Bioorganic & Medicinal Chemistry Letters 28 (2018) 1708–1713

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Targeting lysine specific demethylase 4A (KDM4A) tandem TUDOR domain – A fragment based approach

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ARTICLE INFO

Article history:
Received 17 January 2018
Revised 17 April 2018
Accepted 18 April 2018
Available online 19 April 2018

Keywords:
Fragment based drug discovery
2D-NMR based fragment screening
KDM4A tandem TUDOR domain

ABSTRACT

The tandem TUDOR domains present in the non-catalytic C-terminal half of the KDM4A, 4B and 4C enzymes play important roles in regulating their chromatin localizations and substrate specificities. They achieve this regulatory role by binding to different tri-methylated lysine residues on histone H3 (H3-K4me3, H3-K23me3) and histone H4 (H4-K20me3) depending upon the specific chromatin environment. In this work, we have used a 2D-NMR based fragment screening approach to identify a novel fragment (**1a**), which binds to the KDM4A-TUDOR domain and shows modest competition with H3-K4me3 binding in biochemical as well as *in vitro* cell based assays. A co-crystal structure of KDM4A TUDOR domain in complex with **1a** shows that the fragment binds stereo-specifically to the methyl lysine binding pocket forming a network of strong hydrogen bonds and hydrophobic interactions. We anticipate that the fragment **1a** can be further developed into a novel allosteric inhibitor of the KDM4 family of enzymes through targeting their C-terminal tandem TUDOR domain.

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Methylation of lysine residues on histone proteins plays an important epigenetic role in the regulation of gene expression by recruiting different transcription factors to the chromatin.[1–4] Homeostasis of these methylation patterns by different histone lysine methyl transferase and histone lysine demethylase enzymes is of key importance to maintain proper cellular development and to enable cellular differentiation.[3,5] Dysregulation of histone lysine methylations have been linked to aberrant gene expression and malignant transformations.[3,6–8] Removal of the histone lysine methylation marks are catalyzed by two families of enzymes. The first family is comprised of two enzymes namely; lysine specific demethylase 1 (LSD1) and 2 (LSD2). These enzymes belong to the family of flavin dependent amine oxidases.[9–11] The second family is comprised of several enzymes, which are members of the Jumonji C (JmjC) domain containing Fe^{2+} and alpha-ketoglutarate dependent di-oxygenase enzymes.[12,13] These enzymes are also known as JmjC domain containing histone lysine demethylases (JHDMs) and are further classified into seven sub-families based on their sequence similarities and domain architectures.[13]

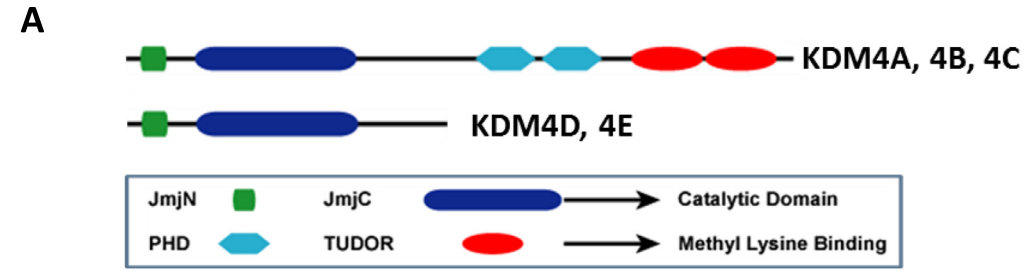
The KDM4 enzymes, which are expressed as five different isoforms in the human genome (KDM4A, KDM4B, KDM4C, KDM4D and KDM4E), constitute one of the seven sub-families of JHDMs and catalyze demethylation of H3K9 and H3K36 methylation marks.[13–16] Over-expression of KDM4A, 4B and 4C enzymes have been implicated in various cancers making them novel targets for developing anti-cancer drugs.[17–19] All of these enzymes contain the catalytic JmjC domain in their *N*-terminal half, which harbors the conserved Fe^{2+} and alpha-ketoglutarate binding motif. The first three family members (KDM4A, 4B and 4C) also contain a tandem PHD and tandem TUDOR domain in their C-terminal region (see Fig. 1A), which are absent in the KDM4D and KDM4E isoforms. Recent studies have indicated that the tandem TUDOR domains in the KDM4 family of enzymes play a critical role in regulating chromatin localizations and enzymatic function by binding to specific lysine methylation marks on histone proteins (H3-K4me3, H3-K23me3 and H4-K20me3).[6,20–23] Disruption of the TUDOR domain binding to the tri-methylated lysine residues on the histone tail may therefore serve as an allosteric mechanism of antagonizing KDM4 functions under specific chromatin environments.

