Chapter 8

Inhibitors of JmjC-containing Histone Demethylases

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Abbreviation

Bpy	4'-carboxy 2,2'-bipyridine
H3	Histone H3
HMT	Histone methyltransferase
8HQ	8-hydroxyquinoline
JmjC	Jumonji-C
KDM	Lysine Demethylase
Kme _n	Methylated lysine (<i>n</i> =1, mono-; <i>n</i> =2, di-; <i>n</i> =3, tri-)
NOG	N-oxalylglycine
2OG	2-Oxoglutarate
2,4-PDCA	2,4-pyridine dicarboxylate
PHD-finger	Plant Homeodomain finger

Keywords

JmjC histone demethylases, 2OG oxygenases, KDMs

Abstract

Histone demethylases (KDMs) catalyse the removal of N-methyl marks on histones and play important roles in epigenetic regulation. Abnormal histone methylation and dysregulation of KDMs have been linked to multiple diseases, and KDMs are emerging as promising therapeutic targets. This Chapter provides an overview of JmjC-domain containing KDMs (JmjC-KDMs), with a particular focus on recent advances in JmjC-KDM inhibitor development from a structural perspective.

8.1. Introduction

Histone demethylases (KDMs) catalyse the removal of N-methyl marks on histones and play important roles in epigenetic regulation. Abnormal histone methylation and dysregulation of KDMs have been linked to multiple diseases. The majority of KDMs are JmjC-domain containing KDMs (JmjC-KDMs) that belong to the superfamily of Fe(II) and 2-oxoglutarate (2OG) dependent oxygenases. JmjC-KDMs are emerging as promising therapeutic targets, in particular, in cancer. The availability of crystal structures has provided understanding of the mechanism, function and selectivity of JmjC-KDMs at the molecular level and has aided structure-based inhibitor design. This Chapter provides an overview of recent advances in JmjC-KDM inhibitor development from a structural perspective.

8.1.1 JmjC-domain containing histone demethylases

Post-translational modifications on histones, including lysine methylation, have key roles in transcriptional control and cellular function. Lysines on histone tails can be mono- (me1), di- (me2), or tri-(me3) methylated, and dynamically regulated by histone methyltransferases (HMTs) and lysine demethylases (KDMs) (Klose and Zhang 2007).

There are two distinct families of KDMs that catalyse the demethylation of *N*-methyllysines on histones. KDM1/LSDs belong to the flavin adenine dinucleotide (FAD) dependent monoamine oxidase superfamily, and Jumonji-C (JmjC) domain containing KDMs (JmjC-KDMs), the larger of the two KDM families, belong to the Fe(II) and 2-oxoglutarate (2OG) dependent oxygenase superfamily. There are at least six human subfamilies of JmjC-KDMs (KDM2-7), based on homologies of their catalytic JmjC-domains, with each having distinct sequence and *N*-methylation state selectivity (**Table 1**, see (Højfeldt 2013) for reviews on function and substrate selectivities of KDMs).

Trimethylated as well as mono- and di-methylated lysines can be demethylated by JmjC-KDMs, unlike KDM1 subfamily which cannot demethylate trimethylated lysines due to the required formation of an imine (**see Chapter 7**). The mechanism of demethylation by JmjC-KDMs requires 2OG and dioxygen as co-substrates and Fe(II) as a cofactor. Evidence shows that demethylation is via known 2OG oxygenase catalysis in which 2OG and the substrate, followed by dioxygen, bind to the active site (**Figure 8.1**). A highly reactive Fe(IV)-oxo intermediate, formed through oxidative decarboxylation of 2OG, can subsequently abstract a hydrogen atom from the *N*-methyl group. This results in an unstable hemiaminal which can fragment to give demethylated lysine and formaldehyde.

Name	Synonyms	Substrate ⁺	PDB (ligand)	Fe(II) metal binding residues		
KDM2A	FBXL11, JHDM1A	H3K36me2/1	4QX7 (2OG, Ni(II), H3K36me2) ¹	H212	D214	H284
KDM2B*	FBXL10, JHDM1B	H3K36me2/1	-	H211	D213	H283
KDM3A*	JMJD1A, JHDM2A	H3K9me2/1	-	H1120	D1122	H1175
KDM3B	JMJD1B, JHDM2B	H3K9me2/1	4C8D (2OG, Mn(II))	H1560	D1562	H1689
JMJD1C	KDM3C, JHDM2C		5FZO (Mn(II))	H2336	E2338	H2466
KDM4A	JMJD2A, JHDM3A	H3K9me3/2, H3K36me3/2	5TVR (2OG, Ni(II))	H188	E190	H276
KDM4B	JMJD2B, JHDM3B	H3K9me3/2, H3K36me3/2	4LXL (24PDCA, Ni(II), H3K9me3)	H189	E191	H277
KDM4C	JMJD2C, JHDM3C	H3K9me3/2	4XDO (2OG, Fe(II))	H190	E192	H278
KDM4D	JMJD2D, JHDM3D	H3K9me3/2	4HON (2OG, Ni(II), H3K9me3)	H192	E194	H280
KDM5A	JARID1A, RBP2	H3K4me3/2	5IVB (2OG, Mn(II))	H483	E485	H571
KDM5B	JARID1B, PLU1	H3K4me3/2	5FUP (2OG, Mn(II))	H499	E501	H587
KDM5C	JARID1C, SMCX	H3K4me3/2	5FWJ (4, Mn(II))	H514	E516	H602
KDM5D*	JARID1D, SMCY	H3K4me3/2		H514	E516	H602
KDM6A	UTX	H3K27me3/me2	3AVR (2OG, Ni(II), H3K27me3)	H1146	E1148	H1226
KDM6B	JMJD3	H3K27me3/me2	2XUE (2OG, Fe(II))	H1390	E1392	H1470
KDM6C	UTY		3ZLI (2OG, Fe(II))	H1014	E1016	H1094
KDM7A	KIAA1718, JHDM1D	H3K9me2/1, H3K27me2/1	3KVA (2OG, Fe(II))	H282	D284	H354
KDM7B	PHF8, JHDM1F	H3K9me2/1	3KV4 (2OG, Ni(II), H3K4me3K9me2)	H247	D249	H319
PHF2	KDM7C, JHDM1E		3PU8 (2OG, Fe(II))	H249	D251	Y321

Table 8.1. Comparison of residues binding to metal centre in JmjC-KDMs. The crystal structures of human JmjC-KDMs reveal three residues that coordinate the active site Fe(II). * where the metal chelating residues are identified from sequence alignments (Klose and Zhang 2007). Note that the KDM nomenclature is not used when histone demethylase activity has not been independently verified. PDB is a representative from the protein when more than one structure is available. ⁺ Independently verified consensus assignment in vitro and in cells. Other putative substrates have been identified in vitro. Ligands are Ni(II) and NOG unless otherwise stated. All PDBs are from Homo sapiens except where indicated: ¹Mus musculus

Functional assignments for some JmjC-proteins remain controversial, with both hydroxylase and demethylase activities being reported (e.g. MINA53 and NO66, JMJD5/KDM8, JMJD6). Recent work has revealed that some JmjC-KDMs can catalyse the demethylation of methylated lysines on non-histone proteins, as well as methylated arginines, thus extending the possibility of a much wider biological role beyond histone lysine modulation (Lu et al. 2010; Solá et al. 2011; Walport et al. 2016a).

