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Inhibitors of histone demethylases

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ABSTRACT

Methylated lysines are important epigenetic marks. The enzymes involved in demethylation have recently been discovered and found to be involved in cancer development and progression. Despite the relative recent discovery of these enzymes a number of inhibitors have already appeared. Most of the inhibitors are either previously reported inhibitors of related enzymes or compounds derived from these. Development in terms of selectivity and potency is still pertinent. Several reports on the development of functional assays have been published.

1. Introduction

A major mechanism in the dynamic regulation of gene expression is the reversible posttranslational modification of the N-terminal tails of histones. Several different histone residues can be covalently modified, and this will eventually affect the ability of RNA polymerase II to access transcription start sites. Thus, histone modifications are believed to constitute important regulatory marks involved in the control of gene transcription. The modifications include acetylations, phosphorylation, methylations, hydroxylations, ubiquitination, SUMOylation, and biotinylation.[1,2] Strict regulation of gene expression patterns is crucial for the normal cell function, in particular during development and differentiation, and in these processes histone-modifying enzymes play a central role. Consequently, malfunctions related to these regulatory marks seem also to play a central role in a range of diseases, and for this reason several of these enzymes are strong candidate drug targets.[3–5]

While the processes of phosphorylation and acetylation of the histone (Fig. 1) have been studied for a long time, the enzymes involved in methylation have only been known for a decade, and enzymes involved in demethylation for even shorter time.[6,7] The first examples of demethylase inhibitors are now appearing along with several new assays and X-ray crystallographic protein structural studies. Here we will cover the recent development in design and development of histone demethylase inhibitors. First, a brief introduction to demethylases and their demethylating mechanisms will be followed by a discussion of the current available assays. Then, coverage of the known inhibitors as well as the general biostructural features of the enzymes and their relevance for drug design will follow. Finally, some therapeutic prospects are outlined emphasizing the need for inhibitors.

2. Histone demethylation

Both lysine and arginine residues of histone tails have been found to be methylated in vivo (Fig. 1). Multiple methylations are possible, adding complexity as compared to other post-translational modifications like phosphorylation and acetylation.[8] Furthermore, histone acetylation of lysines and phosphorylation of threonines and serines leads to transcriptional activation in part via formation of euchromatin due to change of charge, but methylation does not lead to a charge shift. Consequently, the outcome of methylation is often more ambiguous and can lead to both activation and repression.[9] In general, methylation of H3K4 (histone 3, lysine 4), H3K36 and H3K79 are found in regions with transcriptional activity, whereas H3K9me2/me3, H4K20me3 and H3K27me2/me3 are associated with transcriptionally silenced chromatin. While methylations are performed by Histone Methyl Transferases (HMTs) using the ubiquitous methyl donor *S*-adenosyl-methionine, two different oxidation mechanisms for Histone DeMethylases (HDMs) have been shown to lead to demethylation.

Lysine Specific Demethylase 1 (LSD1/KDM1) was the first HDM to be discovered. Like other Flavin Adenine Dinucleotide (FAD) dependent amine oxidases, LSD1 oxidises the lysine methyl ammonium group by catalysing the overall transfer of hydrogen from the methyl amino group to FAD (Fig. 1) generating a methyliminium ion.[6] For mechanistic reasons LSD1 is thus able to demethylate mono- and dimethylated lysines only. LSD1 is specific for H3K4me1/me2 as a component of the CoREST/Bcor1 co-repressor

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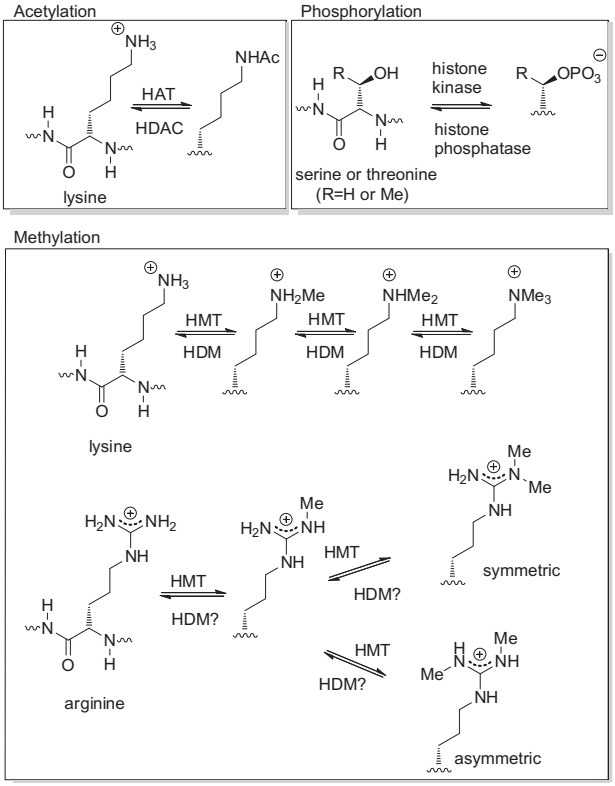

Figure 1. Posttranslational modifications of histones involved in epigenetic regulation: acetylation, phosphorylation and methylation.

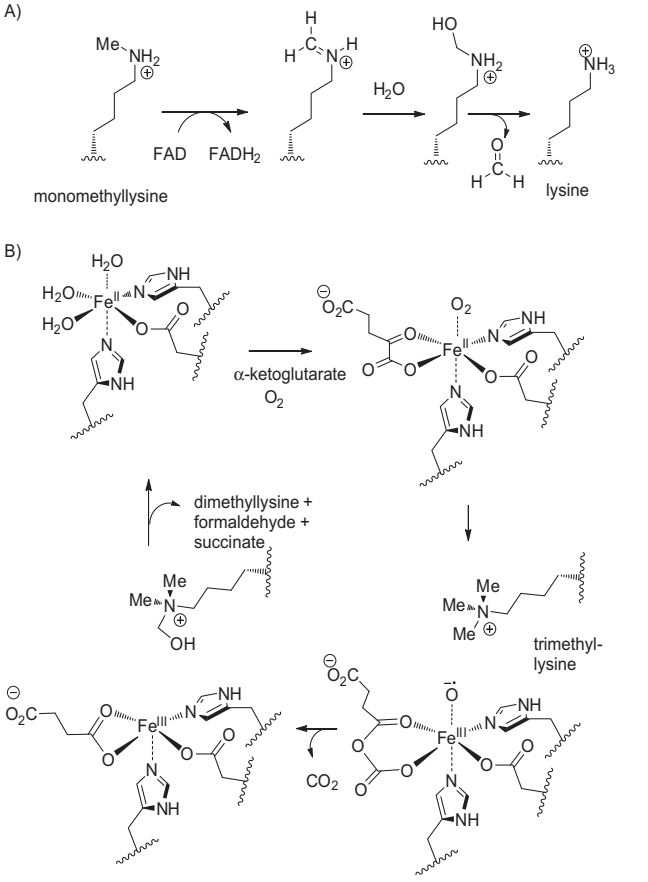

Figure 2. Reaction mechanisms for the demethylation of lysines at histones. (A) FAD dependent LSD demethylases and (B) Fe(II) and α-KG dependent JmjC demethylases.

complex, but it has been reported that in presence of the Androgen Receptor (AR) the specificity of LSD1 changes from H3K4 to H3K9. Demethylation of H3K9 now activates the transcription of AR target genes *KLK2* and *KLK3*.[10] Recently, an orthologue of LSD1 was reported and termed LSD2 (KDM2). LSD2 has specificity for H3K4me1/me2 like LSD1, however LSD2 does not form a stable complex with CoREST and is likely to have a role that is distinct from LSD1.[11]

All other known HDMs so far are from the Jumonji protein family, characterized by containing the JmjC domain. The first one was discovered in 2006[7] and in the same year several other oxygenases from the same family were reported to catalyze histone lysine demethylation.[12–16] There are currently 28 different JmjC domain proteins known to be encoded by the human genome, of which 15 so far have been published to demethylate specific lysines in the H3 tail. Demethylation as it is outlined in Fig. 2, likely occurs via the same mechanism as for other Fe(II) and α-KetoGlutarate (α-KG) dependent oxygenases established from crystallographic, spectroscopic, and isotope incorporation studies.[17] The exact order of the mechanistic events may however vary but all lead to consumption of oxygen and α-KG followed by release of CO_2, succinate, formaldehyde, and a lysine residue with one methyl group less. Although many of the JmjC proteins exhibit lysine demethylase activity they are oxygenases that can give other products as well. Thus, JMJD6 was first reported to be a histone arginine demethylase,[18] but recent studies suggest that the role is to catalyse lysine hydroxylation.[19] We will focus on the reported demethylases in this review. Figure 7 gives an overview of JmjC proteins that have reported lysine demethylase activity.