Various small molecule inhibitors targeting the alpha-ketoglutarate binding site in the catalytic JmjC domain of the KDM4 family of enzymes have been reported with modest potency and

Abbreviations: NMR, nuclear magnetic resonance; ITC, Isothermal Titration Calorimetry; JmjC, Jumonji C; JHDM, JmjC domain containing histone lysine demethylase; CSP, chemical shift perturbation.

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https://doi.org/10.1016/j.bmcl.2018.04.050
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B

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KDM4A-TUDOR  895  ----ALQSITAGQKVISKHKNGRFYQCEVVRLTTETFYEVNFDDGSFSDNLYPEDIVSQD 950
KDM4B-TUDOR  915  ----LLRAVSLGQVVITKNRNGLYYRCRVIGAASQTCYEVNFDDGSYSDNLYPESITSRD 970
KDM4C-TUDOR  875  ----CEKVISVGQTVITKHRNTRYYSCRVMAVTSQTFYEVMFDDGSFSRDTFPEDIVSRD 930
                       : :: ** **:*::*  :* *.*:  :::* *** *****:* : :**.*.*:*

KDM4A-TUDOR  951  CLQFGPPAEGEVVQVRWTDGQVYGAKFVASHPIQMYQVEFEDGSQLVVKRDDVYTLDEEL 1010
KDM4B-TUDOR  971  CVQLGPPSEGELVELRWTDGNLYKAKFISSVTSHIYQVEFEDGSQLTVKRGDIFTLEEEL 1030
KDM4C-TUDOR  931  CLKLGPPAEGEVVQVKWPDGKLYGAKYFGSNIAHMYQVEFEDGSQIAMKREDIYTLDEEL 990
                  *:::***:***:*::::*.**::* **:..*   ::**********:.:** *::**:***
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Fig. 1. (A) Domain architecture of KDM4A, 4B, 4C, 4D and 4E enzymes. (B) Sequence alignment of the KDM4A, 4B and 4C TUDOR domains.

