Setting-up HTRF technology for G9a & DNMT1 High-Throughput Screening

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Introduction

•**Epigenetic modifications** play a crucial role in **human diseases**. Epigenetic phenomena have gained increased attention in the field of cancer research, with many studies indicating that they are significantly involved in tumor establishment and progression.

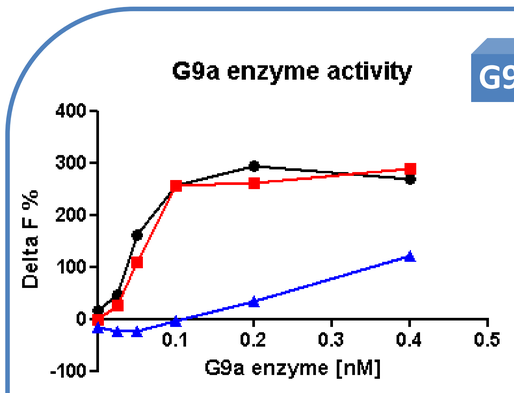
•**Histone methyltransferases** (HMTs) are a large group of enzymes that specifically methylate protein lysine and arginine residues, especially in histones, using S-adenosyl-methionine (SAM) as the methyl donor. DNA methylation is also an important epigenetic mark in eukaryotes, and aberrant pattern of this modification is involved in numerous diseases such as cancers. Therefore, there is a need for identifying new small inhibitors of **DNA methyltransferases** (DNMTs).

•However, in general, HMTs have no widely accepted **high-throughput screening (HTS) assay format**, and reference inhibitors are not available for many of the enzymes. Moreover, despite the development of numerous in vitro DNMT assays, there is a lack of reliable tests suitable for high-throughput screening, which can also give insights into inhibitor mechanisms of action.

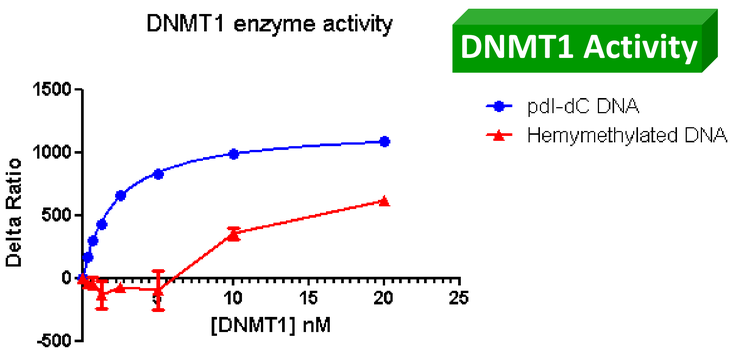
•We describe the setting up of a G9a enzyme activity assay using HTRF® technology in a HTS 384-well plate format which can also give insights into inhibitor mechanisms of action.

•A homogeneous HTRF assay for DNMT1 enzyme activity was also optimized in a HTS 384-well plate format which could be used for identifying new inhibitors fulfilling the requirements of robustness and reproducibility.