3. Pharmacological assays

The number of assays available to study the enzyme kinetics of HDMs is still somewhat limited and more work in this area is needed. The development of new assays have been quite challenging due to the HDMs low stability and turn-over numbers in comparison to other enzyme families. The most widely used assay is the Formaldehyde DeHydrogenase (FDH) coupled assay, in which the formaldehyde that is formed in the demethylation of the histone tails is detected indirectly.[20] FDH catalyzes the oxidation of formaldehyde to formate with concomitant reduction of Nicotinamide Adenine Dinucleotide (NAD^+) to NADH. The amount of NADH, which is directly correlated with the amount of formaldehyde released in the demethylation step, is then detected by fluorescence spectroscopy.[21] Since this method is based on fluorescence, it is highly sensitive and ideal for low turnover enzymes like the HDMs. The FDH assay is an excellent tool for gaining information about substrate activity and inhibition kinetics and the FDH assay is also well suited for High Throughput Screening (HTS) and a miniaturized version of the FDH assay has been described where it was run in a 4 μL 1536-well format with real-time fluorescence detection.[22] However, there are issues with the FDH assay that needs special attention. It cannot be used if the screened ligands are fluorescent themselves, since this will give false positive signals. Detergents present in the buffers used when purifying the recombinant proteins can influence the assay, for example, 0.005% SDS inhibits the enzymatic generation of formaldehyde by LSD1 with 89%. Preservatives can also have a negative effect on the signal generation, for example, 0.09% of azide in the assay buffer reduced the signal for LSD1 by 20% and 35.3% for JMJD2A (KDM4A).

Attempts to detect the formaldehyde directly via NMR have also been made.[23] Unfortunately, formaldehyde could not be unambiguously observed—presumably due to the relatively low concentra-

tion compared to other constituents in the samples. Addition of dimedone converts formaldehyde to two different adducts that more readily can be detected, but this is again an indirect measure of the amount of formaldehyde.

Another approach is the detection of the demethylated histones or histone tails via Matrix-Assisted Laser Desorption/Ionization-Time Of Flight Mass Spectrometry (MALDI-TOF MS).[7,24] This provides detailed information about the number of methyl groups removed from the histones by the HDM's. The relative intensities of different methylation states observed in the mass spectra were then used to calculate percentage demethylation. MALDI-TOF was also used to determine IC_{50}-values from the variation in demethylation-states at different inhibitor concentrations.[25] However, this technique is quite challenging and time consuming and not amenable to larger series of compounds. It should also be mentioned that the MS techniques like MALDI-TOF are sensitive to detergent which distorts the signals. Immunodetection of histones using antibodies can also be used to detect the methylation level of histones after exposure to JmjC's.[7,13] Others have combined these techniques in order to gain more specific information on modifications and inhibitors impacts in cells, through cross-referencing results gained from quantitative mass spectrometry and immunoblotting.[26]

A luminescence based assay has been developed that determines catalytic activity of and binding to the enzymes via the application of Amplified Luminescent Proximity Homogeneous Assay (ALPHA) technology.[27] The assay is built on the following principle: histonetails are coupled to 'donor beads' which contain a photosensitizer. Upon excitation with a laser this photosensitizer converts ambient oxygen to an excited O_2 singlet oxygen, and if an 'acceptor bead' is proximate (~200 nm), energy transfer occurs, producing a luminescent signal. In the absence of an acceptor bead, singlet oxygen returns to its ground state and no signal is detected.[28] The acceptor bead is functionalized either with a methylation-state selective antibody or a HDM. In the first instance luminescence (or lack thereof) provides information about the methylation-state of the histone after exposure of the donor bead to a HDM, and in the second instance luminescence is a direct measure of a given compounds ability to disrupt the complex between the HDM and the substrate. Other studies on HDM binding interactions with histone tails have employed techniques such as Isothermal Titration Calorimetry (ITC),[29] but this technique is hampered by low throughput rates and high enzyme and substrate consumption. In principle the ALPHA-assay can be adjusted to a high-throughput format, and should therefore be useful for providing affinities for larger series of compounds to HDM's.

LSD1 demethylates H3K4 via formation of the corresponding imine, see Figure 2A. This occurs via a FAD/$DADH_2$ mediated reduction of O_2 to H_2O_2. Thus, the activity of LSD1 can be monitored via detection of the amount of H_2O_2 formed. This is done by horseradish peroxidase which reduces H_2O_2 to H_2O using Amplex Red as an electron donor. The resulting product (resorufin) is highly fluorescent at 590 nm and thus easily detected.[30,31] In analogy to the FDH-assay this is an indirect measure of the activity of the enzyme. Several kits based on this setup are available.

Clearly, a number of useful assays have been developed for both screening purposes and for understanding the inhibitory action of HDM inhibitors, however not many are suited for HTS.

4. Medicinal chemistry of KDM inhibitors

Development of potent and selective inhibitors for the HDMs is crucial for understanding the role of HDMs and for drug development. A number of inhibitors have already been reported for both classes of HDMs. Many of the inhibitors are developed from compounds known to inhibit related enzymes, and therefore selectivity is a critical issue.

4.1. LSD1/2 inhibitors

The close homology to the MonoAmine Oxidases MAO-A and MAO-B suggested that mechanism-based or suicide inactivators could be used on LSD1. Indeed, phenelzine (**1**),[32] Tranylcypromine (PCPA, **2**, Fig. 3),[32–34] and Pargyline (**3**)[10] were some of the first LSD1 inhibitors to be reported, although it has later been disputed whether Pargyline can inactivate LSD1.[32,35] PCPA (**2**, also known as Parnate), is in clinical use as an antidepressant and has been used as lead structure for further development to increase potency and specificity. Gooden et al. made a series of PCPA analogues with aromatic substitutions and heteroaromatic exchanges without significant improvement of LSD1 inhibition.[36] Ueda et al. reported compounds **4** and **5** inhibit LSD1 with an IC_{50} around 2 μM and high selectivity compared to MAO-A/B.[37] The compounds were also active in cell cultures affecting H3K4 methylation and inhibiting growth of several cancer cell lines with GI_{50} (50% Growth Inhibition) values ranging from 6.0 to 67 μM. Binda et al. prepared a series of 40 analogues of which compounds **6–8** displayed a K_i around 1–2 μM at LSD1 with comparable potency towards MAO-A and MAO-B but an order of magnitude lower potency towards LSD2.[38] Compound **8** was investigated further and shown to be cell-active against acute promyelocytic leukemia, and displayed unprecedented synergistic activity with antileukemia drugs. Mimasu et al. employed structure-based design to get **9–11** (IC_{50} = 9–14 μM) that upon further optimisation yielded **12** and **13** with K_i = 0.61 and 1.5 μM, and k_{inact}/K_i = 4560 and 2990 M^{-1} s^{-1}, respectively.[39] It was verified that treatment of HEK293T cells with these compounds resulted in a dose-dependent increase in the level of H3K4me2.