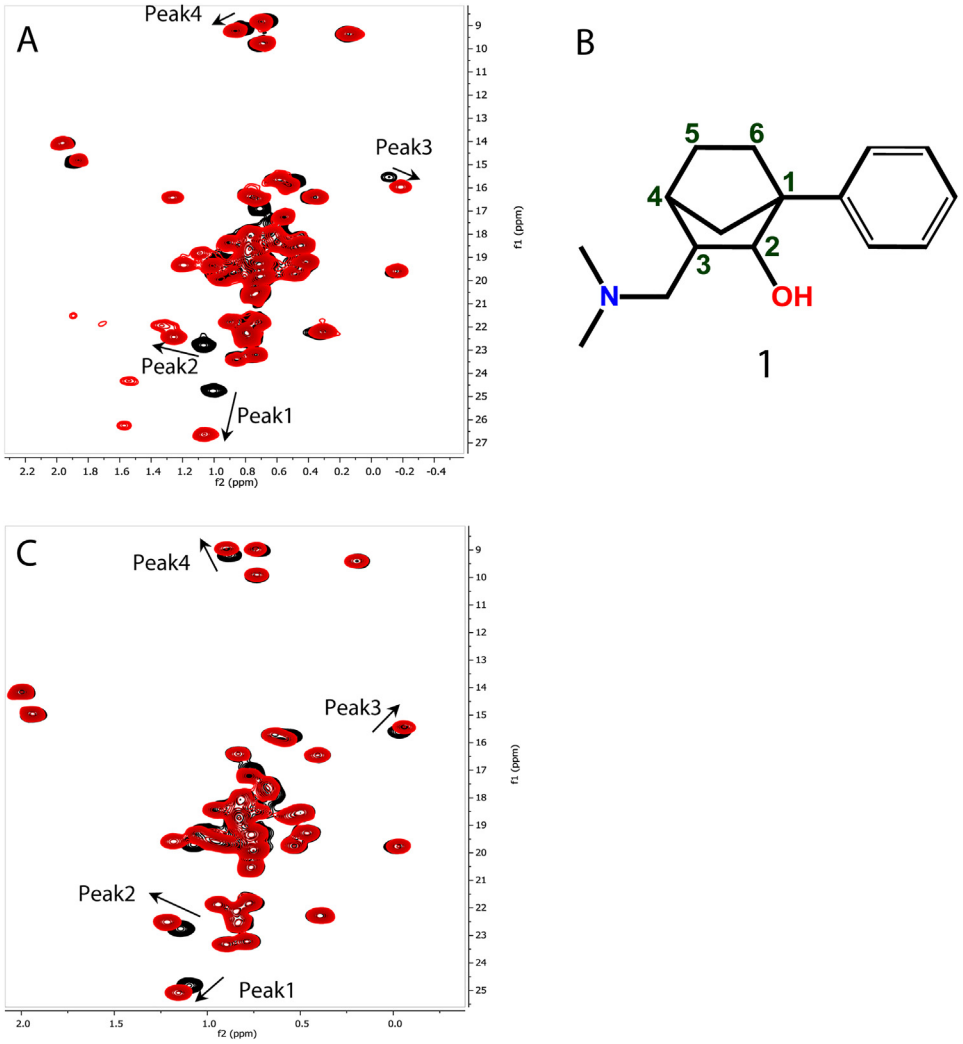


Fig. 2. (A) 13C-HSQC spectra of KDM4A tandem TUDOR domain: Protein only (Black) and with H3(1–10)K4me3 peptide (Red). (B) Fragment **1**: [3-((dimethylamino)methyl)-1-phenylbicyclo[2.2.1]heptan-2-ol]. (C) 13C-HSQC spectra of KDM4A tandem TUDOR: Protein only (Black) and with 1 (Red).

