, uncontrollable cellular proliferation and drug resistance [2,3].

Recent data derived from Chromatin Immunoprecipitation (ChIp) sequencing has allowed us to pursue a model that locates and partitions super-enhancers at the interfaces of repressed heterochromatin and opened euchromatin states of DNA. Furthermore, this model suggests that through a unique combination of real time post-translational modifications, oncogenic super enhancers are constantly balanced between these heterochromatin and euchromatin interfaces to accordingly express downstream oncogenic proteins:



The heterochromatin interface is commonly marked with an extremely repressive trimethylated mark at H3K9, while the euchromatin interface is commonly marked with an hyperactivating acetylation mark at H3K27 [2]. In cancer, low levels of lysine methylation at H3K9 protect oncogenic SEs from being converted to a repressed heterochromatin state, while high levels of lysine acetylation at H3K27 allows the SE to be maintained in a euchromatin state. In tandem, these markings allow nucleic proteins such as bromodomain and extraterminal domain proteins (BET) to bind and act in a matter which increases the oncogenic activity of the SE, promoting the transcription of downstream oncogenic genes [2].

KDM4 is a histone lysine-demethylase that catalyzes the oncogenic demethylation of the heterochromatin interface of the SE [1]. Its amino-terminus contains the catalytic domain, the Jumanji-domain, which displays the demethylase activity that converts K9 to a lower methylated state on histone 3, while the carboxy-terminus contains a conserved double tudor domain (DTD) [1]. We hypothesize that this DTD is a reader domain that allows the KDM4 protein to interact with and demethylate H3K9me3 by first reading key lysine residues such as H3K4me1 [1]. H3K4me1 is a key SE-associated histone modification, and it must be read in order for KDM4 to catalytically demethylate H3K9me3.

The aim of this project is to define the double tudor domain (DTD) of the KDM4 protein as a novel therapeutic target that when inhibited, can interfere with oncogenic SE function by encouraging it to be converted to a repressed heterochromatin state. This would prevent the SE from upregulating key downstream oncogenic genes that define a cell's identity as cancer.

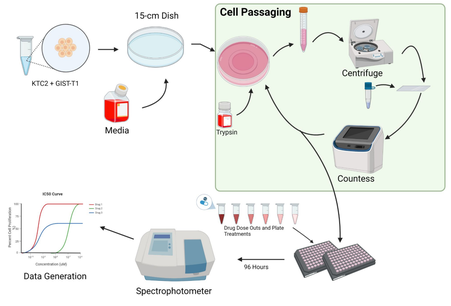
OBJECTIVES AND METHODS

Objectives

1. Test 149 small molecule inhibitors for their potential inhibitory effect against the reader-module (DTD) of the KDM4 histone lysine demethylase family
2. Perform inhibitor dose-outs of identified interaction inhibitors and determine the IC_{50} of the compound
3. Test drug synergy between these identified KDM4 inhibitors and known BET inhibitors
4. Test the potential KDM4i inhibitor for specificity to the KDM4 protein as well as to its Double Tudor Domain (DTD) via binding disassociation to KDM4
5. Use relevant pharmacological data to guide an *in-silico* binding model of the DTD of KDM4 to inspire a novel therapeutic approach

Methods

1. Since no full-length experimental structure exists for KDM4A, a homology model was constructed using YASARA. This generated a hybrid model using the best portions from experimental structures as templates, followed by an empirical force-field based energy minimization [4].
2. Once the model was constructed, a high-throughput virtual screening was conducted using Schrodinger's Glide to screen millions of small molecules for their ability to bind KDM4A's DTD and generate a list of 150 top candidates [4].
3. Two cancerous cell lines were selected for pharmacological testing: KTC-2 (anaplastic thyroid cancer) and GIST-T1 (gastrointestinal stromal tumor), were cultured and passaged on 15 cm dishes.
4. On day 1 cell lines were trypsinized, centrifuged, and counted using Countess 3 FL. Cells were then plated at ~2,000-3000 cells per well on a 96-well plate.
5. On day 2, inhibitors were dissolved in DMSO, at a concentration of 10μM and incubated for 5 days.
6. After incubation time, cells were washed with PBS, and frozen at -80°C. Cells were lysed using CyQuant buffer, where dye was used to mark DNA content from the cells. Then a spectrophotometer was used to read absorbance values which correlated DNA content to cell count.



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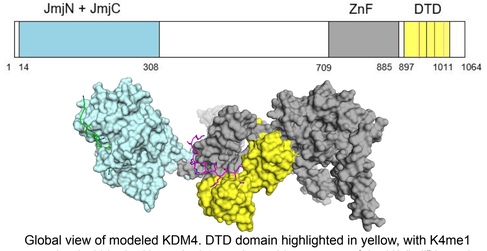
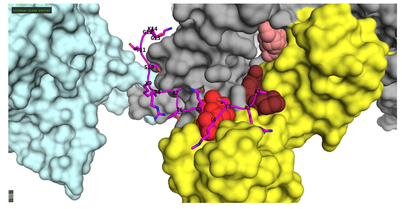
RESULTS AND RELEVANT FIGURES

In-Silico Modeling of KDM4's Double Tudor Domain Using Relevant Pharmacological Data