A series of inhibitors based on a peptide (**14**, Fig. 4) made of the last 21 amino acids of the H3 tail has been reported.[40–42] Exchanging K4 with methionine (**15**) yields a potent inhibitor of LSD1 with

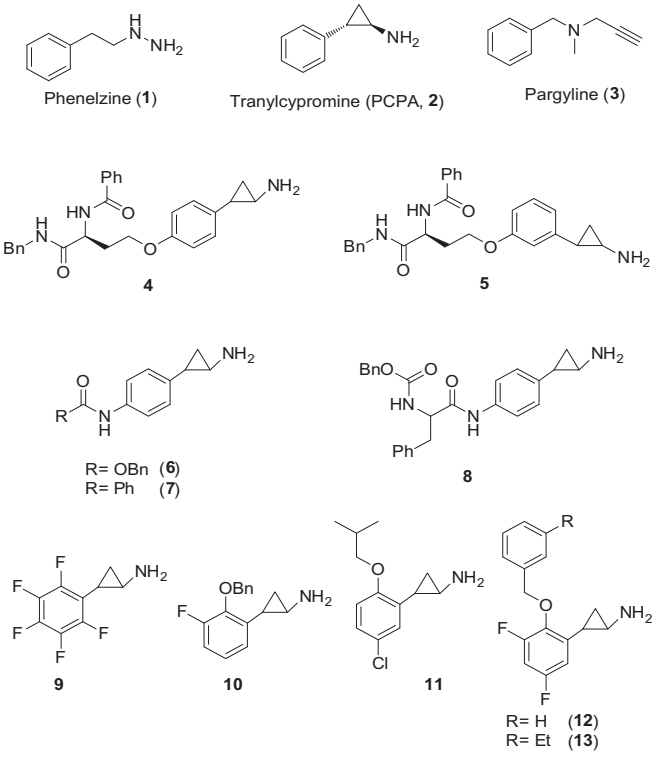

Figure 3. Chemical structures of mechanism-based irreversible inhibitors of LSD1.

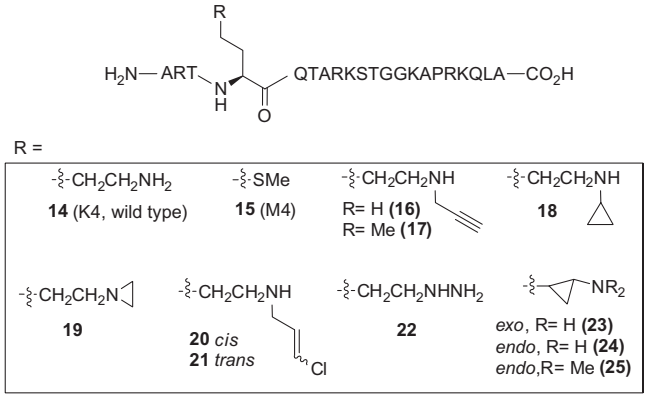

Figure 4. Structures of substrate-based inhibitors of LSD1.

a K_i = 0.05 μM, compared with a K_i = 1.8 μM of the wild-type peptide **14**. Truncation significantly reduces the affinity. Inspired by pargyline an irreversible inhibitor was designed by adding a *N*-propargyl group to the distal amine of K4 (**16**) and monomethylated K4 (**17**) with K_i ranging from 8.3 to 16.6 μM and a k_{inact} from 0.25 to 0.70 min^{-1}, respectively.[40,42] These data were determined with recombinant LSD1 produced in baculovirus transfected Sf9 insect cells, however a K_i of 0.29 μM was determined for **16** using GST tagged LSD1 produced in *Escherichia coli*, which is the most common used source.[44] In contrast to the propargyl analogues, the cyclopropyl (**18**) and aziridine (**19**) analogues are reversible inhibitors with a K_i of 2.8 μM (GST-LSD1) and 15.6 μM (Baculo-LSD1), respectively.[42,44] These results spurred the synthesis of an expanded series of suicide inhibitors that were characterised using GST-LSD1.[41] In this series other reactive moieties were added to K4 giving **20–21** with a K_i of 0.9 and 0.7 μM and a k_{inact} at 0.5–0.1 min^{-1}. Inspired by phenelzine peptide **22** was synthesized and displayed a K_i of 4.4 nM and 0.25 min^{-1} compared to a K_i of 17.6 μM for phenelzine in this assay making **22** the most potent LSD1 inhibitor described yet. In contrast to tranylcypromine, cyclopropyl analogues **23–25** were reversible inhibitors with K_i ranging from 2.7 to 24 μM compared to a K_i of 357 μM for the irreversible inhibition by tranylcypromine.

Several studies have investigated the mechanism of LSD1 inactivation by the suicide inhibitors using kinetic and structural approaches and showed how they react with FAD through radical oxidation reactions which also provides insight to the demethylation mechanism.[33,37,38,42,43]

Another class of LSD 1 inhibitors is a number of bisguanide and bisguanidine polyamine analogues (Fig. 5) that were tested as inhibitors of LSD1. This was inspired by the considerable homology

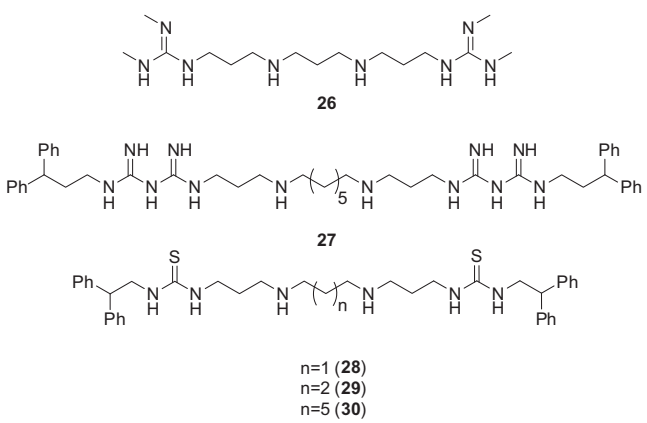

Figure 5. Bisguanide and bisguanidine polyamine inhibitors of LSD1.

LSD1 shares with FAD-dependent polyamine oxidases, including SperMine Oxidase (SMO/PAOh1) and by the fact that guanidines have been shown to inhibit both SMO/PAOh1 and other polyamine oxidases.[44–46] Nine compounds were found to inhibit demethylase activity by >50% at 1 μM and two compounds **26** and **27** were examined further and displayed non-competitive inhibition kinetics at concentrations <2.5 μM. Furthermore, the compounds were cell-active in human colon carcinoma cells and also leading to increased H3K4me2 and acetyl-H3K9 marks, decreased H3K9me1 and H3K9me2 repressive marks.[45] The series were expanded further to include (bis)urea and (bis)thiourea analogues (**28–30**)[47] with similar potency at LSD1 and the ability to inhibit growth of human Calu-6 lung carcinoma cells with GI_{50} values in the 10–40 μM range.

4.2. JmjC HDM inhibitors

Despite the relatively recent discovery of the JmjC containing histone demethylases several reports of inhibitors have already appeared. Several of them are well-established inhibitors of Fe(II) and α-KG dependent oxygenases, such as *N*-oxalyl glycine (**31**, Fig. 6) which is an analogue of α-KG and also inhibits human Hypoxia-Inducible Factor (HIF) prolyl hydroxylase PHD2 with K_i of 8 μM, and the asparaginyl hydroxylase factor-inhibiting-HIF (FIH) with a K_i of 1.2 mM.[48–50] The IC_{50} for **31** was determined by Rose et al. to 78 μM for JMJD2E employing a FDH coupled assay in one of the first reports of a collection of JmjC HDM inhibitors.[51] In that study it was further demonstrated that the structure of **31** can be elaborated to *N*-oxalylated D-amino acids and both a benzyl group (**32**) and a phenethyl group (**33**) can be accommodated in the binding pocket with IC_{50} = 320 μM and 100 μM, respectively. This was inspired by the X-ray crystal structure of **31** in JMJD2A that clearly implies that such substitutions are possible. Compound **33** inhibits FIH with K_i = 83 μM but is a much less potent inhibitor of PHD2, with IC_{50} >1 mM. The study also showed that Histone DeACetylase (HDAC) inhibitors may inhibit JmjC HDM. Many of the HDAC inhibitors are hydroxamic acids with the ability to coordinate the Zn^{2+} ion in the binding pocket and has a lipophilic stretch imitating the hydrophobic part of lysine, and these compounds may fit the binding pockets of JmjC HDMs and coordinate Fe^{2+} instead. Indeed, SuberoylAnilide Hydroxamic Acid (SAHA, **34**) is an inhibitor of JMJD2E with IC_{50} = 540 μM (14 μM with 30 min pre-incubation). In the same family, hydroxamic acid **35** inhibited JMJD2E with 28 μM (4.8 μM with pre-incubation) while methoxy- (**36**) and phenoxy-substituted (**37**) analogues were less potent. Finally, the study included several pyridine carboxylates of which some were known inhibitors of collagen or HIF prolyl hydroxylases with