In addition to the catalytic JmjC-domain, many JmjC-KDMs have ancillary domains that are involved in recognising histone modifications (e.g. reader domains such as PHD-finger domains or Tudor domains) or DNA binding domains (e.g. AT-rich interacting (ARID) domains, CXXC zinc-finger domains). In some cases, these ancillary domains can allosterically modulate the KDM catalysis and influence substrate specificity (Horton et al. 2010; Torres et al. 2015). There are also biological functions of KDMs that are independent of catalytic activity, as exemplified by Jarid2, the founding member of JmjC-family. Jarid2 is predicted to be catalytically inactive, but associates with polycomb proteins and has essential roles in ES cell differentiation and development (Landeira and Fisher 2011).

8.1.2. JmjC-KDM Biology

There is now strong evidence that changes in the expression levels of some lysine demethylases can lead to the onset and progression of certain cancers. This occurs either through activating expression of oncogenes, repressing expression of tumour suppressors, disrupting chromosomal stability, altering DNA mismatch repair or interacting with hormonal receptors that control cellular proliferation (Young and Hendzel 2012; Young et al. 2013; Black et al. 2013). Overexpression of KDM4s has been linked to various cancers such as lung, breast, esophageal, prostate cancers and lymphoma (Yang et al. 2000; Italiano et al. 2006; Vinatzer et al. 2008; Liu et al. 2009; Coffey et al. 2013). KDM5 proteins are also associated with several cancers such as acute myeloid leukemia (Wang et al. 2009), hepatocellular carcinoma (Liang et al. 2013), lung (Teng et al. 2013), gastric (Zeng et al. 2010), melanoma (Roesch et al. 2010), breast (Angela et al. 2002), ovarian (Wang et al. 2015) and testicular cancer (Madsen et al. 2003), and are essential for the survival of multiple drug-tolerant cancer cells (Blair et al. 2011; Rasmussen and Staller 2014). KDM3 is involved in controlling chemoresistance and tumorigenic growth in human colorectal cancer stem cells through activating Wnt target gene transcription (Li et al. 2017).

KDMs also have important roles in development such as KDM5B which blocks differentiation of embryonic and hematopoietic stem cells (Dey et al. 2008) and KDM5C which has a role in neuronal development (Cellot et al. 2013). KDM2A (FBXL11) inhibits osteo/dentinogenic differentiation in mesenchymal stem cells (Juan et al. 2012) and also regulates cell proliferation in human stem cells from apical papilla (Gao et al. 2013). It also has a role in the regulation of NF- κ B through lysine demethylation of the p65 subunit (Lu et al. 2009). KDM6A (UTX) point and deletion mutations have been associated with Kabuki Syndrome, a rare congenital

8.2. Mechanism and Structure of JmjC-KDMs

The catalytic core of JmjC-domain in JmjC-KDMs is made up of eight anti-parallel β -strands (β I-VIII) arranged in a distorted double stranded β -helix (DSBH) core fold (**Figure 8.2**). Long β -strands I, VIII, III and VI (major β -sheet) on one face and short β -strands II, VII, IV and V (minor β -sheet) on the other face result in an asymmetric barrel-like DSBH fold, to form an active site. The JmjC-KDMs have a characteristic extended N-terminal loop between DSBH and the N-terminal anti- β strand, as well as an extended insertion between strands IV and V of the DSBH which is involved in substrate binding (Markolovic et al. 2016).

8.2.1.1 2OG cofactor binding site

The JmjC-KDMs have structurally similar active sites in line with their catalytically similar mechanisms, where 2OG co-factor binds to the catalytic Fe(II) centre through bidentate coordination of the keto-acid (**Figure 8.1**). In all KDM structures, the metal ion is positioned through interaction with a triad of residues that forms a HxD/E...H motif (**Table 8.1**). Note that catalytically inactive 2OG analogue, N-oxalylglycine (NOG), and Ni(II) or Mn(II) are often used for JmjC-KDM crystallography. The two His residues are positioned distally on β II, β VII or β VIII and the Asp or Glu residue is located on β II/ β III loop (**Figure 8.2**). For KDM2A/B, KDM3A/B, KDM7A/B, two histidine and an aspartic acid coordinate to the metal centre. For KDM4 (JMJD2A, B, C and D), KDM5 (JARID1A, B, C and D) and KDM6 (JMJD3, UTX and UTY), the acidic aspartate residue is replaced with a glutamic acid. PHF2 (KDM7C) is anomalous with a histidine, aspartic acid and tyrosine residues coordinating to the metal centre. The three metal coordinating residues are essential for catalysis, and Ala mutants have been shown to abolish the enzyme activity, and often used as catalytically inactive controls (Hatch et al. 2017). Due to the conserved catalytic mechanism involving Fe(II), the majority of JmjC-KDM inhibitors bind through a monodentate or bidentate coordination to the metal centre and compete with 2OG (**Figure 8.3**).

The other main interaction at the 2OG-binding site is the salt-bridge formed between the terminal carboxylate of 2OG and residues in the active site of KDMs. Inhibitors of KDMs therefore commonly contain a carboxylic acid or a bioisosteric replacement to mimic 2OG binding and form an ionic interaction with a lysine in the KDM binding pocket (**Figure 8.3**). Although the lysine is found on different β -strands, it is orientated so that the terminal amino group overlaps in different KDM structures. Unlike all other KDMs, JMJD1C (KDM3C) contains a glutamine rather than a lysine at this position in the sequence which overlaps well with the analogous lysine interaction. Other key residues interacting with the carboxylate include tyrosine and threonine. KDM2A, KDM3C and KDM7A/B /C have a threonine within hydrogen bonding distance to the carboxylate. KDM6A/B/ C have a threonine and asparagine residues in addition to the lysine that can hydrogen bond to the carboxylate (**Table 8.2**).

Unlike the other KDMs, KDM4 (JMJD2) and KDM5 (JARID1) contain a tyrosine which interacts with the carboxylate of 2OG. This key structural difference in the binding site can be exploited in inhibitor design to give KDM4/5 inhibitors that are selective over the other KDMs. However, selectivity between KDM4 and KDM5 is difficult to achieve due to identical residues in the primary sphere of the binding pocket. Differences in the second sphere of amino acids can confer different conformations to the binding residues which can result in improved selectivity. Structural information obtained from different co-crystal structures with inhibitors has shown that this key tyrosine residue is more flexible in KDM5 than KDM4. Some

selectivity for KDM5 has therefore been observed with cyanopyrazoles, as the tyrosine side chain can be displaced to allow binding in KDM5 (Vinogradova et al. 2016).

Strands	N-terminal anti- β strands	DSBH							
		β I	β II	β III	β IV	β V	β VI	β VII	β VIII
KDM2A			T209		K229				
KDM3B			T1557						K1699
JMJD1C			T2333						Q2476
KDM4A	Y132				K206				
KDM4B	Y133				K207				
KDM4C	Y134				K208				
KDM4D	Y136				K210				
KDM5A	Y409				K501				
KDM5B	Y425				K517				
KDM5C	Y440				K532				
KDM6A		K1137	T1143	N1156					
KDM6B		K1381	T1387	N1400					
KDM6C		K1084	T1090	N1103					
KDM7A			T279	Y292	K299				
KDM7B			T244		K263				
PHF2				Y258	K266			T323	

Table 8.2. Comparison of residues interacting with carboxylate in KDM structures on different β strands. All JmjC-KDMs have a lysine residue (green) that forms a salt bridge to a carboxylate in 2OG and inhibitors, with the exception of KDM3C which has a glutamine (grey). Further hydrogen bonds to the substrate / inhibitor carboxylate are made by threonine (salmon), tyrosine (yellow), and asparagine (blue).

Figure 8.3. Polar interactions of JmjC-KDMs with C5-carboxylate of 2OG (or NOG) (A) KDM3B/C binding mode; (B) KDM4/5 binding mode; (C) KDM6A/B/C binding mode; (D)

KDM2A, KDM7A, B and C binding mode; (E) 2OG mimicking JmjC-KDM inhibitor scaffold.