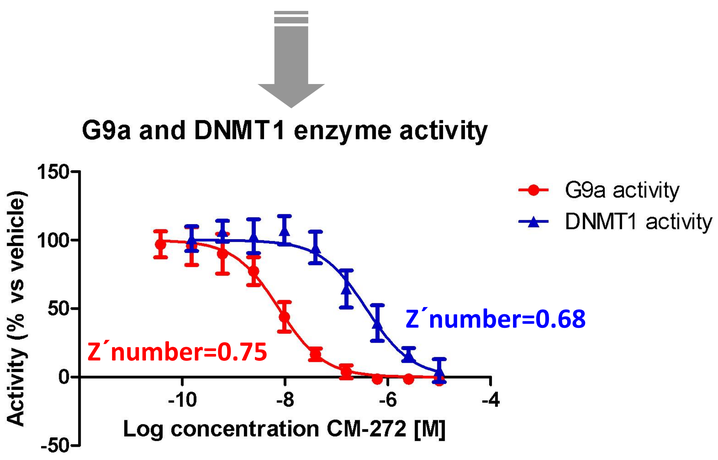
G9a & DNMT1 Optimization



- The biochemical assay to measure G9a enzyme activity relies on time-resolved fluorescence energy transfer (TR-FRET) between europium cryptate (donor) and XL665 (acceptor). TR-FRET is observed when biotinylated histone H3K9 peptide is incubated with cryptate-labeled anti-histone H3K9 antibody (Cisbio Cat# 61KB2KAE) and streptavidin XL665 (Cisbio Cat#610SAXLA). Enzyme activity assay was carried out in a white 384-well plate in a final volume of 20µl of assay buffer (50mM Tris-HCl, 10mM NaCl, 4mM DTT, 0.01% Tween-20 pH9) containing 20µM SAM and 40nM biotinylated histone substrates. After 1 hour of incubation at room temperature, reaction was stopped adding cryptate-labeled anti-histone H3K9 antibody and streptavidin XL665 conjugate.

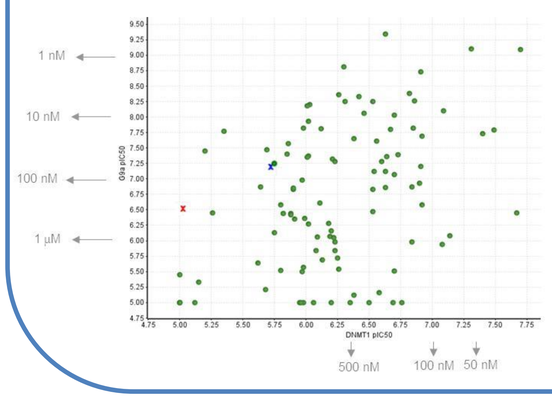


- The biochemical assay to measure DNMT1 enzyme activity relies on time-resolved fluorescence energy transfer (TR-FRET) between terbium cryptate (donor) and d2 (acceptor) using the EPIgeneous™ methyltransferase assay (Cisbio Cat#62SAHPEB). TR-FRET is observed when antibody specific to SAH labeled with terbium cryptate is incubated with d2-labeled S-adenosylhomocysteine. Enzyme activity assay was carried out in a white 384-well plate in a final volume of 20µl of assay buffer (50mM Tris-HCl, 1mM EDTA, 1mM DTT, 0.1% Triton X-100, 5% glycerol pH 7.5) containing 1µM SAM and 1µg/ml of DNA substrates. After 20 minutes at 37ºC, reaction was stopped and processed as indicated in the Kit instructions.



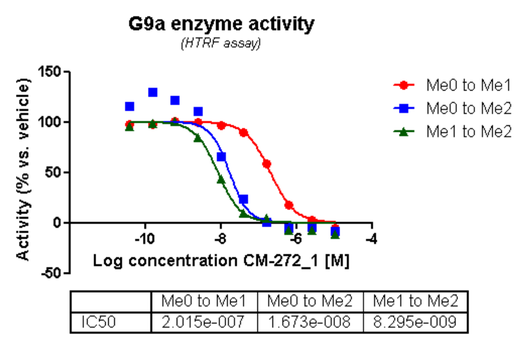
- DNMT1 enzyme activity was carried out during 20 minutes at 37ºC. G9a was carried out during 60 minutes at room temperature using biotinylated monomethylated histone H3K9 peptide and product was detected with cryptate-labeled anti-dimethylated histone H3K9 antibody

Biochemical profiling of compounds in G9a and DNMT1 activities

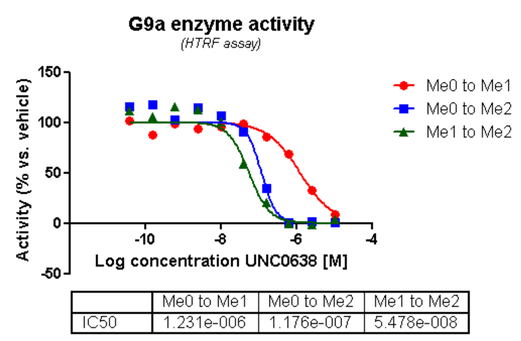


Mode of action studies

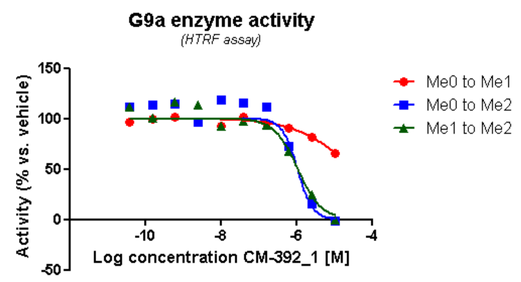
Mode of action (I): Processivity of G9a activity