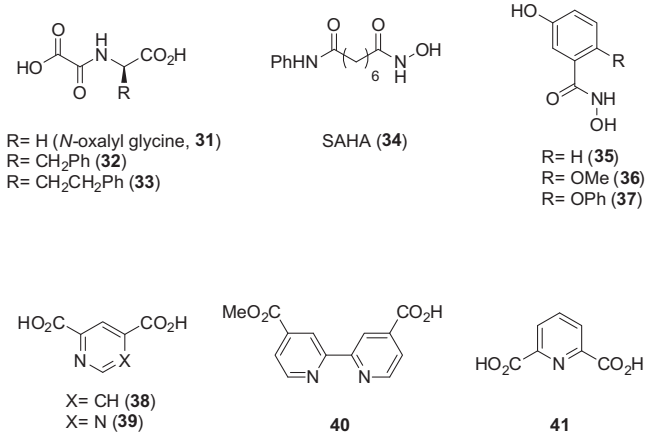

Figure 6. Inhibitors of JMJD2E with Fe-binding ability.

Fe-binding capability, including the most potent inhibitor in the study pyridine-2,4-dicarboxylic acid (**38**) with K_i = 914 nM. However, this compound is known to target other oxygenases such as collagen prolyl-4-hydroxylase in similar concentration.[48,52] Insertion of a ring nitrogen gave a significantly less potent pyrimidine analogue **39**, whereas the bipyridine analogue **40** was nearly equipotent to **38**. The 2,6-substituted **41** is a potent inhibitor of collagen prolyl-4-hydroxylase, but is 2 orders of magnitude less potent than **38** towards JMJD2E.

The series of *O*-oxalylated D-amino acids were later expanded (Fig. 5). In particular derivatives of *N*-oxalyl D-tyrosine were potent inhibitors. The most potent inhibitors were *O*-phenyl sulfonated *N*-oxalyl D-tyrosine (**42**) and *para* substituted analogues **43–45** displaying affinities in the 5–15 μM range with the most potent being **45** with IC_{50} = 5.4 μM. Furthermore, the *O*-benzylated *N*-oxalyl D-tyrosine derivatives (**46–48**) were potent with favorable 2-benzyl substitution with electron withdrawing groups. A selection of compounds was characterized at PHD2 with no detectable activity and compounds **43** and **45** at FIH showing activity albeit with an order of magnitude lower potency compared to JMJD2E. Interestingly, a disulfide exchange based dynamic combinatorial-mass spectrometry screen of *N*-oxalyl D-cysteine mixed with five different thiols at 15 μM was performed. The phenyl sulfide **49** and the *ortho* methoxy analogue **50** were captured in this experiment, and the corresponding benzyl sulfides **51** and **52** also displayed activity although in the 200–300 μM range. A crystal structure of **46** was obtained and docking **45** in this structure suggested that additional hydrogen bonds were picked up by sulfonate group.

A class of N-substituted *N*-oxalyl glycine derivatives **53** (Fig. 7) was also designed from inspection of the crystal structure of JMJD2A complexed with **31** and shown to be JmjC HDM inhibitors of JMJD2A, C and D.[53] However, the compounds were only characterized in an assay probing the reduced demethylation of methylated peptides with antibodies at 1 mM and 3 mM concentrations. Interestingly, **54** a dimethyl ester prodrug of **53** (n = 1) was designed that exerted histone lysine methylating activity in cellular assays; however, it was shown that **54** was inhibiting a JHDM different from the JMJD2 class.

The same group reported another compound class **55** (Fig. 7) with an Fe coordinating hydroxamic acid group combining the

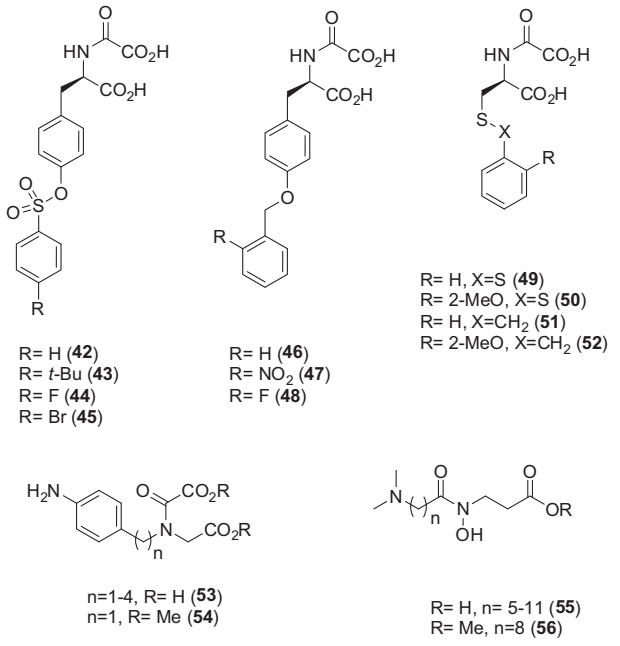

Figure 7. Inhibitors of JMJD2 HDMs derived from *N*-oxalyl glycine.

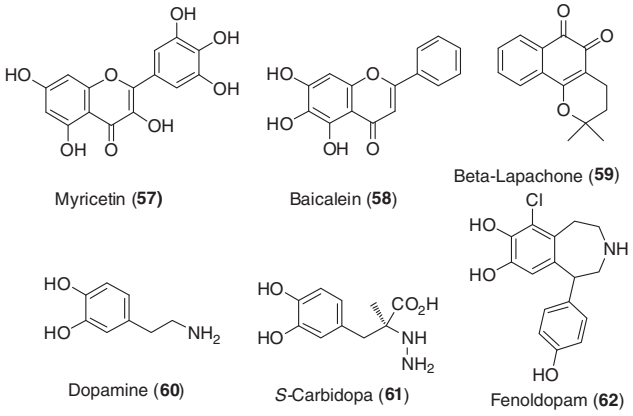

Figure 8. Flavanoids and catechols with inhibitory effects on JMJD2E.

N-alkylated with the carboxylic acid tail of succinate or α-KG and varying length of the lipophilic stretch of lysine (n = 5–11).[54] This class was again designed from crystal structure of JMJD2A with the specific aim of avoiding activity at PHD1 and PHD2. In general, the activities were in the same range 1–10 μM with n = 8 as the optimal chain length and indeed no PHD activities were observed. The alkyl group at the amine was also varied with little effect on the activity. Compound **55** (n = 8) and the corresponding methyl ester **56**, was evaluated at human prostate cancer cell line LNCaP with no effect, however the compounds acted synergistically with **4** on LNCaP, PC, and HCT116 cells.

A range of flavenoids and catechols were reported as demethylase inhibitors by Sakurai et al. (Fig. 8).[22] Myricetin (**57**) and baicalein (**58**) and beta-lapachone (**59**) are naturally occurring flavenoids that can inhibit JMJD2E with IC_{50} values of 3–10 μM. Also catechols like dopamine (**60**), (*S*)-carbidopa (**61**) and fenoldopam (**62**) could inhibit JMJD2E with similar potency. Catechols and flavenoids already have been noted for their iron-binding properties[55] thus the compounds have the potential to bind to iron ions at the active site and/or in solution. This was probed in the studies by testing the compounds in presence of ferrous ammonium sulfate but no firm conclusions could be derived. Kinetic analysis revealed that **59**, **62** and **58** were non-competitive inhibitors of JMJD2E with respect to α-KG (K_i = 3.55 μM, K_i = 1.92 μM, and K_i = 4.33 μM, respectively), while **61** appears to be an uncompetitive inhibitor of JMJD2E with respect to α-KG (αK_i = 1.98 μM). In general, these compounds are known to have other biological effects and therefore should be considered scaffolds for further development.