8.2.1.2. Histone substrate binding

Despite the high conservation in the active site of JmjC-KDMs, the individual subfamilies have distinct substrate specificities (**Table 8.1**). Structural information is available for some JmjC-KDMs complexed with histone substrates, which provide some insight into the unique features of these family of proteins (**Table 8.3**).

KDM(JmjC)	Ligands	PDB	References
Kdm2A	H3K36me1, Ni(II), NOG	4QXH	(Cheng et al. 2014b)
	H3K36me1, Ni(II), 2OG	4QWN	(Cheng et al. 2014b)
	H3K36me2, Ni(II), NOG	4QXC	(Cheng et al. 2014b)
	H3K36me2, Ni(II), 2OG	4QX7	(Cheng et al. 2014b)
	H3K36me, Ni(II), NOG	4QXB	(Cheng et al. 2014b)
	H3K36me3, Ni(II), 2OG	4QX8	(Cheng et al. 2014b)
KDM4A	H3K9me3, Ni(II), 2OG	2OQ6	(Ng et al. 2007; Couture et al. 2007)
		2Q8C	
	H3K9me2, Ni(II), 2OG	2OX0	(Ng et al. 2007)
	H3K9me1, Ni(II), 2OG	2OT7	(Ng et al. 2007)
	H3K36me2, Ni(II), succinate	2Q8D	(Couture et al. 2007)
	H3K36me3, Ni(II), 2OG	2OS2	(Ng et al. 2007; Couture et al. 2007)
	H3K36me3, Ni(II), 2OG	2P5B	(Chen et al. 2007)
	H3K36me3, Ni(II), 2OG	2Q8E	
	H3K36me1, Fe(II), 2OG	2PXJ	(Walport et al. 2016a)
	H4R3me2s, Ni(II), 2OG	5FWE	(Williams et al. 2015)
	H3K27me3, Ni(II), 2OG	4V2W	
	H3K9me3, Ni(II), 24PDCA	4LXL	(Chu et al. 2014)
KDM4B			
KDM4D	H3K9me3, Ni(II), 2OG,	4HON	(Krishnan and Trievel 2013)
KDM6A	H3K27me3, Ni(II), 2OG,	3AVR	(Sengoku and Yokoyama 2011)
Kdm6B	H3K27me3, Ni(II), NOG	4EZH	(Kruidenier et al. 2012a)
KDM6B	H3A21M, Fe(II), 2OG	5OY3	(Jones et al. 2018)
KDM7B	H3K4me3K9me2, Ni(II), 2OG	3KV4	(Horton et al. 2010)

Table 8.3. Structures of JmjC-KDMs in complex with histone peptides. Note all are human JmjC-KDMs except Kdm2A and Kdm6B which are from *Mus musculus*.

Recognition of methylated lysines. Structures of KDM4A in complex with H3K9me3/2/1 revealed that the size of the active-site region can, to some degree, confer methylation state selectivity in KDM4A (Ng et al. 2007) (**Figure 8.4, A-C**). The tri-Nε-methylated lysine (Kme3) fits tightly in the active site pocket composed of Y177, E190, S288, N290, and one methyl group projects towards the catalytic metal (position **b**), whereas the other two point towards Y177 (position **c**) and N290 (position **a**). In di-Nε-methylated lysine (Kme2) structures, the methyl-group can

either occupy a productive conformation pointing towards the metal or a non-productive conformation towards Y177 and N290. In the case of mono N ϵ -methylated lysine, the water molecules are positioned to direct the methyl-group away from the metal, stabilising the unproductive over productive mode, in agreement with the lack of activity (Ng et al. 2007). Similar Kme3 interactions are observed in KDM6A, whereby the H3K27me3 tri-methyl group points towards the active site metal (**Figure 8.4D**). The tri-methyl group is stabilised by Y1135, and G1128 forming H-bonded interactions with methyl groups, and E1148 forms electrostatic interaction with methylammonium group to stabilise the orientation of methyl group for demethylation (Sengoku and Yokoyama 2011). From the peptide co-crystal structures available, the Tyr which stabilises the methyl group of Kme3 is conserved across all trimethyllysine demethylases (Y177(KDM4A)/Y1135(KDM6A)/Y1380(KDM6B)), but it is absent in KDM2A/KDM7A dimethyl-lysine demethylases. Thus it is proposed that this Tyr may be important in Kme3 recognition (Sengoku and Yokoyama 2011).

In KDM7B, a N ϵ -methyl group of H3K9me2 points towards the aromatic ring of Y234, and the other towards D249 and N333 forming hydrogen bonds. The lone pair from the terminal dimethylated nitrogen forms a hydrogen bond with an oxygen atom of 2OG (NOG in crystal structure), thus a steric clash prevents accommodation of a tri-methyl lysine (Horton et al. 2010) (**Figure 8.4E**).

In contrast, the crystal structure of mouse Kdm2A in complex with H3K36me3/me2/me1 reveals that the spatial constraint within the methyl-lysine binding pocket is not the sole determinant of methylation state selectivity in all JmjC-KDMs (Cheng et al. 2014b). Kdm2A is a H3K36me2 di-/mono-demethylase, which is structurally very similar to KDM7s. In H3K36me2 complexed structure, the lysine side chain binding is stabilised by van der Waals interactions with aromatic (F215, Y199) and hydrophobic interactions (L201, I144), with dimethyl-ammonium group directed towards D214, N298 and NOG (**Figure 8.4F**). However, H3K36me3, which is not a substrate of Kdm2A, can also bind in the same catalytic pocket in a similar manner. Intriguingly, for all NOG-complexed structures, NOG coordinate the metal in an equatorial coordination (also referred as “in-line” mode), ie. C1 carboxylate oxygen is positioned opposite His1 (H284), in plane at equatorial position, whereas all 2OG-complexed structures have axial mode (Hausinger 2004), ie. where C1 carboxylate oxygen coordinates at axial position (also referred as “off-line” mode) (**Figure 8.5**). In the 2OG complexed structures, the side-chain of Y222 within the active site can adopt two alternative positions, with major conformer directed towards the metal, and minor conformer away from the metal (**Figure 8.4G**). The minor conformer of Y222 reflects the equatorial coordination (as observed in NOG complexes) and only observed with H3K36me1/2 and not with H3K36me3. It has been proposed that the steric constraints of H3K36me3 binding could prevent the 2OG transitioning from axial to equatorial coordination, thus H3K36me3 cannot be demethylated by Kdm2A despite its binding (Cheng et al. 2014b).

Figure 8.4. Crystal structures of JmjC-KDMs in complex with NOG/2OG and histone H3 substrates. (A-C) Different orientations of methyl-groups are observed for KDM4A complexed with (A) H3K9me3 (PDB 2OQ6), (B) H3K9me2 (PDB 2OT7) and (C) H3K9me1 (PDB 2OTQ). (D) KDM6A in complex with H3K27me3, and (E) KDM7B in complex with H3K9me2 in the JmjC-domain active sites. (F,G) KDM2A in complex with H3K36me2 with NOG (F) and 2OG (G). NOG adopts an equatorial binding mode to H212, whereas 2OG co-crystal structures can also adopt axial binding mode relative to H212. Two different conformers are observed for Y222 with H3K36me1/2 but only axial for H3K36me3. Histone methyl-lysines, NOG/2OG, metal and water molecules are in green, yellow, orange and red (sphere) respectively.

Figure 8.5. Equatorial and axial mode of 2OG binding. Two different coordination modes of 2OG. Equatorial coordination of 2OG to His1 is catalytically productive, whereas axial coordination of 2OG cannot bind O_2 / substrate. Carbons on 2OG are numbered. NOG has nitrogen instead of C3 position. The first and second histidine in the HxD/E...H motif are numbered in blue.