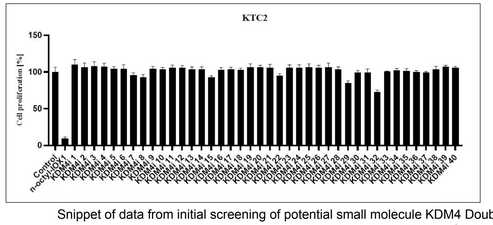


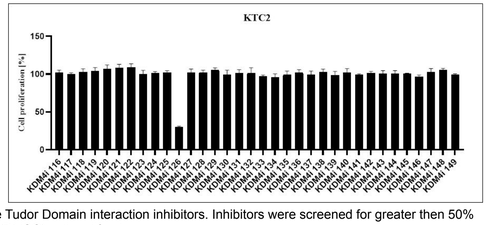
Global view of modeled KDM4. DTD domain highlighted in yellow, with K4me1 contained within the N-terminal 14-amino acid sequence of the H3 "tail" bound as reference. JMJ domain is highlighted in cyan with a green bound-H3 tail.



Potential binding pockets within the DTD domain of KDM4A highlighted in red, burgundy, and pink. H3K4me1 present as reference for docking.

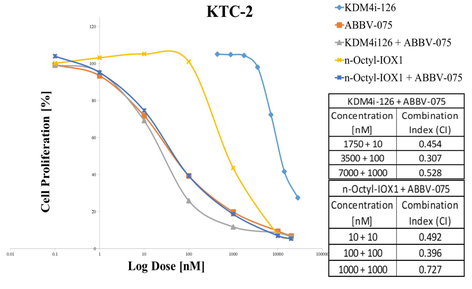
Initial Drug Screening on KTC-2 of Small Molecule Inhibitors Hypothesized to Target KDM4's Double Tudor Domain





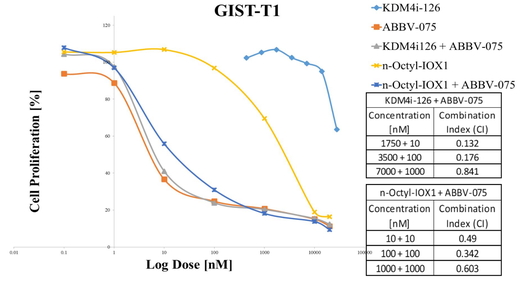
Snippet of data from initial screening of potential small molecule KDM4 Double Tudor Domain interaction inhibitors. Inhibitors were screened for greater then 50% inhibition of control (DMSO) cell proliferation

IC_{50} Curves with Hypothesized Inhibitor of KDM4's Double Tudor Domain, Known Inhibitor of KDM4's Jumanji Domain, and Known BET inhibitor



KDM4i-126 + ABBV-075	
Concentration [nM]	Combination Index (CI)
1750 + 10	0.454
3500 + 100	0.307
7000 + 1000	0.528

n-Octyl-IOX1 + ABBV-075	
Concentration [nM]	Combination Index (CI)
10 + 10	0.492
100 + 100	0.396
1000 + 1000	0.727



KDM4i-126 + ABBV-075	
Concentration [nM]	Combination Index (CI)
1750 + 10	0.132
3500 + 100	0.176
7000 + 1000	0.841

n-Octyl-IOX1 + ABBV-075	
Concentration [nM]	Combination Index (CI)
10 + 10	0.49
100 + 100	0.342
1000 + 1000	0.603

CONCLUSIONS

The identified compound KDM4i-126 inhibited cell proliferation in cell lines GIST-T1 and KTC2. Based on *in-silico* modeling and small molecule dynamics, this indicates that 126 may be an interaction inhibitor between the DTD domain of KDM4 and H3K4me1.

Based on synergy experiments between BETis and KDM4i-126, as well as comparing results to previous data patterns regarding KDM4 enzymatic inhibitors and BETis, KDM4i-126 is a promising compound that could be developed into a BETi adjuvant.

FUTURE DIRECTIONS

1. Continue to conduct synergy experiments between KDM4-126 and other BET inhibitors
2. Set up a thermophoresis experiment that detects the motion of fluorescent molecules along a microscopic temperature gradient, which reflects changes in the molecular hydration shell, charge, or size.
   - Use these experiments to characterize KDM4i-126 binding specificity via dissociation constants (K_d)
3. Conduct a histone peptide microarray study to determine the binding of H3K4me1 to HaloTagged-DTD. Test KDM4i-126 to further characterize and understand if this small molecule disrupts DTD-binding to key lysine residues on H3.
4. If future experiments are indicative of KDM4i-126 binding specificity to the DTD, we will begin to plan and carry out *in-vivo* experiments.

ACKNOWLEDGEMENTS

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REFERENCES

[1] Su Z, Wang F, Lee J-H, et al. Reader domain specificity and lysine demethylase-4 family function. Nature communications. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5114558/. Published November 14, 2016. Accessed July 28, 2022.

[2] Crump NT, Ballabio E, Godfrey L, et al. BET inhibition disrupts transcription but retains enhancer-promoter contact. Nature News. https://www.nature.com/articles/s41467-020-20400-z. Published January 11, 2021. Accessed July 28, 2022.

[3] Sengupta S, George RE. Super-Enhancer-Driven transcriptional dependencies in cancer. Trends in cancer. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5546010/. Published April 2017. Accessed July 28, 2022.

[4] Land, H.; Humble, M. S., YASARA: A Tool to Obtain Structural Guidance in Biocatalytic Investigations. Methods Mol Biol 2018, 1685, 43-67.