Despite the relative recent discovery of HDMs a number of inhibitor scaffolds has been reported. However most of these scaffolds originate from other enzymes that are mechanistically related and indeed many of the compounds has the ability to inhibit several different enzymes. Thus, one of the great challenges will be to develop highly specific and also subtype selective compounds in order to achieve useful pharmacological tools for understanding the roles of individual HDMs.

5. Structural studies and implications for inhibitor design

A number of X-ray crystal structures have been disclosed since the recent discovery of HDMs. These structures provide information on biological and mechanistic aspects of demethylation, but have also been important for guiding the design of inhibitors.

5.1. LSD1/2 demethylases

In 2006 human LSD1 structures were determined independently by four different groups. These structures were: (1) A

complex of LSD1 residues 171–852 in complex with a C-terminal fragment of CoREST residues 293–482 including one of the SANT2 domains and the linker region to the other SANT2 domain,[56] (2) LSD1 residues 166–835,[57] (3) LSD1 residues 172–833[58] and (4) the LSD1 SWIRM domain residues 169–279.[59] The structures revealed a catalytic domain closely related to that of the classical FAD-dependent amine oxidases and with high structural similarity to maize polyamine oxidase, where the structure was determined back in 1999,[60] and to human MAOs.[61] The two halves of the catalytic domain is separated by a 92 residue helical insertion. The CoREST fragment was found to bind to this insertion and the LSD1:CoREST complex in conjunction with a DNA:SANT2 structure thus enabled the construction of a model for the ternary LSD1:CoREST:Nucleosome complex[56] with many biological implications.

Recently three new mammal specific isoforms of LSD1 have been discovered. They arise from the combinatorial inclusion of two short exons encoding two (exon 2a) and four amino acids (8a), respectively. The crystal structure of the neuron specific exon 8a containing isoform has been determined.[62] Even though the insertion is in the amine oxidase domain it does not seem to affect the kinetic parameters of the enzyme. It does, however, affect LSD1 repressor activity on a reporter gene.

It has proven difficult to co-crystallize inactivated LSD1 with substrate analogue peptides, probably due to the low affinity required to form a transient complex. Such a complex can give valuable information for the design of specific ligands. This problem was elegantly solved through the synthesis of a substrate-like peptide with a replacement of one of the methyl groups of the peptide ARTK(me2)QTARKSTGGKAPRKQLA with a propyn group (**17**, Fig. 4) and subsequently forming a covalent link to the N5 of the flavin group of the FAD moiety using $NaBH_4$. The crystal structure of this complex showed that the peptide adopts three consecutive g-turns that enable optimal side-chain spacing.[43] It was also seen that the active site cannot accommodate more than three residues on the N-terminal side of the methylated lysine. This explains the H3K4 specificity of LSD1.

Attempts to use the structures in the design of new ligands have so far been focused on the catalytic domain and the use of known MAO inhibitors as lead compounds. Small molecules containing cyclopropylamine or propargylamine moieties are well known irreversible inhibitors of MAOs. They covalently modify the FAD co-factor.

The active site cavity of LSD1 is much larger than that of the MAOs since LSD1 has to accommodate a peptide substrate. Accordingly, PCPA (**2**, Fig. 4) is a weaker inhibitor of LSD1 than of MAO. The structure of the LSD1:PCPA complex has been determined.[33] It was clear that the FAD moiety was modified, but the specific modification was not clear from the electron density. This was later resolved in a crystal structure to higher resolution.[63] It was found that two different reaction products were found one with a five-membered ring comprising the N5 and C4 atoms of the flavin ring and one with a 3-phenyl propanoyl substituent on the N5 atom. Six crystal structures of PCPA analogues covalently bonded to the flavin ring of LSD1 have recently been determined.[38] All compounds show inhibition of LSD1 but lack of specificity towards MAO A, whereas some selectivity towards MAO B was observed. Other structures of PCPA derived complexes has been determined and used in the design of inhibitors selective towards MAO B.[39]

5.2. JmjC domain containing demethylases

The 28 members of the JmjC family can be divided into seven different subfamilies based on phylogenetic analysis and domain architecture.[64] All of them contain the catalytic active JmjC domain, but in addition a number of different DNA binding domains, zinc finger binding domains and uncharacterized domains are present in the different subfamilies. In addition to domains known in the Pfam and SMART domain databases, the family is characterized by long stretches of sequence being either disordered or containing unidentified domains. The function of these regions is currently not known. Currently, structural information from the JmjC family members is available for the ARID domain present in the KDM5 subfamily, different PHD domains from the PHF8 and KDM5 subfamily, Tudor domains from the KDM4 family and the JmjN and JmjC from the KDM2, KDM4, KDM7 and PHF8 subfamilies. Domains with known structure are marked with thick borders in Figure 9.

The ARID domains comprise approximately 100 amino acids, and proteins containing this domain are implicated in cell growth, development and tissue specific gene expression. Within the JmjC family the ARID domain is present in JARID2 and in all four members of the KDM5 subfamily and structural studies have been performed on all five family members using NMR spectroscopy The core structure of the ARID domain consists of 6 α-helices and members of the ARID domain family, Bright, Dead ringer (Dri) and Mrf-2 proteins have been reported to bind to AT-rich sequences of DNA.[65] Their interaction with DNA involves a loop between helix 4 and 5, giving rise to a Helix-Turn-Helix (HTH) DNA binding motif. In addition to the 6 α-helix core structure, the structure of KDM5C shows the presence of 2 additional short α-helices[66] in the N-and C-terminal part, respectively, whereas in JARID2 an additional C-terminal helix is present.[67] The DNA binding properties have only been explored for JARID2, KDM5A and KDM5B which showed non-specific binding of AT rich sequences to JARID2 and identified binding to a CCGCCC motif for KDM5A and B.[67,68] Even though the exact role of the ARID domain remains to be elucidated, studies performed from deletion mutants of the ARID domain indicated that the ARID domain is important for the catalytic function of the histone demethylases. In three different reports, deletion of the ARID domain did not result in reduced levels of H3K4me2/3, indicating that an intact ARID domain is necessary for demethylase activity.[69–71]

The Tudor domains contain a conserved protein structural motif consisting of approximately 50 amino acids. In the JmjC domain family it is present in the KDM4 subfamily as a tandem repeat. The tandem repeats have been reported to bind H3K4me3 and H4K20me3, and structures have been obtained of the Tudor tandem repeat of KDM4A in complex with both substrates.[72,73] A structure of the apo-tandem Tudor repeat has also been solved for KDM4C (PDB ID 2XDP). The overall secondary structure of the tandem Tudor domains from KDM4A and KDM4C is identical. The two Tudor domains fold into a hybrid structure in which each Hybrid Tudor Domain (HTD) consists of 4 β-strands; β-strand 1 and 2 from one Tudor domain and β-strand 3 and 4 from the other Tudor and vice versa. This hybrid folding of the Tudor domains makes each of the HTD's adopt the canonical Tudor fold. The tandem Tudor domains are necessary for demethylase activity in vivo, and the dual binding mode allows selective binding. This binding mode has been speculated to be involved in the recruitment of KDM4A to chromatin.[73] Binding of histone peptides have been studied for KDM4A and showed that binding of the trimethylated lysine occurs in a hydrophobic pocket comprised of F932, W967 and Y973.[72,73] This pocket is different from the catalytic active site and could possibly be exploited as a new binding site for small molecule inhibitors.