Histone recognition and binding pocket. The structural overlay of representatives from KDM2/4/6/7 demonstrate that the histone binding orientation (N-to C-terminal orientation of peptides) in the active-site pocket is the same for all histone H3 peptide co-crystal structures of JmjC-KDMs except for KDM7B (**Figure 8.6A**). However, in all cases, the methyllysines are positioned in the active site accessed from the same face. Interestingly, Kme3 for KDM4/6s are positioned slightly differently and further away from the catalytic centre compared to the Kme1/2 in KDM2/7s, which may in part account for the inherent differences in the Kme3 and Kme2 demethylases (Sengoku and Yokoyama 2011).

Histone H3 peptides make multiple interactions with JmjC-KDM proteins, both via their side-chains and backbones. In KDM6A, residues surrounding H3K27me3 make interactions with the JmjC-domain, contributing to its substrate specificity and catalytic activity, in addition to the Kme3 binding (Sengoku and Yokoyama 2011). Extended interactions of H3 peptide beyond the JmjC-domain are also observed, whereby the zinc-binding domain of KDM6A/B, located C-terminal to JmjC-domain, provide additional affinity and specificity (Sengoku and Yokoyama 2011; Jones et al. 2018) (**Figure 8.6B**). This region undergoes a conformational change upon H3 peptide binding, to expose hydrophobic patches that interact with H3(17-21) region of the peptide. These key interactions have been supported by kinetic studies on mutations to H3 and KDM6A (Sengoku and Yokoyama 2011), providing evidence that multiple factors contribute towards defining substrate binding and catalysis.

For other binding domains, such as the PHD-fingers, their spatial positioning and distance relative to JmjC-domains can also influence the histone H3 binding sequence specificity. PHD-fingers of KDM7 subfamily recognise the H3K4me3 mark, and in the case of KDM7B, the PHD-finger interacts with H3K4me3 in H3K4me3K9me2 to allow demethylation at K9me2 site by JmjC-domain, while it has negligible binding and catalysis with H3K9me2 alone (Horton et al. 2018). The crystal structure of KDM7B in complex with H3K4me3K9me2 reveal that the histone H3 bind across the PHD-finger and JmjC-domain interface in an extended conformation (H3(1-9)), and forms extensive interactions with both domains or individual domains (Horton et al. 2018) (**Figure 8.6C**). KDM7A, on the other hand, has

a longer linker between the PHD-finger and JmjC-domains and cannot demethylate at the K9 site of H3K4me3K9me2, while it can demethylate at the K27 site of H3K4me3K27me2 (Horton et al. 2018). The ancillary reader domain and linker length can thus contribute significantly to the substrate selectivity of the KDM7s. Other JmjC-KDMs, such as the KDM4s and KDM5s, are also allosterically regulated by their ancillary reader domains (PHD-fingers, Tudor domains)(Wang et al. 2009; Klein et al. 2014; Zhang et al. 2014; Torres et al. 2015; Pack et al. 2016). It is anticipated that the histone binding complex structures of multi-domain KDM5s and KDM4s will provide molecular insight into the substrate binding and cross-talk between the reader and catalytic domains.

Figure 8.6. Histone H3 peptide binding to JmjC-KDMs. (A) Overlay of representative crystal structures of JmjC-KDMs in complex with histone H3 peptides. Methyllysines on histone peptides (cartoon) bind in the active-site pocket, where it projects towards the catalytic metal coordinated by Hx/D/E...H residues on JmjC-KDMs. The first and the second His are labelled H1 and H2. Methyllysines are projected from different angles for trimethyl-demethylases (KDM4A/6A) relative to dimethyl-demethylases (KDM2A/7A). Overlaid PDB IDs: 4QXH, 2OQ6, 3AVR, 3KV4. (B) Histone H3 peptide binds across the JmjC- and Zinc binding domain in KDM6A. H3 peptide binding induces conformational change at the zinc-finger domain upon binding to KDM6A (PDB 3AVR). (C) The K4me3 of H3K4me3K9me2 peptide binds to the PHD-finger and places K9me2 at the catalytic site of JmjC-domain in KDM7B (PDB 3KV4). (D) "Molecular threading" of histone H3(30-39) peptide through KDM2A, with K36me3 directing towards the catalytic centre (PDB 4QXB).

The histone H3 binding surfaces can also differ between the JmjC-KDMs. In KDM2A, the H3 peptide “threads” through a narrow channel that can only be accommodated by Gly residues (Cheng et al. 2014a) (**Figure 8.6D**). This steric constrain in the channel provides selectivity towards a unique GG motif at H3K36 region (A29-PATGG-V35). Methylated H3K36 then inserts into a deep cavity (‘cavity insertion’) and locks the H3K36me2 peptide substrate into the active site pocket (Cheng et al. 2014a). In contrast, in KDM4A, the same H3 peptide sequence binds at the protein surface, and K36me3 inserts into a surface groove (‘surface groove’ recognition) (Ng et al. 2007; Couture et al. 2007; Cheng et al. 2014a). This binding mode can account for the ability of KDM4s to demethylate multiple histone substrates (Williams et al. 2015; Walport et al. 2016b), but KDM2A is highly specific for the H3K36me2.

In summary, the histone substrate recognition and selectivity are achieved through a combination of unique features and mechanisms by JmjC-KDMs, including distinct methyl-lysine binding pockets, intermolecular side-chain and backbone interaction network, different substrate binding channels, and interplay with other domains.

8.3. Development of JmjC-KDM inhibitors

There has been significant progress in the development of JmjC-KDM inhibitors since the first inhibitors were reported in 2008 (Rose et al. 2008). The overwhelming majority target the catalytic domain and inhibit the enzyme activity via chelating the active-site Fe(II), often competing with 2OG binding. Due to the similarity in the active-site pockets of JmjC-KDMs, achieving selectivity has proved challenging, not only between different JmjC-KDM subfamilies but also across a wider 2OG oxygenases superfamily (Joberty et al. 2016). The recent availability of crystal structures of JmjC-KDM has facilitated medicinal chemistry efforts and enabled the generation of several chemical probes for the JmjC-KDMs. In the following sections, we describe the recent progress, focusing on the development of JmjC-KDM inhibitors based on structural rationalization. For a general overview of JmjC-KDM inhibitors, the readers are referred to recent reviews (McAllister et al. 2016; Kaniskan et al. 2017).

8.3.1. Inhibitors targeting the 2OG binding site

8.3.1.1. Dual KDM4/5 inhibitors

Several inhibitors of KDM4/5 have been developed using 2,4-pyridinedicarboxylic acid (2,4-PDCA), a micromolar pan-KDM inhibitor, as a starting point (**Figure 8.3E**). 3-amino pyridine carboxylic acid **1** was optimized as a potent inhibitor of KDM4 ($IC_{50} < 100$ nM) and KDM5C ($IC_{50} = 100 - 125$ nM) in a MS-based biochemical assay (Westaway et al. 2015) (**Figure 8.7**). The compound demonstrated good selectivity (>50 fold) over KDM6B and EGLN3. Some cellular activity

against KDM4C was observed ($IC_{50} = 6 - 8 \mu M$) with a significant drop in potency from the biochemical assay which was likely due to the poor cell penetration of the carboxylate group. Low cell activity was also observed with overexpressed KDM5C.

A crystal structure of 4-methylthienyl analogue **2** in KDM4D (PDB:5FP8) confirmed binding in the 2OG site through a monodentate coordination of pyridine *N* to the active site metal (**Figure 8.8A**). The carboxylate hydrogen bonded directly to K210 and Y136 while the 4-methylthienyl ring was positioned in a hydrophobic pocket comprised of Y136, A138 and K245.