	Me0 to Me1	Me0 to Me2	Me1 to Me2
IC50	2.015e-007	1.673e-008	8.295e-009



	Me0 to Me1	Me0 to Me2	Me1 to Me2
IC50	1.231e-006	1.176e-007	5.478e-008



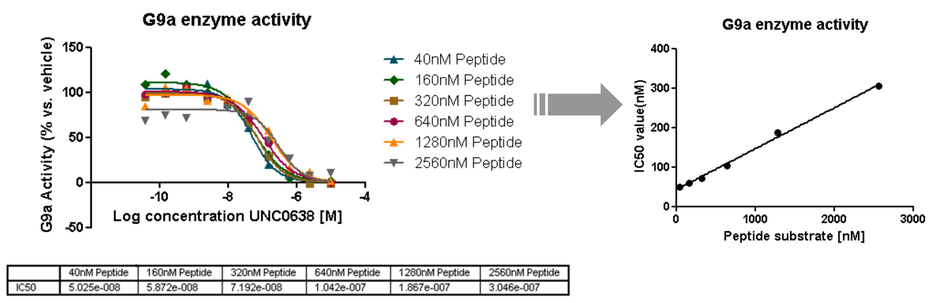
	Me0 to Me1	Me0 to Me2	Me1 to Me2
IC50	>1.00E-05	1.047e-006	1.074e-006

G9a inhibition IC_{50} values of tool compounds in different steps of the methyltransferase

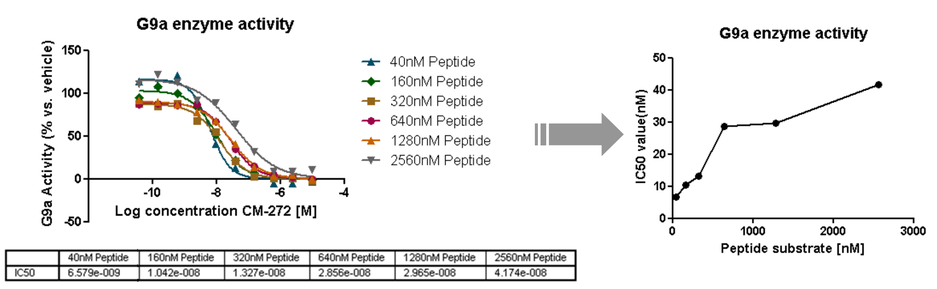
Compound ID#	Me0 to Me1	Me0 to Me2	Me1 to Me2
CM-272_1	2.02E-07	1.67E-08	8.30E-09
UNC0638	1.23E-06	1.18E-07	5.48E-08
CM-392_1	>1.00E-05	1.05E-06	1.07E-06

- Enzyme activity assay was carried out in a white 384-well plate in a final volume of 20µl of assay buffer (50mM Tris-HCl, 10mM NaCl, 4mM DTT, 0.01% Tween-20 pH9) containing 20µM SAM and 40nM biotinylated unmethylated or monomethylated histone K3K9. After 1 hour of incubation at room temperature, reaction was stopped adding cryptate-labeled anti-monomethylated histone H3K9 antibody or anti-dimethylated histone H3K9 histone to detect different products species, and streptavidin XL665. IC_{50} values were calculated with the 4 parameters non-lineal regression model of GraphPrism software

Mode of action (II): Competition assays of G9a activity



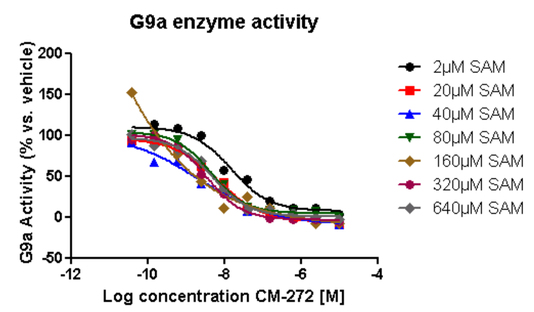
	40nM Peptide	160nM Peptide	320nM Peptide	640nM Peptide	1280nM Peptide	2560nM Peptide
IC50	5.025e-008	5.872e-008	7.192e-008	1.042e-007	1.867e-007	3.046e-007