The PHD fingers are zinc finger binding domains derived from the family of Plant HomeoDomain Protein (PHD). The core structure comprises a classical zinc finger consisting of a two-stranded β-sheet and an α-helix. Two zinc ions are coordinated through conserved histidine and cysteine residues. In the JmjC family, PHD fingers are present in most of the subfamilies, including, KDM2, KDM4, KDM5, KDM7, JARID2 and PHF8. 3 structures have been

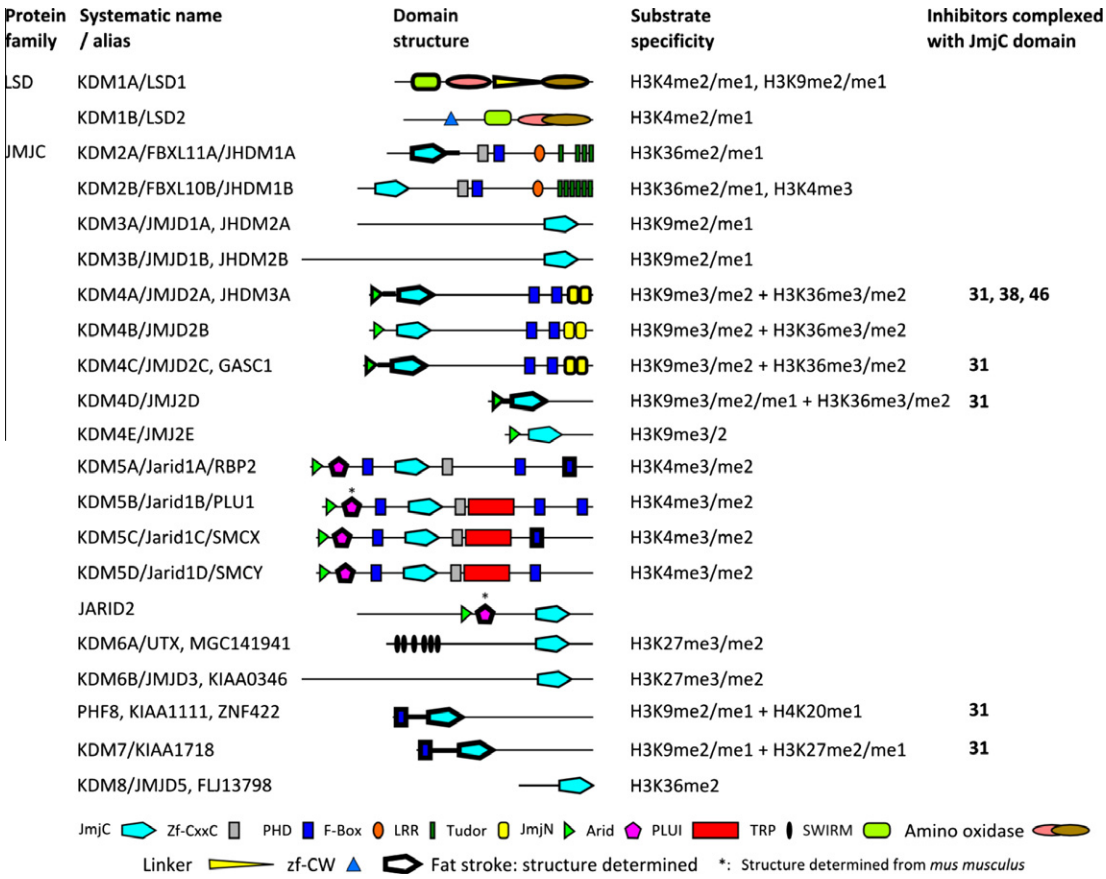

Figure 9. Domain architecture within the human KDM family. Domains for which structural data are available are marked with thick borders. Proteins are named according to the Protein Knowledgebase (UniProtKB).

solved, and cover the PHD finger from KDM7,[29] PHF8 (PDB ID 1WEP)[29] and the C-terminal PHD fingers in KDM5A[74] and KDM5D (PDB ID 2E6R). Of particular interest is the structure solved of the C-terminal PHD finger from KDM5A. Using both NMR-solution spectroscopy and X-ray diffraction the binding properties of the H3K4me3 peptide to this domain have been mapped. The C-terminal PHD finger of KDM5A (PHD3) has been shown to only interact with H3K4me2/3. In contrast, the N-terminal PHD finger only showed interaction with H3K9me3/2/1. This was also shown for the N-terminal PHD finger of KDM5C, which only interacted with H3K9me3,[75] and neither N-terminal PHD fingers from KDM5A or C showed interaction with H3K4me2/3.[74,75] This implies that although there are structural and sequential similarities between the different PHD fingers in the KDM5 family, they selectively bind to different methyl marks. The structure of the PHD finger from PHF8 has been solved both alone (from the mouse homologue, PDB ID 1WEP) and in complex with the JmjC domain.[29] Also a complex structure of the PHD and the JmjC domains from KDM7 has been solved.[29] The interaction with H3K4me3 has been studied in both complexes, and will be discussed further below.

The JmjC domain is present throughout the Jumonji family, and it is the domain responsible for catalytic activity. In the KDM4 subfamily, this domain is further expanded with the JmjN domain. The structure of the JmjC domain has been determined for members representing 4 of the different subfamilies; KDM2, KDM4, KDM7 and PHF8.[12,29,76–81] The structures all contain the conserved jelly-roll like fold (Fig. 10), characteristic for members of the Cupin superfamily.

The KDM4A JmjC domain was the first structure to be solved, and the majority of the structures available are from the KDM4 subfamily. These include complexes with different substrates, co-factors and inhibitors, and represent JmjC domains within the subfamily of KDM4A, C and D. The structures from the KDM4 subfamily are almost identical except for the C-terminal region in which KDM4D shows a different helical conformation. The KDM4 structures reveal a structural arrangement of the JmjN domain interacting with the JmjC and the presence of a C-terminal domain. Between the C-terminal domain and the JmjC domains a novel fold for zinc binding was observed.[12] This zinc finger motif consists of a histidine and a cysteine from the JmjC domains and two cysteines from the C-terminal domain. The zinc finger motif provides stabilization of the core structure between the JmjC domains and the C-terminal domain, and has been suggested to form a structural arrangement necessary for substrate recognition.[12]

KDM4A demethylates H3K9me2/3 and H3K36me2/3 and three different papers have synergistically elucidated the structural basis for substrate selectivity in KDM4A.[76,77,79] The discrimination between H3K9 and H3K36 peptides is obtained when the peptides adopt different curved conformations, allowing them to fit the substrate binding pockets without steric clashes. The curved conformation of the H3K4 peptide relies on the two glycine residues positioned +3 and +4 relative to K4 in the sequence, whereas in H3K36 the curved conformation is achieved from the proline positioned +2 relative to K36. A model for the discrimination between the different methylated states of the substrates was proposed by Ng et al., suggesting that side specific occupation at 2 of 3 potential methyl sites relative to the catalytic Fe(II) is responsible for the demethylation of H3K4me2/3.[79] The model also explains why the H3K4me3 is the preferred substrate, as this substrate always will have 2 methyl groups in the preferred sites.

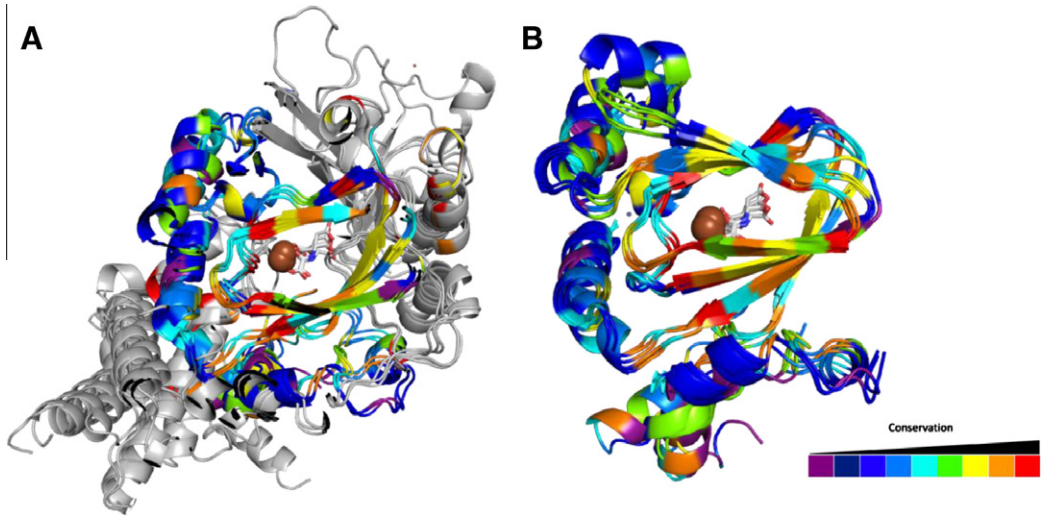

Figure 10. (A) Overlay of KDM4A (PBD ID 2OQ7),KDM4C (PDB ID 2XML), KDM4D (PDB ID 3DXU), KDM2A (PDB ID 2YU1), PHF8 (PDB ID 3K3O) and KDM7 (PDB ID 3KV5). (B) Overlay of the JmjC domain only. The residues are colored according to structural conservation as calculated with ConSurf. The structure guided sequence alignment was calculated with PRALINE. The color bar shows the conservation with purple being the least conserved and red being the most conserved.