Figure 8.7. Structures of dual KDM4/KDM5 inhibitors

Figure 8.8. Crystal structures of KDM4/5 in complex with inhibitors. A) Crystal structure of **2** bound to KDM4D (PDB:5FP8); B) Crystal structure of **3** bound to KDM4A (PDB:5VGI); C) Crystal structure of **4** bound to KDM5B (PDB:5A3T); D) Crystal structure of (R)-**6** (blue) and (S)-**6** (green) bound to KDM5A (PDB:6BH0 and 6BH1); E) Crystal structure of **8** bound to KDM4A (PDB:5F3I); F) Crystal structure of **9** bound to KDM5A (PDB:5IW0).

Using the pan-KDM inhibitor 2,4-PDCA as a starting point, Celgene Quantical reported 3-aminoisonicotinic acid **3** (QC6352) as a potent inhibitor of KDM4 family (Chen et al. 2017). Preliminary SAR showed that (*R*)-tetrahydronaphthalene was the active enantiomer while the (*S*)-enantiomer was relatively inactive. Compound **3** was identified as the most potent inhibitor against KDM4C (IC_{50} = 35 nM) and showed cell activity against KYSE-150 cells with an EC_{50} of 3.5 nM. Cellular on-target engagement using the same cell-line was determined by measuring the increase in H3K9me3 and H3K36me3 through time-resolved fluorescence assay and mass spectroscopy detection to give a H3K36me3 mechanism of action (MOA) EC_{50} of 1.3 nM. The significant improvement in cellular potency from previously reported pyridine carboxylic acids could be attributed to the greater cell permeability from the addition of the lipophilic tetrahydronaphthalene and phenyl rings. Good selectivity (>1000 fold) was achieved over other KDM families such as KDM2B, KDM3, KDM6B and KDM7B. However, selectivity over KDM5B was moderate (IC_{50} = 750 nM) so some cellular activity may be contributed to KDM5B inhibition. **3** was also efficacious *in vivo* in breast and colon cancer PDX models showing a reduction in the rate of tumour growth.

A co-crystal structure of **3** with KDM4A (PDB:5VGI) revealed a similar binding mode involving pyridyl *N* coordinating to Ni(II). Key hydrogen bonding interactions of carboxylic acid with Y132 and K206 were observed (**Figure 8.8B**). The methyl group of the methylaniline pointed towards N86 and carbonyl of H240 while the phenyl group is positioned in the R239/H240 loop.

An analogue of 2,4-PDCA, **4** (KDM5-C49), in which the 2-position is substituted with an amino methyl group, was identified as a potent nanomolar inhibitor of KDM5 family (KDM5A/B/C/D IC_{50} 7/4/13/15 nM) with ~50 fold selectivity over closely related KDM4C and >50 fold selectivity over KDM2, 3 and 6 (Johansson et al. 2016; Tumber et al. 2018). **5** (KDM5-C70) was designed as an ester pro-drug to permeate the cell and be hydrolysed in the cell to the active carboxylic acid. This compound showed growth inhibition in several breast cancer cell lines and also exhibited increased expression of *MTIF* and *MTIH* genes in comparable levels observed upon KDM5B knockdown (Horton et al. 2016).

4 binds to KDM5B (PDB:5A3T) in the 2OG binding site with bidentate coordination of pyridine *N* and aminomethyl *N* to the metal (**Figure 8.8C**). As with other pyridine carboxylate derivatives, there are key hydrogen bonding interactions with K517 and Y425. Extensive polar interactions are observed with K517, Y425, N509, E509, R98 and Y488 as well as hydrophobic interactions with W486, V99 and F496. Similar key binding interactions were observed when **4** was crystallised in KDM4 (PDB:5FPV) which resulted in only moderate selectivity.

Chiral enantiomers (*R*)- and (*S*)-2-((2-chlorophenyl)(2-(piperidin-1-yl)ethoxy)methyl)-1*H*-pyrrolo[3,2-*b*]pyridine-7-carboxylic acid, (*R*)-**6** and (*S*)-**6**, were active inhibitors of KDM5A with binding affinities of 220 nM and 60 nM respectively and greater than 20-fold selectivity over KDM4A (Horton et al. 2018). Cellular activity was not observed, even at high concentrations, with the methyl ester cell-permeable

pro-drugs of the racemic compound. The compounds were 2OG competitive with the carboxylic acid ring interacting with K501, N575 and Y409 and pyridine nitrogen coordinating to the metal.

Overlay of the structures of (*R*)-**6** and (*S*)-**6** bound in KDM5A showed differences in their binding and consequently on their potencies ((*R*)-**6** PDB code 6BH0 and (*S*)-**6** PDB code 6BH1) (**Figure 8.8D**). The (*S*)- enantiomer engaged in extensive van der Waals interactions between the chlorine of chlorophenyl with A411, Y409 and Y472. The piperidine nitrogen of (*R*)-**6** hydrogen bonds with Y472 while the corresponding nitrogen in (*S*)-**6** interacts with carboxylate ions of D412. The greater binding affinity of (*S*)-**6** could be attributed to ionic bonding with D412 if the piperidine nitrogen is protonated. A displacement of D412 was observed between the (*R*)-**6** and (*S*)-**6** structures.

A series of pyrido[3,4-*d*]pyrimidin-4(3H)-one inhibitors were reported by GSK as potent KDM4 and KDM5 inhibitors as a replacement for pyridine-4-carboxylates which suffer from poor cell permeability (Westaway et al. 2016). Several pyrazoloxo analogues showed good potency in biochemical assays ($pIC_{50} \sim 6.4$) and some cellular assays ($pIC_{50} \sim 5.3$) against KDM4C. The best compound **7** also showed activity against KDM5C ($pIC_{50} \sim 7.2$) but were selective over KDM6B and EGLN3. **7** was crystallised in KDM5B (PDB:5FUN) which engaged in similar key interactions via a monodentate coordination of pyridine nitrogen with the active site metal and hydrogen bonding interactions of amide with K517 and N591. Substitution at positions 5 and 6 was unfavourable due to good shape complementary at these positions. There were two conformations of Y425 which enabled flexibility for binding to KDM5B depending on the orientation of the phenyl group.

Superimposition of 3-aminoisonicotinic acid **3** and 2-pyridopyrimidinone **7** revealed that their cores were positioned in the same plane (**Figure 8.9**). The carboxylic acid and the amide of pyridopyrimidinone overlap well to interact with the key lysine residue however Y425 was not necessary for binding with **7**, unlike with **3**, so could rotate out of the pocket.

Figure 8.9. Overlay of 3-aminoisonicotinic acid **3** in co-crystal of 2-pyridopyrimidinone **7** in KDM5B (PDB:5FUN).

The Institute of Cancer Research and the Structural Genomics Consortium reported 8-(1H-pyrazol-3-yl)pyrido[3,4-*d*]pyrimidin-4(3H)-one scaffolds as equipotent inhibitors of KDM4 and KDM5 subfamilies (Bavetsias et al. 2016). One of the best compounds incorporated a dichloro-phenylpiperidine moiety **8** and showed an IC_{50}

of < 50 nM against KDM4B and KDM5B. Some cellular inhibition of H3K9me3 and H3K4me3 demethylation was observed but only at high compound concentrations.

A crystal structure of dichloro-phenylpiperidine **8** bound in KDM4A was obtained (PDB:5F3I) (**Figure 8.8E**). The compound bound in the 2OG binding site and engaged in a bidentate coordination to the active site metal via the pyridine nitrogen and pyrazole N2 nitrogen. The carboxylate engaged in favourable interactions with K206 and Y132 and extending the pyrazole with a phenylpiperidine linker resulted in access to the histone peptide binding pocket for interactions with D135 and Y175. The piperidine ring had an important role in rigidifying the pyrazole substituent from different rotational conformations and ensuring good selectivity over KDM2, KDM3 and KDM6. A hydrophobic interaction was observed between the chlorine and V313 in the *m*-dichlorophenyl analogue which could explain the improved KDM4A/B inhibition compared with the *p*-chlorophenyl analogue in which the chlorine was too distal for an interaction.