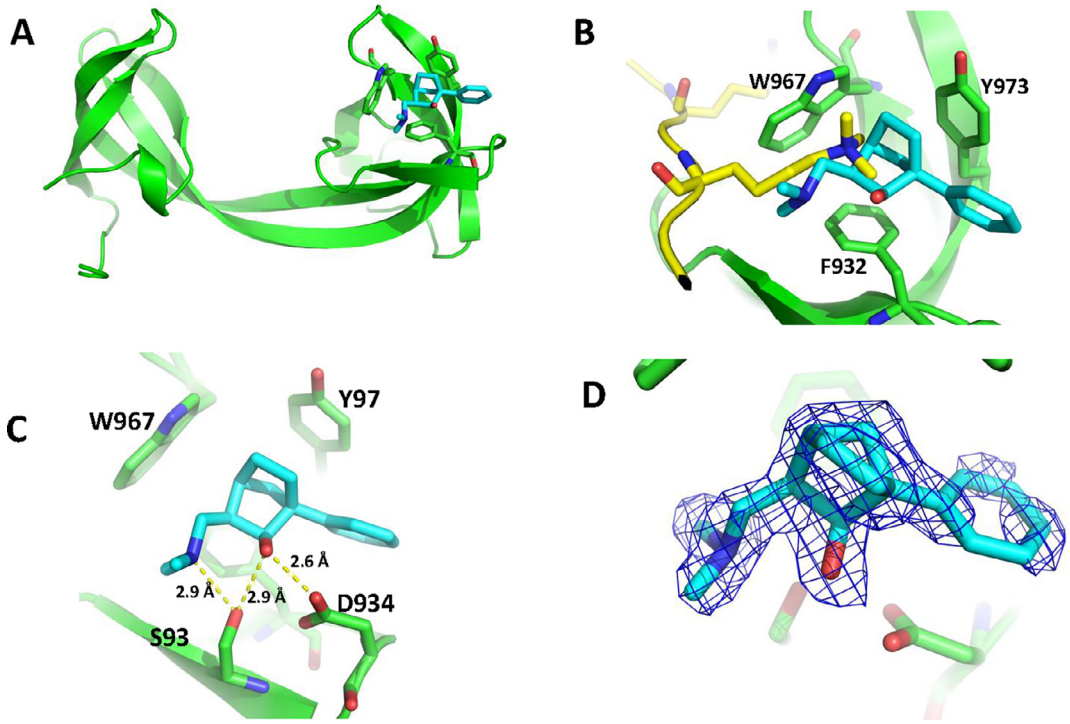


Fig. 3. (A) Crystal structure of KDm4A-tandem TUDOR domain in complex with fragment **1a** (shown in cyan). (B) Superimposed structures of H3-K4me3 peptide (yellow) and **1a** (cyan) bound KDM4A-TUDOR domain. The hydrophobic bicyclic core of **1a** fits into the aromatic cage formed by W967, F932 and Y973. The phenyl ring positions sideways outside of the aromatic cage. (C) Fragment **1a** bound stereo-specifically to the conserved tri-methyl lysine binding pocket through strong H-bonding and hydrophobic interactions. (D) Only (2*S*,3*R*)-3-((dimethylamino)methyl)-1-phenylbicyclo[2.2.1]heptan-2-ol (**1a**) enantiomer of **1** could be fit into the observed electron density (displayed at 1.0 σ).

Table 1
X-ray diffraction and refinement statistics for the KDM4A tandem TUDOR domain in complex with 1a (PDB ID: 5VAR).

Property	Value
Space group	*P*1211
Cell dimensions	
a, b, c (Å)	33.27, 39.84, 44.30
α, β, γ (°)	90.00, 101.61, 90.00
Resolution (Å)	32.59–1.83
Data completeness (%)	96.8
Rmerge	8.5%
Multiplicity	3.9
R_{work}/R_{free} (%)	19.4/24.3
Anisotropy	0.408
Estimated twinning fraction	No twinning to report
Number of atoms (protein/water)	869/74
Mean B-factor (Å^2)	28.55
r.m.s.d bonds (Å)	0.010
r.m.s.d angles (°)	1.030
Ramachandran	
Favored (%)	99.06
Allowed (%)	0.94
Outlier (%)	0

selectivity.[24–26] However, a major challenge in developing active site specific inhibitors for JmjC domain containing histone lysine demethylase (JHDM) enzymes is maintaining selectivity between different families to minimize undesired side effects.[26] Furthermore, inhibitors targeting the catalytic domain of JHDM enzymes potentially can also inhibit other Fe^{2+} and alpha-ketoglutarate dependent oxidases, which share a structurally similar catalytic domain.[26–29] The alternative approach of targeting the regulatory domains of the JDHM family of enzymes may therefore lead to the discovery of novel chemical probes or allosteric inhibitors for this class of epigenetic targets with better selectivity.[13]

