Domainex
Enrich your Medicines Pipeline

Biochemical lysine methyltransferase assay – studying low-turnover enzymes

White Paper

domainex.co.uk

Introduction

Lysine methyltransferases (KMTs) are enzymes that epigenetically regulate gene expression by the covalent modification of histones. They catalyse the transfer of methyl groups from S-Adenosyl methionine (SAM) to lysine residues on histone proteins. There is a multitude of literature which supports the role of KMTs in mechanisms of carcinogenesis making them attractive oncology targets.[1-4] KMTs have two binding sites accessible to small-molecule inhibitors: the substrate binding site and the cofactor (SAM) binding site. In this study Domainex sought substrate-competitive inhibitors: this may be a means to achieve better selectivity between KMT family members than addressing the SAM site, and this approach means that inhibitors do not have to compete with high intracellular SAM levels. Here, we describe the development of a biochemical assay to measure KMT activity and perform mechanism of action studies on selected compounds.

Assay Development

PerkinElmer's AlphaLISA technology is a homogenous, non-radioactive proximity assay that is ideal for the assay of low-turnover enzymes such as KMTs (https://www.perkinelmer.com/lab-products-and-services/application-support-knowledgebase/alphalisa-alphascreen-no-wash-assays/alphalisa-alphascreen-no-washassays-main.html#AlphaLISAAlphaScreenno-washassays-Assayprinciple). This bead-based assay relies on transfer of singlet oxygen from a donor to an acceptor bead when they come in close proximity, ultimately producing a luminescent/fluorescent signal. The intensity of the signal is directly proportional to the number of donor/acceptor bead pairs within the diffusion range of the excited oxygen species.

Unless otherwise stated, all assays were performed using commercially available EZH2 enzyme complex (BPS Cat # 51004), with SAM concentration at its apparent K_m (10 µM), and a biotinylated H3 peptide substrate comprising amino acids 1-45 (13.4 nM). Apparent Km's were determined using the Michaelis-Menten equation (Figure 1A). Optimal enzyme concentration and assay duration were determined prior to performing screening assays and mechanism of action work. In addition, Domainex developed methylation assays using as the substrate biotin-tagged full-length H3/H4 histone tetramers (produced in house). pIC_{50} values for the published EZH2 inhibitor EPZ6438 obtained using tetramer and peptide assays were within 3-fold of each other (Figure 1B).

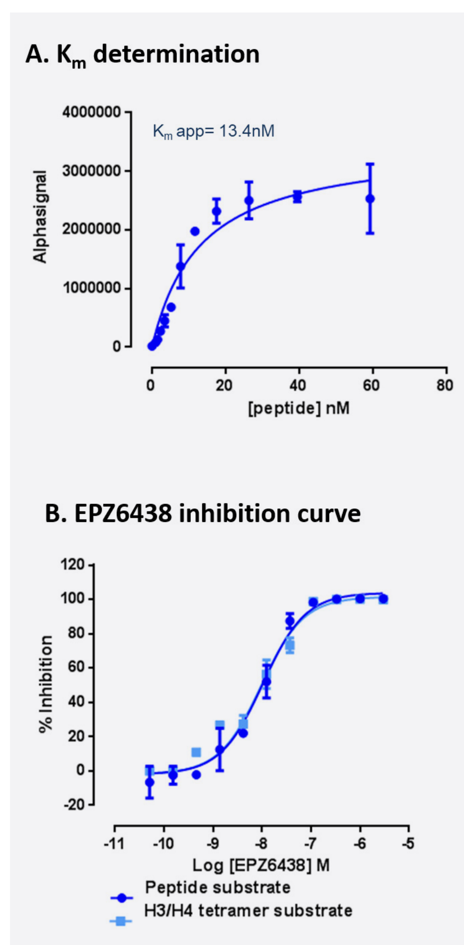

Figure 1: Assay development: (A) Example 1-45 H3 peptide substrate titration against fixed concentration of EZH2 enzyme. AlphaLISA signal plotted against substrate concentration. (B) Example pIC_{50} curve of EZH2 inhibitor EPZ6438 using either 1-45 H3 peptide or full length H3/H4 tetramer as substrate. Serial dilution of EPZ6438 was incubated with fixed concentration of enzyme, SAM and substrate at K_m. Percentage inhibition plotted against inhibitor concentration.