Pyrazolylpyridines were designed based on structural information from the known KDM inhibitors (Nie et al.). The best compound in this series, **9**, showed nanomolar activity against KDM5 (IC₅₀ KDM5A/B = 13/2 nM) as well as KDM4C (IC₅₀ = 41 nM) but did exhibit good selectivity across the other KDM families. An increase in H3K4me3 was observed in a breast cancer cell line, ZR-75-1, and *in vivo*, in a MCF7-breast cancer xenograft PK/PD model upon treatment with **9**.

9 was co-crystallised with KDM5A (PDB:5IW0) confirming bidentate coordination of pyrazolyl and pyridyl nitrogens with the metal centre (**Figure 8.8F**). As previously observed, the carboxylate group interacts with K501 and Y409 while the pyridyl group π -stacks with F480. The benzyl ether also engaged in π -stacking interactions with Y409 resulting in improved selectivity over the other KDMs.

8.3.1.2. Selective KDM5 inhibitors

More recently, compounds have been designed that are more potent and selective towards KDM5 using structural information from crystal structures of known KDM5 inhibitors (**Figure 8.10**).

Figure 8.10. KDM5 selective inhibitors.

Aminomethylpyridine **10** (KDOAM-25), in which the carboxylic acid of KDM5-C49 was replaced with a primary amide, showed excellent activity on KDM5B (IC_{50} = 19 nM) and also showed improved selectivity (>1000 fold) over KDM4C as well as other 2OG-oxygenases (Tumber et al. 2018). The compound was partially competitive with 2OG which may explain the low cellular activity (~50 μ M) observed in an immunofluorescence assay measuring the increase in H3K4me3 levels upon incubation with **10**.

KDOAM-25 binds to KDM5B (PDB: 5A3N) in a similar pose to KDM5-C49 via metal chelation of the pyridine nitrogen (**Figure 8.11A**). The main difference is the displacement of Y425 to become a hydrogen bond acceptor with the carboxamide rather than a hydrogen bond donor with the carboxylate. A water molecule can occupy the space created by Y425. The flexibility of Y425 in KDM5B explains the selectivity over KDM4 which has a more rigid tyrosine.

Figure 8.11. Crystal structures of KDM5 in complex with selective inhibitors. A) Crystal structure of **10** bound in KDM5B (PDB:5A3N); B) Crystal structure of **11** bound in KDM5A (PDB:5CEH); C) Crystal structure of **13** bound to KDM5A (PDB:5K4L); D) Crystal structure of **14** bound to KDM5A (PDB:5V9T).

11 (CPI-455) was identified by Genentech and Constellation as a potent inhibitor of the KDM5 subfamily with IC_{50} of 10 nM against KDM5A in enzymatic assays. The compounds were 200-fold selective over KDM4C and 700-fold selective over KDM7B and showed no inhibition against KDM2B, KDM3B or KDM6A. A cellular assay measuring global increase in levels of H3K4me3 in PC9 cells after incubation with the compound resulted an EC_{50} of 5.2 μ M (Vinogradova et al. 2016). Crystallisation of **11** in KDM5A (PDB:5CEH) identified a monodentate interaction between the nitrile and the active site metal and a hydrogen bond between carbonyl oxygen and N575 (**Figure 8.11B**). A π - π stacking interaction was observed between the core and Y472 and F480 aromatic side chains as well as an edge:face interaction with W503. Although K501 and partially charged N1 of CPI-455 are positioned in

close proximity, hydrogen bonding distance was not observed. As with other KDM inhibitors, an acidic hydrogen was required which was demonstrated by the loss of activity upon alkylation of 4-nitrogen or replacement of pyrimidine with triazene. 5-phenyl substituent interacted through an edge-face contact with Y409 but otherwise was solvent exposed. Substitution of the isopropyl group was not tolerated due to its tight binding pocket defined by Y409 and S478. CPI-455 binds in the 2OG binding site and demonstrated competitive inhibition with 2OG in biochemical assays.

This compound showed no selectivity over other KDM5 isoforms due to identical amino acids in its binding site. Selectivity over KDM6B could be explained through collisions between the isopropyl and phenyl substituents and K1381, F1328 and T1387 residues. Y409 in KDM5A has conformation freedom to rotate out of the pocket to allow CPI-455 binding whereas F1328, on a β -sheet next to ARID and PHD1 domains in KDM5A, projects into the pocket in KDM6B. The residues in KDM4C only differ in the second sphere of amino acids that do not directly interact with the compound. Selectivity over KDM4C could be explained through replacement of KDM5A A583 with KDM4C S290. The presence of I73 in KDM4C compared to R73 in KDM5A could result in a displacement of Y409.

Further optimisation of **11** lead to pyrazolo[1,5-a]pyrimidin-7(4*H*)-one **12**; a potent and selective inhibitor of KDM5 with improved cell potency (PC9 H3K4Me3 EC₅₀ = 0.34 μ M) which could be explained by a more balanced human plasma protein binding (hPPB) and cell permeability (Liang et al. 2016). **12** showed good selectivity over the other KDM families (1A, 2B, 3B, 4C, 6A, 7B) with IC₅₀ > 1.9 μ M and also against 300 kinase panel with no activity >50% at 10 μ M. The compound demonstrated a good pharmacokinetic profile in mice so was suitable for further profiling in vivo.

Superimposition of KDOAM-25 **10** and CPI-455 **11** in KDM5A shows that the cyanopyrazole core is tilted from the plane of the pyridine carboxamide (**Figure 8.12**). The core in CPI-455 is offset by the nitrile group coordinating to the metal centre which improves π - π stacking interactions with Y472 and 480. Y409 is displaced away from the active site when CPI-455 is bound which highlights the improved selectivity of CPI-455 over KDM4 in which the rigid tyrosine blocks the isopropyl group from binding.

Figure 8.12. Overlay of **10** (green) bound in KDM5B (green) and **11** (blue) bound in KDM5A (blue).

A hybrid compound of the pyridopyrimidinone and cyanopyrazole scaffolds was developed by Genentech to give a series of 1,7-naphthyridone inhibitors that were potent against KDM5 and showed good selectivity over KDM4C and KDM2B (Labadie et al. 2016). Various substitutions were trialled at C-3 to extend into the region occupied by isopropyl group of the cyanopyrazoles with KDM5. Introductions of electron withdrawing groups at C-3 resulted in an increase in potency for KDM5A due to improved interactions with K501 and N575. To lower the pKa of the ionisable proton and improve the cell permeability, C-3 carboxamides were explored. Only small alkyl groups on the nitrogen were tolerated due to the tight binding pocket created by S478 and Y409. **13** was identified as the most potent inhibitor against KDM5A ($IC_{50} = 50$ nM) with excellent MCDK permeability, however no cellular activity was observed in a PC9 cell based assay measuring H3K4me3 levels.

A co-crystal structure of **13** in KDM5A (PDB:5K4L) confirmed that 1,7-naphthyridones were 2OG competitive with monodentate metal coordination of N7 (**Figure 8.11C**). π - π stacking interactions were observed between the ring system and Y472 and F480 and the carbonyl oxygen engaged in hydrogen bonding to Ne of K501. Van-der-Waals interactions were observed between N-ethyl of C-3 amide with S478 and Y409. N1 was solvent exposed and therefore alkylation was not tolerated due to unfavourable hydrophobic interactions. The selectivity over KDM2B was attributed to the loss of aromatic stacking interactions through replacement of Y472 with a leucine. KDM4 does not differ in the direct binding residues so selectivity over this family was likely due to changes to the second shell of amino acids.