	40nM Peptide	160nM Peptide	320nM Peptide	640nM Peptide	1280nM Peptide	2560nM Peptide
IC50	6.579e-009	1.042e-008	1.327e-008	2.856e-008	2.965e-008	4.174e-008

- G9a enzyme activity was carried out in in a white 384-well plate in a final volume of 20µl of assay buffer (50mM Tris-HCl, 10mM NaCl, 4mM DTT, 0.01% Tween-20 pH9) containing 20µM SAM and different concentrations of biotinylated monomethylated histone H3K9 substrate. After 1 hour of incubation at room temperature, reaction was stopped adding cryptate-labeled anti-dimethylated histone H3K9 antibody and streptavidin XL665 beads.

Competitive inhibition of UNC0638 was confirmed by cristalography studies: 3RJW.pdf file



	2µM SAM	20µM SAM	40µM SAM	80µM SAM	160µM SAM	320µM SAM	640µM SAM
IC50	1.557e-008	7.051e-009	2.256e-009	4.591e-009	~ 1.155e-013	3.542e-009	5.129e-009

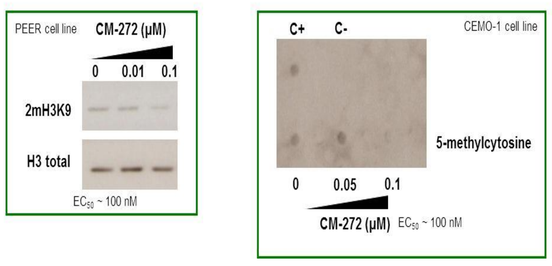
- G9a enzyme activity was carried out in in a white 384-well plate in a final volume of 20µl of assay buffer (50mM Tris-HCl, 10mM NaCl, 4mM DTT, 0.01% Tween-20 pH9) containing 40nM of biotinylated monomethylated histone H3K9 substrate and different concentrations of SAM. After 1 hour of incubation at room temperature, reaction was stopped adding cryptate-labeled anti-dimethylated histone H3K9 antibody and streptavidin XL665 conjugate.

Functional profiling of tool compounds

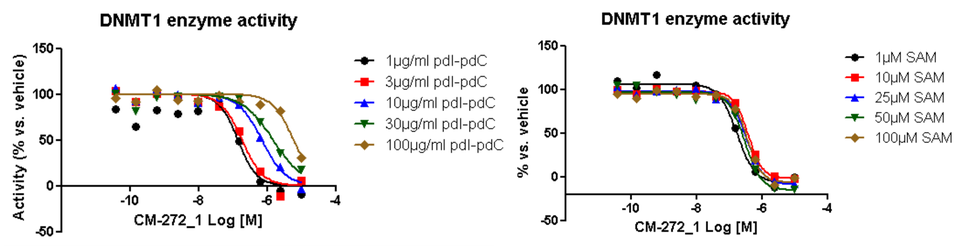
- Selected, from our proprietary chemical series, as pharmacological tool compound: **CM-272**

G9a	IC_{50} (nM)	9
DNMT1	IC_{50} (nM)	347

- **Epigenetic marks,** *in-vitro* cellular



Mode of action (III): Competition assays of DNMT1 activity



	1µg/ml pdI-pdC	3µg/ml pdI-pdC	10µg/ml pdI-pdC	30µg/ml pdI-pdC	100µg/ml pdI-pdC
IC50	1.427e-007	1.881e-007	7.130e-007	1.526e-006	5.968e-006

	1µM SAM	10µM SAM	25µM SAM	50µM SAM	100µM SAM
IC50	1.753e-007	3.886e-007	3.375e-007	3.008e-007	3.584e-007

- DNMT1 enzyme activity was carried out during 20 minutes at 37ºC in the presence of different DNA (left panel) or SAM (right panel) concentrations.

CONCLUSIONS

- The key methyltransferases enzymes activities G9 and DNMT1 have been optimized in a 384-well plate format able to be used with screening purposes
- The assays permitted to find and characterize reversible inhibitor compounds with a high potency and selectivity on these targets, confirmed by direct measurements of cellular epigenetic marks
- The assays could be used to explore mechanisms of action such as processivity and compound profiling on different steps of G9a activity, and substrate competition analysis in both epigenetic enzymes

Acknowledgments

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We thank Cisbio for providing kits for assay optimization for this study.