To identify fragment binders to the KDM4A tandem-TUDOR domain we used 2D-NMR based fragment screening. This screening technique utilizing ^{15}N or ^{13}C-Heteronuclear Single Quantum Correlation (^{15}N- or ^{13}C-HSQC) spectroscopy, has been shown to be a very powerful tool in fragment based drug discovery.[30–32] The tandem TUDOR domain present in KDM4A, 4B and 4C enzymes shares high overall sequence homology and a conserved aromatic cage constituting the tri-methylated lysine binding pocket (Fig. 1B). Purified tandem TUDOR domain from the KDM4A enzyme bound H3(1–10)-K4me3 and H4(16–25)-K20me3 peptides with similar affinities as measured by Isothermal Titration Calorimetry (ITC) (See Fig. S1). We used ^{13}C-labeled (only terminal methyl groups of the Ile, Leu, Val and Met residues) KDM4A tandem TUDOR domain protein (see Supplementary materials and methods section) to perform a 2D-NMR based fragment screen. As shown in Fig. 2A, comparison of the ^{13}C-HSQC spectra of the protein itself (black) with that of the protein upon addition of H3(1–10)-K4me3 peptide (red) showed chemical shift perturbations (CSPs, indicated as Peak1 to 4 with arrows), which were used as indicators of binding. Using this we identified a total of six fragments from a library of ~3700 rule of three compliant fragments with a poor hit rate of less than 0.2%.[33,34] Only fragment **1** [3-((dimethylamino)methyl)-1-phenylbicyclo[2.2.1]heptan-2-ol] (Fig. 2B) exhibit binding with a modest affinity of nearly 80 μM and showed CSPs of all the four major cross peaks (Peak1 to 4 in Fig. 2C) that were shifted upon H3(1–10)-K4me3 peptide binding, suggesting a competitive binding mode that is desired for a functional antagonist. The other five fragments exhibit much weaker binding (affinities ranging from 0.5 to 1.5 mM as measured using 2D-NMR) and were not pursued further.

In order to further understand the binding mode of fragment **1** to KDM4A TUDOR domain, we have solved the co-crystal structure of KDM4A-TUDOR domain in complex with **1** at 1.86 Å resolution