A novel series of KDM5 inhibitors was discovered by Genentech and Constellation (Liang et al. 2017). Optimisation of a high throughput screen hit led to a single enantiomer **14** with a core pyrazole which showed a potent IC_{50} of 45 nM against KDM5A and good selectivity over KDM1, KDM2, KDM3, KDM6 and KDM7 and 91-fold selectivity over most closely related KDM4C. The compound had reasonable cellular potency of 960 nM and demonstrated good *in vivo* PK properties.

Co-crystallisation of **14** with KDM5A (PDB:5V9T) revealed a bidentate coordination of pyrazole N1 and carbonyl oxygen with the active site metal (**Figure 8.11D**). A strong hydrogen bonding interaction was observed between pyrazole N2 and E485 while the cyclopropyl carbonyl oxygen engaged in an interaction with K501. As observed with other KDM5 inhibitors, the compound did not require a weakly acidic proton to engage in an ionic interaction with K501, which resulted in improved cell permeability. Most of the compound was positioned in the 2OG binding site while the isopropyl group resided in the histone peptide binding site. Competition with histone peptide was also confirmed using ^{19}F NMR which represent a novel mechanism for KDM5A inhibition.

8.3.1.3. KDM6 inhibitors

A large screening campaign by GSK led to **15** (GSK-J1), a potent ($IC_{50} = 60$ nM) inhibitor of KDM6B (**Figure 8.13**) (Kruidenier et al. 2012b). The carboxylic acid was necessary for binding however its high polarity resulted in poor cell permeability. Ethyl ester analogue, **16** (GSK-J4), was developed as a cell penetrant pro-drug

which is rapidly hydrolysed by macrophage esterases to give GSK-J1. **16** inhibited loss of nuclear H3K27me3 by KDM6B in FLAG-KDM6B-transfected HeLa cells and showed increased nuclear H3K27me3 levels in untransfected cells. Although GSK-J1 showed marginal inhibition against KDM4 and other closely related proteins, it was found to be only five to ten-fold selective over KDM5B and KDM5C (Heinemann et al. 2014). Similar IC_{50} s (3-7 μ M) were also obtained with GSK-J4 in cells transfected with KDM6B, KDM4C and KDM5B. As a result, inhibition by GSK-J1 is likely a combination of activity towards H3K4me3/me2 and H3K27me3/me2. Despite this, GSK-J1 is the most-selective KDM6B inhibitor and is a useful chemical tool for studying KDM6B biology.

Figure 8.13. Structures of GSK-J1, GSK-J2 and GSK-J4

The key binding interactions were determined from a co-crystal structure of GSK-J1 **15** bound to KDM6B (PDB:4ASK) (**Figure 8.14**). GSK-J1 bound in the 2OG binding site with the propanoic acid interacting with K1381, T1387 and N1480 and bidentate coordination of the pyridyl-pyrimidine biaryl with catalytic metal. The pyridine regio-isomer **17** (GSK-J2) was a suitable inactive control as it was not able to engage in the key bidentate coordination to the metal so was much less active ($IC_{50} > 100 \mu$ M). The catalytic metal in KDM6B was shifted by 2.34 Å away from the HHE triad which was not observed in the other KDM structures. This resulted in the displacement of a water molecule which could engage in a water-mediated interaction between H1470 and the metal. As a result, selectivity could be achieved through designing compounds that can induce this displacement of the metal centre. The tetrahydrobenzazepine ring is positioned in the narrow cleft between R1246 and P1388 which is occupied by P30 in the histone peptide. Despite this, GSK-J1 was found to be only 2OG competitive and not peptide substrate competitive. **15** was also co-crystallised in KDM5B (PDB:5FPU) which showed similar key interactions as in KDM6B with bidentate coordination to the metal and hydrogen bonding of carboxylate to K517. The main difference is the lack of a second bonding interaction with the carboxylate in KDM5B as the flexible Y425 is projected towards the tetrahydrobenzazepine ring.

Figure 8.14. Co-crystal of GSK-J1 in KDM6B (left-PDB:4ASK) and in KDM5B (right-PDB:5FPU).

8.3.2. Substrate competitive inhibitors

8.3.2.1 Histone peptide derived inhibitors

As discussed in **Section 8.2.1.2**, histone peptide sequences and methylation states can provide remarkable selectivity towards individual JmjC-KDMs/ subfamilies. However, the affinities of histone peptides are generally weaker for the JmjC-domains (as reflected in their K_M values ($10^{-5} - 10^{-4}M$)), compared to ‘reader’ binding domains, where the K_d values can be in the sub-micromolar range. Thus, efforts have been made to combine the histone peptides with metal-chelating inhibitors to achieve both selectivity and potency. Woon *et al.* used dynamic chemistry (using multiple thiols) linked to mass spectrometry analysis to identify optimal positions to conjugate a 2OG co-factor mimic, N-oxalylcysteine (NOC), to histone peptides (Woon *et al.* 2012). H3K9me3 peptides with Cys at varying positions along the histone peptide was incubated with L-NOC or D-NOC, and various products were tested. H3K9me3T11C was identified to be most stabilising, and a crystal structure (PDB:3U4S) revealed the optimal bidentate coordination of NOC projecting from T11C to Fe(II), as well as H3K9me3 positioning. Replacing the disulphide bond with CH₂-S bond using thiol-ene coupling yielded a demethylase inhibitor **18** that was potent for KDM4A/E ($IC_{50} = 0.09-0.27\mu M$) and >100 fold selective over KDM2A/KDM3A/KDM6B/KDM7B and other 2OG oxygenases (**Figure 8.15**). Interestingly, **19** developed using similar approach from H3K36me3, a substrate for KDM4A/B/C but not KDM4D/E (Hillringhaus *et al.* 2011) conferred selectivity for KDM4A over KDM4E, demonstrating that selective inhibition can be achieved, even within the subfamily, using peptides (Woon *et al.* 2012). In a different approach, Lohse *et al.* identified minimal histone peptide lengths that maintained demethylase activity for KDM4A/C (H3(7-11)K9me3) and conjugated a bromouracil at the K9 position to chelate the iron (ARK_(BRU)ST) (**20**) had $K_i = 27\mu M$ for KDM4C (Lohse *et al.* 2011). While a co-crystal structure was not obtained, the study highlights that a small peptide fragment can confer potency and selectivity for JmjC-KDMs (Lohse *et al.* 2011).

Figure 8.15. KDM4 inhibitors derived from histone H3 peptides.

8.3.2.2 Substrate binding pocket targeted peptide inhibitors

mRNA-display based cyclic peptide library screening against KDM4A generated potent and selective inhibitors for the KDM4A/B/C family (Kawamura et al. 2017). Interestingly, the binding cyclic peptides were distinct sequences with no homology to histone H3. A co-crystal structure of KDM4A with CP2 (**21**, PDB:5LY1), a cyclic peptide inhibitor ($IC_{50} = 42 \text{ nM}$ and binding potency $K_d = 30 \text{ nM}$ against KDM4A), revealed that CP2 occupies the substrate-binding pocket (**Figure 8.16**). CP2 forms a β -sheet with two turns; in the β -turn pointing towards the active site pocket, Arg (at position 6 of CP2 (CP2R6)) extends towards the Fe(II) and occupy the same sub-pocket as tri-methyl lysine of H3K9me3 / H3K36me3 in KDM4A. Similar to the lysines, the positively charged guanidino group makes hydrogen bonding interactions to KDM4A (Y177, S288, N290). In addition, Y175 and R309 shift to accommodate CP2 binding, inducing a conformational change in these regions of KDM4A. CP2 demonstrate high selectivity towards KDM4A-C, with >100 fold selectivity over other KDMs and 2OG oxygenases tested, and even over KDM4D/E. This selectivity was attributed to the intermolecular interactions that CP2 makes with KDM4A side chains. CP2 interacts with 14 residues in KDM4A, of which five are different between KDM4A/B/C and KDM4D/E. Three (N86, Q88, R309) of these five residues have been identified to be crucial for H3K36me3 binding to KDM4A/B/C (Hillringhaus et al. 2011). Thus, subtle differences in the substrate binding pockets can be utilised for the generation of selective and potent inhibitors against JmjC-KDMs. Structure and MS guided modifications of CP2 yielded peptides that retained *in vitro* potency and selectivity, and increased cellular permeability and cellular KDM4A target engagement, providing promising cellular tools for the KDM4s.