Routine Screening and Selectivity Assessment

For routine screening 10-point concentration-response curves were measured in assay buffer containing 1% DMSO, and 'no-enzyme' controls were used for normalisation of inter-plate variability. EZH2 enzyme complex and cofactor SAM were pre-incubated for 30 min to form the enzyme-substrate complex followed by addition of compounds and a further pre-incubation for 15 min at room temperature (RT). The reaction was initiated by addition of the 1-45 H3 peptide substrate at its K_m and was allowed to proceed for 3 hours, before the extent of mono- and di-methylation of Histone 3 Lysine 27 (H3K27me2-1) was quantified using the AlphaLISA assay. To stop the reaction and detect the methylated lysine, AlphaLISA Streptavidin Donor beads and Anti-di/mono-methyl-Histone H3 Lysine 27 (H3K27me2-1) AlphaLISA Acceptor beads were added in high salt buffer and incubated for 30 min at RT. The AlphaLISA signal was measured on a PheraStar instrument.

A G9a selectivity assay was carried out as described for EZH2 except that the reaction was stopped after 45 min and anti-dimethyl-Histone H3 Lysine 9 (H2K9me2) AlphaLISA Acceptor beads were used.

For an assay to pass quality control criteria, the Z' for each plate had to be >0.7 and positive control compound measurements had to be within 3-fold of historic values.

Domainex identified several EZH2 inhibitors with more than 10-fold selectivity over G9a (Figure 2). The identified EZH2 inhibitors did not show interference in an AlphaLISA TruHits assay. Finally, the Kinase Detection Reagent was added and incubated for 30 min prior to measuring the resulting luminescence signal on a PheraStar instrument.

For an assay to pass, Z' of each plate had to be >0.7 and included controls had to be within 3-fold of historic values.

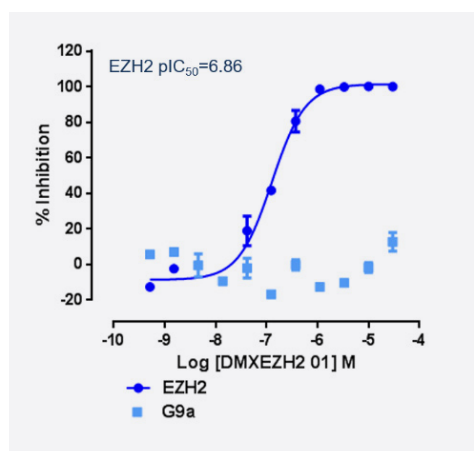

Figure 2: EZH2 selectivity: Inhibition curve of one EZH2 inhibitor against EZH2 and G9a. The compound shows good activity against EZH2 but no activity against G9a.

Mechanism of Action Studies

To investigate whether the identified EZH2 inhibitors were competitive with the cofactor SAM or the histone substrate, pIC_{50}s were determined in the presence of 1x and 10x concentration of cofactor or substrate (Figures 3A and 3B). These studies revealed that the pIC_{50} values of our inhibitors are independent of SAM concentration but dependent on peptide concentration, suggesting that they are peptide-competitive inhibitors.

To show that our inhibitors do not interfere with the enzyme, pIC_{50}s were determined in the presence of 1x or 5x enzyme concentration (Figure 3C). The observed pIC_{50}s were independent of enzyme concentration, suggesting that they are true enzyme inhibitors.

Reversibility studies (Figure 3D) were carried out by incubating 10-fold IC_{50} concentration of inhibitors with enzyme at 100-fold the normal concentration for 30 min, followed by dilution to normal assay conditions. Control experiments were run with 10-fold and 0.1-fold IC_{50} of inhibitors under normal assay conditions. EZH2 inhibitors showed no residual inhibition after dilution, which is suggestive of reversible binding.