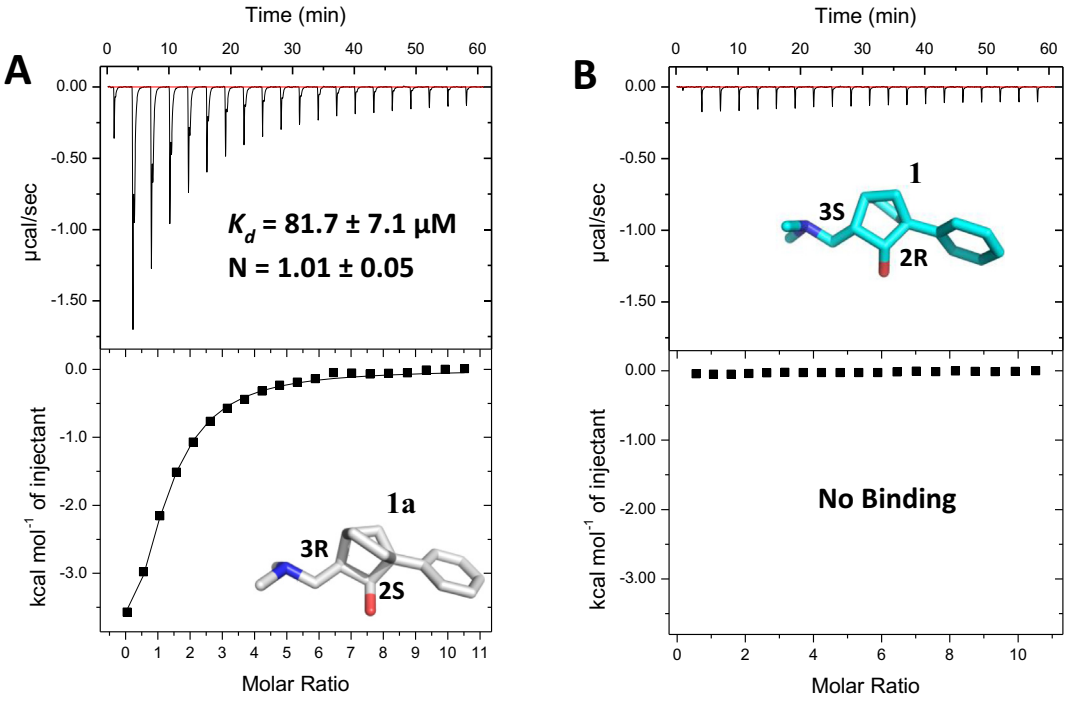


Fig. 4. (A) ITC binding data of **1a** to KDM4A-tandem TUDOR domain. (B) ITC binding data of **1b** binding to KDM4A-tandem TUDOR domain.

Table 2
Summary of ITC K_d values for 1a binding to the KDM4A, 4B, 4C, UHRF1 and 53BP1 tandem TUDOR domains.

TUDOR domains	K_d (uM)	N (Stoichiometry of binding)
KDM4A	81.7 ± 7.1	1.01 ± 0.05
KDM4B	127 ± 16	1.18 ± 0.08
KDM4C	129 ± 22	1.2 ± 0.1
UHRF1	no binding	
53BP1	No binding	

(Fig. 3A, Table 1). The structure shows that the hydrophobic bicyclic portion of **1** binds to the tri-methyl lysine binding pocket formed by the three conserved aromatic residues (F932, W967, Y973) and overlays well with the tri-methyl lysine head group of the H3-K4me3 peptide (Fig. 3B). The binding is mediated through a network of strong hydrogen bonding interactions involving the dimethylamino and hydroxyl group on **1** with D936 and S934 residues and hydrophobic interaction between the bicyclo-heptan moiety with the aromatic cage (Fig. 3C). The phenyl group at position 1 of the bicyclic ring points sideways towards the open end of the cage structure with greater degrees of rotational freedom around the C—C single bond. This is reflected by the slightly disordered electron density of the phenyl ring compared to the rest of the molecule (Fig. 3D). Interestingly, only the 2S, 3R enantiomer of **1** could be fit in the observed electron density (Fig. 3D), although the compound stock used for X-ray crystallography and NMR studies contained two stereo-isomers at an almost equimolar ratio (Fig. S2). In order to gain further insights on the stereo-chemical dependence of the binding, we separated the two stereo-isomers of **1** using preparative chiral chromatography (see methods and Figs. S2B and S2C) and confirmed their stereo-chemical properties

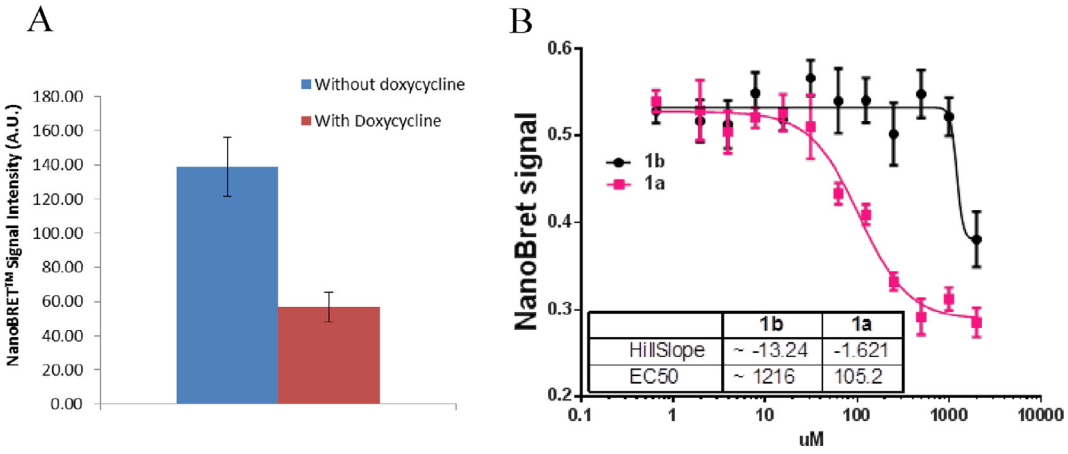


Fig. 5. (A) Validation of the NanoBRET™ assay. Addition of doxycycline in the culture media induces expression of KDM5B, which lowers the global level of H3-K4 methylation in the cell and thereby reduces the NanoBRET™ signal intensity. (B) Cellular Nano-BRET assay data on the inhibition of KDM4A-TUDOR domain binding to H3-K4me3 mark by **1a** and **1b**.