Figure 8.16. Cyclic peptide CP2 binding to KDM4A.

8.3.2.3 Small molecule targeting substrate binding pocket

BIX-01294 (diazepin-quinazoline-amines, **22**) and its analogues are potent inhibitors of H3K9 methyltransferase G9a and G9a-linked proteins (GLP) (Chang et al. 2009). They inhibit by mimicking the HMT bound histone H3 (K4-R8) conformation and bind in the substrate binding groove of HMT. Upadhyay *et al.* tested BIX-01294 and analogues for inhibition against KDM7A, a H3K9me2/1 demethylase (Upadhyay et al. 2012). BIX-01294 was found to be a moderate inhibitor of KDM7A (IC₅₀ = 16.5 μ M), and its analogue E67 (**23**) showed improved potency (**Figure 8.17**). An overlay of the crystal structure of KDM7A.E67.NOG, with KDM7B.H3K4me3K9me2 (PDB:3KV4) show that E67 occupies the same region where histone H3(K4-K9) lie in KDM7B, and that the 5-aminopentyloxyl moiety at the O7-methoxy group extends towards the active site metal. The terminal amino group forms a weak hydrogen bond with carboxyl oxygen of D284, one of the residues that coordinate Fe(II) in the active site. The 3-dimethylamino propyl moiety at 2'-position of E67 extends towards the substrate binding groove on the protein surface. It is worth noting that the E67 binding leads to a different Y292 conformer to the *apo* structure, inducing NOG to adopt an axial coordination mode (**Figure 8.17**). This suggests Y292 in KDM7B, conserved across KDM2/7 subfamilies, may potentially be attractive residue to target to induce non-productive co-factor binding. Further fine-tuning of selectivity against HMTs generated E67-2 (**24**) which removed HMT inhibition and maintained JmjC-KDM inhibition, providing promising opportunities for 'target-hopping' for histone substrate mimicking inhibitors. It is worth noting that inhibitor chemotypes targeting other epigenetic proteins have successfully been designed to inhibit the JmjC-KDMs. Filgastat (**25**), derived from methyllysine reader domain inhibitor (**26**), is a potent and selective inhibitor of KDM2/7 (Gerken et al. 2017). It does not compete with 2OG nor histone H3 peptide, and the mode of inhibition is unclear; however, it demonstrates that selective allosteric inhibition of JmjC-domain is possible.

Figure 8.17. JmjC-KDM inhibitors inspired from inhibitors targeting other methyllysine-associated epigenetic targets. (A) Structures of histone methyltransferase inhibitor BIX-10294 and analogues. (B) Co-crystal structure of KDM7A complexed with E6, NOG and Ni(II) (teal, PDB:3U78) overlaid on KDM7A.2OG.Fe(II) (grey, PDB:3KVA). (C) Structures of methyllysine binding domain inhibitor and Filgastat.

KDM(JmjC)	Ligands	PDB	References
KDM4A	3 , Ni(II)	5VGI	(Chen et al. 2017)
	8 , Zn(II)	5F3I	(Bavetsias et al. 2016)
KDM4D	2 , Co(II)	5FP8	(Westaway et al. 2015)
KDM5A	(R)- 6 , Mn(II)	6BH0	(Horton et al. 2018)
	(S)- 6 , Mn(II)	6BH1	(Horton et al. 2018)
	9 , 2OG, Mn(II)	5IW0	(Nie et al.)
	11 , Ni(II)	5CEH	(Vinogradova et al. 2016)
	13 , Ni(II)	5K4L	(Labadie et al. 2016)
	14 , Ni(II)	5V9T	(Liang et al. 2017)
KDM5B	4 , Mn(II)	5A3T	(Johansson et al. 2016)
	7 , Mn(II)	5FUN	(Johansson et al. 2016)
	10 , Mn(II)	5A3N	(Tumber et al. 2018)
	15 , Mn(II)	5FPU	(Johansson et al. 2016)
KDM6B	15 , Co(II)	4ASK	(Kruidenier et al. 2012b)
KDM4A	H3K9me3T11C, NOC.Ni(II)	3U4S	(Woon et al. 2012)
	21 , NOG, Ni(II)	5LYI	(Kawamura et al. 2017)
	CP2R6Kme3, NOG.Ni(II)	5LY2	(Kawamura et al. 2017)
KDM7A	23 , 2OG.Ni(II)	3U78	(Upadhyay et al. 2012)

Table 8.4 PDB codes for different JmjC-KDMs in complex with small molecule inhibitors (top section) or substrate competitive inhibitors (bottom section) described in this Chapter.

8.4. Conclusions

The JmjC-KDMs are a protein family rich in links to disease pathology and are an exciting new hunting ground of epigenetic targets for drug discovery. This family of enzymes has been extensively characterized by structural biology to explain their function and enable inhibitor design. The KDMs all share a similar mechanism of action and conserved features: Fe(II) chelation, salt bridging and hydrogen bonding to 2OG, but differences in their active sites, substrate binding surface and domain organization allow selectivity for similar methylated lysine substrates.

Initial efforts at inhibitor discovery yielded non-selective compounds by relying primarily on iron chelation and salt-bridging for potency such as NOG, 8HQ, Bpy (**Figure 8.3**). Since those initial inhibitor reports, much effort at inhibitor discovery, aided by thorough characterization of the family with X-ray crystal structures, has yielded new chemotypes that still rely on iron chelation and ionic interactions with the KDMs, but achieve selectivity by exploiting differences in the active sites' first sphere of residues. This led to compounds selective for KDM5 and KDM6 sub-families such as KDOAM25 (**10**), CPI-455 (**11**), and GSK-J1 (**15**). Selectivity for KDM members with similar active sites was then achieved by taking into account subtle differences in active site residues such as CPI-455 (**11**) which is selective for KDM5 members over KDM4 due to the propensity of an active site Tyr to shift more readily in KDM5 than KDM4. Single KDM selectivity is still elusive, although this may be acceptable and even desirable in drug development as many KDM sub-family members, i.e. KDM5A/B/C/D, have identical substrates and redundant function.

A second class of KDM inhibitors are peptide-competitive and achieve selectivity at the methyl lysine binding groove and enzyme surface. CP2 is a cyclic peptide, that has no homology to histone H3 yet is a potent and selective inhibitor of KDM4s (**Figure 8.16**). The HMT inhibitors BIX01294 and E67-2 bind at the peptide site and are selective for KDM7 and the related Filgerstat is KDM2A/7A selective although its binding is not well understood.

The future of KDM drug discovery will involve developing a deeper understanding of KDM biology using the inhibitors described herein. To be successful it will also be necessary to discover new inhibitor chemotypes to continue to improve the potency, selectivity and cellular activity of this important class of epigenetic enzymes.

8.5. References

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