The structure of KDM2A revealed the presence of 10 helices surrounding and stabilizing the Cupin fold.[78] During expression and crystallization trials of the catalytic core, it became apparent that the presence of the C-terminal extension, which is not in close proximity to the α-KG binding pocket, was essential for structural integrity. The C-terminal extension provides hydrophobic residues to shield a hydrophobic patch at α10 of the JmjC domain, reducing unfavorable exposure of hydrophobic surfaces. Whereas the structures from the KDM4 subfamily and the structure of KDM2A share the same Cupin fold, they differ in the presence of the C-terminal extension, and by the presence of the zinc finger motif identified from KDM4 structures. The residues involved in zinc coordination in KDM4 JmjC domains are not conserved in KDM2A, and no zinc finger motif could be identified in the structure of KDM2A. Although the presence of a JmjN domain in KDM2A has not been predicted on the sequence level, the N-terminal part of the KDM2A structure resembles a structural arrangement comparable to the JmjN domain observed in the KDM4 structures. This N-terminal part of KDM2A differs from the JmjN domain in the KDM4 structures by the presence of an extended loop between helix 1 and 2, which runs across the Cupin fold, contributing to additional loop–loop interactions thought to be important for substrate binding.

The structure of the JmjC domain in PHF8 was the third structure of the catalytic domain to be solved.[80,81] The structure shares a high degree of structural similarity with KDM2A, which, besides the Cupin fold, includes the C-terminal extension. The zinc finger motif observed in structures from the KDM4 subfamily is not present. As in the structure of KDM2A, the C-terminal extension forms a 4 helix bundle, however, the helical arrangement within the bundle differs. The arrangement of the C-terminal extension has been speculated to contribute to substrate selectivity, and the differences observed within that region in KDM2A and PHF8 might explain their selectivity towards H3K36me2/1 and H3K9me2/1 respectively.[80] As for KDM2A the C-terminal extension was shown to be essential for catalytic activity.[81]

The structure of the JmjC domain from KDM7 tightly resembles the structure of the close homolog, PHF8. Despite the high structural similarity, a striking difference was identified when structures of the JmjC domains of KDM7 and PHF8, including their respective PHD fingers, were solved.[29] The two structures showed different orientations of the two domains, which resulted in different conformations of the linker between the PHD finger and the JmjC domains. In PHF8 the linker adopted a disordered structure allowing the two domains to interact tightly, whereas the linker between the PHD finger and the JmjC in KDM7 is well ordered. The latter induced an extended conformation between the two domains. Both enzymes demethylate H3K9me2, but the different domain organization in PHF8 and KDM7 provided a structural explanation of why different activities were observed in demethylase assays using a H3K4me3K9me2 substrate. Enzymatic assays had shown increased activity of PHF8 (residue 1–447) upon using a substrate which in addition to the di-methylated K9 also was trimethylated on K4.[29] Interestingly, the opposite effect was observed for KDM7. The structural analysis of PHF8 in complex with the H3K4me3K9me2 substrate showed that the first 11 substrate residues were buried in a hydrophobic cleft formed at the interface of the PHD finger and the JmjC domain in PHF8 promoting K9me2 binding in the catalytic site. In contrast, binding of the K4me3 residue to the PHD finger in KDM7 would place the K9me2 residue far from the catalytic site due to the extended conformation, and thereby abolish catalytic activity. These two structures were the first examples to explain how closely related JmjC enzymes with identical substrate specificity read the epigenetic code.

Common for all the structures of the JmjC domain is the binding pocket for the catalytically essential Fe(II) and the co-factor, α-KG. This pocket is an obvious target for structure-based design of inhibitory compounds. The residues coordinating Fe(II) adopt a HxD/E. . .H motif and are fully conserved in the family. In the absence of α-KG, Fe(II) is coordinated by two additional water molecules, while in the presence of the co-factor, two oxygens from the co-factor supply the last two coordinative atoms. As opposed to the surroundings of the Fe(II) ion, the binding pocket for α-KG shows medium degree of sequence conservation. The variation in the co-factor binding pocket is most pronounced in the hydrogen bonding network of the co-factor and the surrounding residues (Fig. 11). While the highly conserved S196/198/200 in the KDM4 sub family is involved in hydrogen bonding to the co-factor, the corresponding serine from KDM2A, PHF8 and KDM7 is not as a valine residue occupies this spatial position. On the other hand T209/244/279 from KDM2A, PHF8 and KDM7, respectively, structurally aligns with F185/187/189 from the KDM4 subfamily but only the threonine residues are involved in hydrogen bonding. Figure 11 summarizes the conservation in the co-factor binding pocket and the residues involved in hydrogen bonding within the pocket.

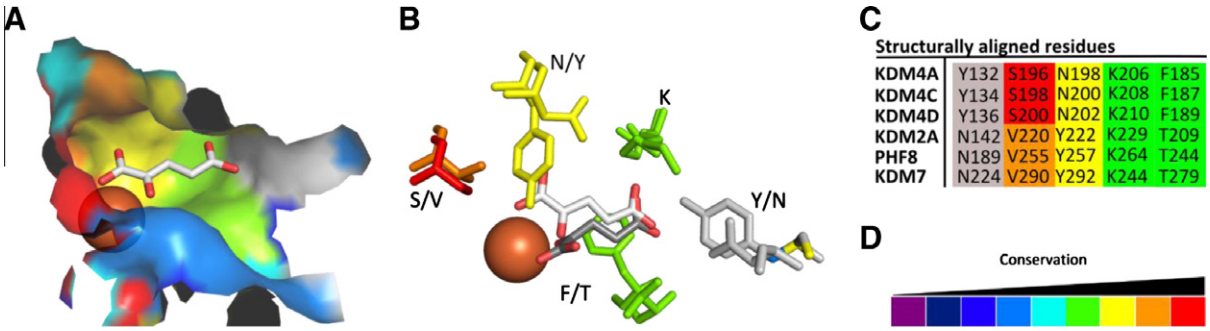

Figure 11. (A) Surface representation of the binding pocket for Fe(II) and α-KG in KDM4C. (B) Stick representation of residues involved in hydrogen bonding with α-KG in KDM4C (light grey) and PHF8 (darker grey). (C) Structurally aligned residues from KDM4A (PBD ID 2OQ7), KDM4C (PDB ID 2XML), KDM4D (PDB ID 3DXU), KDM2A (PDB ID 2YU1), PHF8 (PDB ID 3K3O) and KDM7 (PDB ID 3KV5). Residues are colored as described in Figure 10.