by solving small molecule X-ray structures (see Fig. S3). In the end, we obtained two enantiomers of **1**: (2*S*,3*R*)-3-((dimethylamino) methyl)-1-phenylbicyclo[2.2.1]heptan-2-ol (hereafter referred to as **1a**) and (2*R*,3*S*)-3-((dimethylamino)methyl)-1-phenylbicyclo [2.2.1]heptan-2-ol (hereafter referred to as **1b**).

Consistent with the X-ray crystallographic observations; ITC data presented in Fig. 4A and B shows that only **1a** (not **1b**) exhibits stoichiometric binding to the KDM4A-tandem TUDOR domain with a dissociation constant (K_d) of 81.7 ± 7.1 μM and competes with the H3(1–10)K4me3 peptide binding to KDM4A-TUDOR domain with an apparent K_i of 170 μM (Fig. S4, Table S1). Furthermore, mutating the three aromatic cage residues to alanine (F932A, W967A and Y973A) completely abrogates the binding of **1a** to the KDM4A tandem TUDOR domain (See Fig. S5). These data further corroborate the binding mode observed in the X-ray structure (Fig. 3).

In order to understand the specificity of **1a** towards KDM4 family members as well as other tandem TUDOR domain containing proteins, we have compared **1a** binding to the tandem TUDOR domains of KDM4A, KDM4B, KDM4C, UHRF1 and 53BP1 by ITC. As summarized in Table 2 and Fig. S6, **1a** exhibits similar binding affinities towards KDM4A, 4B and 4C tandem TUDOR domains but does not bind to the tandem TUDOR domains from UHRF1 and 53BP1. This is consistent with high sequence homology among the KDM4 tandem TUDOR domains (See Fig. 1B).

We next wanted to test whether **1a** can inhibit H3-K4me3 binding to the KDM4A TUDOR domain in cellular assays. In order to measure this inhibitory activity, we have developed a NanoBRET™ (Promega Corporation) based cellular proximity assay using NanoLuc®-KDM4A-TUDOR and Histone H3.3-HaloTag® as the two interacting proteins.[35] The HaloTag® on histone H3.3 was covalently modified using HaloTag® NanoBRET™ 618 Ligand (Promega Cat. No. G980A). Addition of the NanoBRET™ Nano-Glo® Substrate (Promega Cat. No. N157A) resulted in a NanoBRET signal, which is dependent on the proximity of the two interacting proteins. In order to validate that the observed NanoBRET signal is truly due to binding of the KDM4A-Tandem TUDOR domain to the histone H3-K4me3 mark, we utilized a cell line with inducible expression of KDM5B upon doxycycline treatment, which decreased the global H3-K4me3 level. As shown in Fig. 5A, addition of doxycycline in the culture media reduces the NanoBRET™ signal intensity by nearly 60%, suggesting that the observed NanoBRET™ signal is indeed dependent on the H3-K4me3 level and therefore drops upon overexpression of KDM5B enzyme. Addition of increasing amounts of **1a** inhibits the binding of KDM4A tandem TUDOR domain to the H3-K4me3 methylation mark in a concentration dependent manner with a concomitant drop in NanoBRET™ signal intensity. The plot of NanoBRET™ intensity vs **1a**-concentration yields an EC_{50} of 105 μM (with a 95% confidence interval of 72 to 154 μM;), whereas it's inactive enantiomer **1b** shows only very weak inhibition with an apparent EC_{50} ~1.2 mM (Fig. 5B).