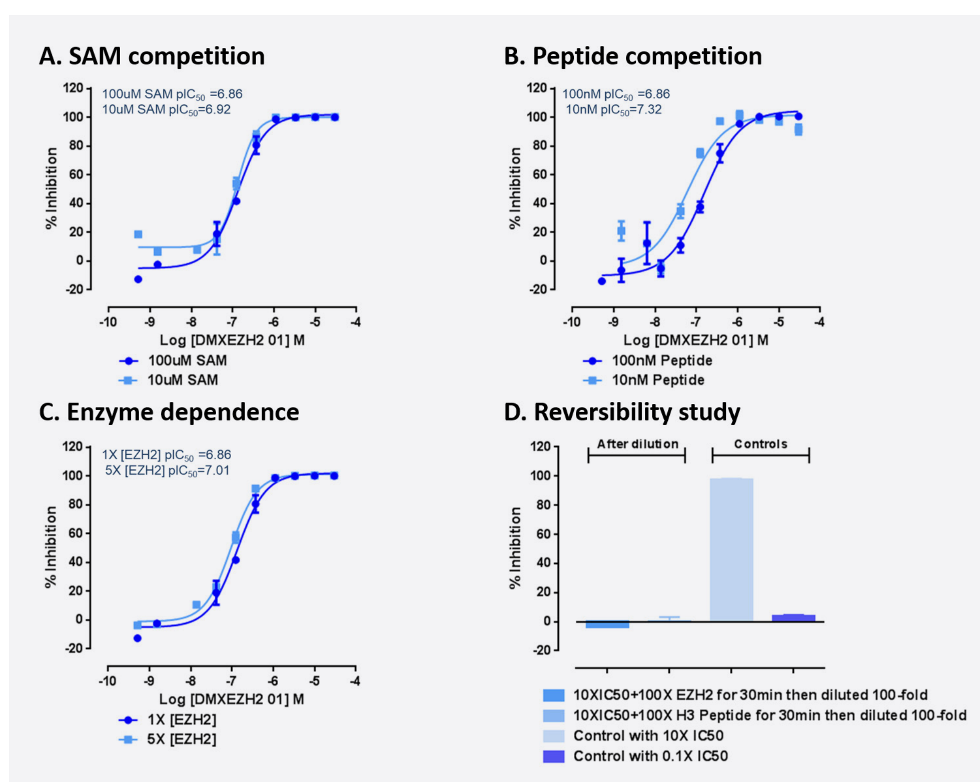

Figure 3: Mechanism of action studies: (A) Inhibition curve of DMXEZH2-01 in the presence of 10μM and 100μM SAM. (B) Inhibition curve of DMXEZH2-01 at 100nM peptide & 10nM peptide. (C) Inhibition curve of DMXEZH2-01 at 1X EZH2 and 5X EZH2 concentration. (D) Reversibility study of DMXEZH2-01.

References

1. Human protein methyltransferases as a target class for drug discovery. Copeland RA, Solomon ME, Richon VM. *Nat. Rev. Drug Discov.* **2009**, 8, 724–732

2. SMYD3 encodes a histone methyltransferase involved in the proliferation of cancer cells. Ryuji Hamamoto, Yoichi Furukawa, Masashi Morita, Yuko Iimura, Fabio Pittella Silva, Meihua Li, Ryuichiro Yagyu and Yusuke Nakamura. *Nature Cell Biology*, **2004**, (6), 731-740;

3. Enhanced SMYD3 expression is essential for the growth of breast cancer cells. Ryuji Hamamoto, Fabio Pittella Silva, Masataka Tsuge,Toshihiko Nishidate,Toyomasa Katagiri, Yusuke Nakamura and Yoichi Furukawa. *Cancer Sci.*, **2006**, (97), 113-118;

4. SMYD3 as an Oncogenic Driver in Prostate Cancer by Stimulation of Androgen Receptor Transcription. Cheng Liu, Chang Wang, Kun Wang, Li Liu, Qi Shen, Keqiang Yan, Xiaoqing Sun, Jie Chen, Jikai Liu, Hongbo Ren, Hainan Liu, Zhonghua Xu, Sanyuan Hu, Dawei Xu andYidong Fan. *J. Natl. Cancer. Inst.*, **2013**, 105, 22, 1719–1728.

Domainex

domainex.co.uk

Enrich your medicines pipeline

Providing drug discovery research brainpower

FEATURED SERVICE

Assay Biology

Quality Data Throughout

Assays built for full compound profiling

- Biochemical
  - Intracellular and membrane targets
  - Potency, selectivity & MOA measurements
- Biophysical:
  - Full suite (MST, GCI/SPR, DSF/nanoDSF, NMR, X-ray crystallography)
  - Affinity, kinetic & structural determinations
- Cellular
  - Cell lines and primary cells
  - Signalling & reporter-based assays
  - On and off target assessments

- Tailored multidisciplinary expertise
- Forming dynamic relationships
- Innovative outcomes for our partners

Based in the Cambridge UK Biocluster

Established in 2001

Contact us today!

enquiries@domainex.co.uk