Histone lysine methylation plays an important role in the epigenetic gene regulation pathway by serving as anchoring points on the chromatin that are recognized in a site and methylation state (mono-, di- or tri-) specific manner by different chromatin modifying proteins, enzymes or multi-protein complexes.[36–38] This recognition takes place through interaction between the specific lysine residue (in different methylated states) on histone proteins and the recognition modules (reader domains) present in different chromatin modifiers. Disruption of this recognition process is considered to be an alternative avenue towards developing novel epigenetic therapeutics with improved specifies and efficacies for treating many cancers and developmental diseases.[39]

Protein lysine methylations are recognized by the plant homeodomain (PHD), WD40 repeat domain, TUDOR domain, chromodomain and MBT domain.[39–41] All of these proteins harbor an aromatic cage structure formed by conserved aromatic residues that mediate pi-cation interactions with the methylated lysine residues. Development of small molecule and peptidomimetic inhibitors targeting many of these reader domains is being pursued by many laboratories around the world with a goal of finding novel therapeutics or chemical probes to delineate the biological functions of the target protein.[39,41–45]

The human genome encodes for 41 TUDOR domain containing proteins.[46] Together, they play important roles in regulating various cellular processes like RNA metabolism, DNA repair, cell division, differentiation and genomic stability.[47,48] Biochemical and biophysical studies have characterized TUDOR domains as reader modules that bind to methylated protein lysine and arginine residues.[47,48] Among the known methyl lysine binders, tandem TUDOR domains present in 53BP1, UHRF1, Spindlin1, SETDB1 and KDM4 enzymes (KDM4A, KDM4B and KDM4C) are the most well characterized.[39,47–49] Development of small molecule inhibitors targeting the TUDOR domains in 53BP1, Spindlin1 and UHRF1 have been reported.[39,50–52] Also, crystal structures of the SETDB1 tandem TUDOR domain in complex with small molecule inhibitors have been deposited in the protein data bank (PDB ID: 5KH6, 5KE2). However, currently, there are no reported inhibitors for the tandem TUDOR domains of the KDM4 family of histone lysine demethylase enzymes in the literature and hence this was a goal of this study.

The fragment **1a** identified in this study, stereo-specifically binds to the tri-methyl lysine binding pocket in KDM4A tandem TUDOR domain. To our knowledge, this is the first crystal structure of KDM4A tandem TUDOR domain in complex with a small molecule inhibitor, which antagonizes its ability to bind to the H3-K4me3 mark in biochemical and cell based assays. Efforts to further improve potency and selectivity of this novel fragment are currently ongoing in our laboratory. This may eventually lead to the discovery of more potent next generation compounds that are suitable as chemical probes to further explore the physiological functions of the KDM4A tandem TUDOR domain.

Accession codes

Atomic coordinates of KDM4A tandem TUDOR domain in complex with **1a** and associated experimental data has been released in protein data bank (PDB ID: 5VAR). Coordinates of **1a** and **1b** X-ray structures are deposited in Cambridge Crystallographic Data Centre under deposition IDs 1835343 (**1a**) and 1835344 (**1b**).

Notes

AKU, RAJ, LL, RP, JS, PMB, VM, RFH, AMP, CS are employees of AbbVie. The financial support for the research of these authors was provided by AbbVie Inc. North Chicago, Illinois, U.S.A.. AbbVie participated in the interpretation of the data, review and approval of the publication.

Acknowledgments

X-ray diffraction data were collected at beamline 17-ID in the facilities of the Industrial Macromolecular Crystallography Association Collaborative Access Team (IMCA-CAT) at the Advanced Photon Source. These facilities are supported by the companies of the Industrial Macromolecular Crystallography Association. Use of the Advanced Photon Source, an Office of Science User Facility operated for the U.S. Department of Energy (DOE) Office of Science by Argonne National Laboratory, was supported by the U.S. DOE under Contract No. DE-AC02-06CH11357.

A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2018.04.050.

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