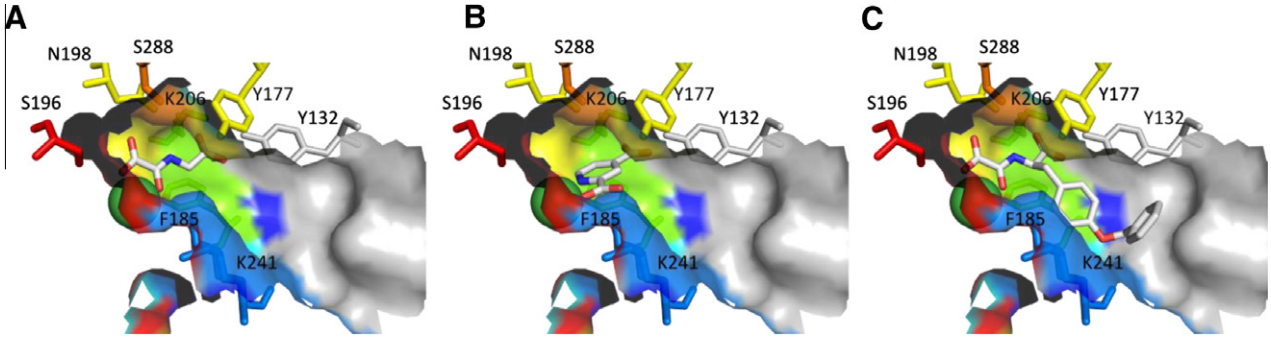

Figure 12. Comparison of the binding mode of **31** (A), **38** (B) and **46** (C) in the catalytic pocket in KDM4A. Residues are colored according to conservation as described in Figure 10. Residues not included in the JmjC domain are colored in grey.

Structural studies of inhibitor complexes of proteins from different JmjC family proteins are currently limited to structures of the JmjC domain in complex with *N*-oxalglycine (**31**, Fig. 6) (PDB ID 2OQ7, 2XML, 3DXU, 3K3O, 3KV5), and 2 structures of KDM4A in complex with 2 other inhibitors: Pyridine-2,4-dicarboxylic acid (**38**, Fig. 6) (PDB ID 2VD7) and *O*-benzyl-*N*-(carboxycarbonyl)-D-tyrosine (**46**, Fig. 7).[25] Analysis of the binding mode of **31** shows little difference compared to α-KG, however, the binding mode of **38** and **46** involves new polar contacts and an exploration of the catalytic site. The structure of the complex with **38** shows polar interaction with Y132, Y177, K241 and K206 and an additional π stacking between F185 and the pyridine ring of **38**. The inhibitor complex with **46** even further expands the network of polar interactions involving S196, S288, N198, K206 and Y132. In addition, the aliphatic residues from the non JmjC domain stabilize the hydrophobic part of **46** (Fig. 12).

It is interesting to compare the binding mode of **38** and **46** to other JmjC domains. One example is the π stacking between F185 and the pyridine ring of **38**. F185 is conserved in the KDM4 and the KDM5 subfamily, whereas in the remaining JmjC enzymes it is more divergent, like the threonine, contributing to the hydrogen bonding network of α-KG in KDM2A, PHF8 and KDM7. Furthermore, the non JmjC residues contributing to the catalytic pocket are less conserved, and could be explored in structure-based drug design of family selective inhibitors. As more structures of the catalytic active pocket from different JmjC family members become available, such rational drug design will be further facilitated.

6. Disease relevance and therapeutic prospects

Since the approval of clinical applications of HDAC inhibitors in the treatment of certain cancers it has become clear that interrupting epigenetic events is a successful strategy not only in cancer but perhaps also in other indications. For this reason many of the enzymes involved in epigenetic regulation are today considered potential drug targets. The histone demethylases are no exception and several of the genes coding for demethylases were known oncogenes prior to the discovery of demethylase function.

KDM1A (LSD1) is involved in the regulation of several different gene expression programs. Being the first demethylase cloned, it has been more thoroughly studied and thus knock-out mice have been generated for both Kdm1a and the other family member Kdm1b. Mice lacking Kdm1a dies early during embryogenesis whereas mice deficient of Kdm1b have a maternal lethal phenotype underscoring the importance of both proteins during development.[82,83] KDM1A has been reported to be highly expressed in certain tumor types and depletion of KDM1A as well as chemical inhibition, can inhibit growth of several different tumor cell lines.[10,84] This indicates that KDM1A could be classified as an oncogene. However, KDM1A has also been reported to repress the metastatic potential of breast cancer cells, thus in this context functioning as a potential tumor suppressor.[85] Because of the possible dual role of KDM1A in cancer, further studies are needed to clarify the precise function of KDM1A in cancer.

KDM2A was the first JmjC domain containing histone demethylase described.[7] KDM2A and its close homologue KDM2B catalyze the demethylation of H3K36me2/me1. Both proteins have been linked to cancer functioning either as tumor suppressor genes or oncogenes depending on cellular context. Thus, both proteins were found in a retroviral insertion screen for genes involved in the induction of lymphomas in rat.[86] In line with this ectopic expression of both proteins results in immortalization of Mouse Embryonic Fibroblasts (MEF) most likely through negative regulation of the tumor suppressor gene *Ink4b*.[86–88] Other data have suggested

that KDM2B can have tumor suppressor function through a mechanism in which loss of KDM2B results in genomic instability and cancer.[89] Several lines of evidence have linked H3K9me3 methylation to cancer through mechanisms involving genomic instability[90] and/or oncogene-induced senescence, where it is accumulated in the DAPI dense structures known as Senescence Associated Heterochromatin Foci (SAHF).[91] E2F target genes are enriched in SAHFs, which are believed to be important for the maintenance of senescence. Supporting a role of H3K9me3 in cancer is the observation that mice, that are heterozygous for the *Suv39H1* gene (the enzyme that catalyzes H3K9 methylation), are prone to cancer.[90,92] Overexpression of an H3K9me3 demethylase could function antagonistically to an H3K9 methylase and thereby promote tumor formation. Indeed several reports have linked the H3K9me3/me2 specific KDM4 family to malignant transformation. Thus, the locus for *KDM4C* a.k.a. Gene Amplified in Squamous Carcinoma 1 (GASC1) is amplified in esophageal carcinomas, medulloblastomas, and breast carcinomas.[93–95] Gene expression profiling has shown that KDM4A, B and C are overexpressed in prostate cancer, and depletion of KDM4B and C affects growth of several different cancer cell lines.[13] Hence, placing the KDM4 family members as candidate oncogenes.

KDM5B is an H3K4me3 specific demethylase. It is expressed specifically in testes of adult mice but has a more broad expression pattern in the developing mouse embryo.[96,97] High expression of KDM5B has been reported in both prostate and breast cancer. Depletion of KDM5B affects the growth of the breast cancer cell line MCF7 making KDM5B a candidate oncogene in breast cancer.[71]

The Polycomb Group (PcG) protein EZH2 catalyzes the trimethylation of H3K27. EZH2 is overexpressed in a variety of different tumor types and several results suggest that it is an oncogene.[98] EZH2 together with other PcG proteins repress the *INK4A-ARF* tumor suppressor locus in normal growing fibroblasts.[98] When normal cells are subjected to cellular stress, as for instance by the activation of oncogenes or by DNA damaging agents, the *INK4A-ARF* locus is transcriptionally activated. During this process the repressive H3K27me3 mark together with the PcG proteins are removed from the locus. The simultaneous recruitment of KDM6B to the *INK4A-ARF* locus is required and its H3K27 demethylation activity is required for the full transcriptional activation in response to stress.[99,100] Interestingly KDM6B is located in a chromosomal region close to p53, which is frequently lost in many types of cancer.[99,100] KDM6A is part of the MLL3/4 complex and is involved in the activation of *HOX* genes in response to retinoic acid.[101–103] Somatic mutations in *KDM6A* have been found in multiple myeloma, esophageael squamous cell carcinoma and renal cell carcinoma.[104,105] Depletion of KDM6A gives an immediate growth advantage to primary human fibroblasts, while over expression of KDM6A results in repression of growth depending on a functional JmjC domain.[106] Taken together these data suggest that KDM6A and KDM6B can function as tumor suppressor genes.

KDM2B, the KDM4 family, and KDM5B are candidate oncogenes that are overexpressed in cancer. This, together with the fact that the growth of several cancer cell lines are negatively affected when these proteins are depleted using siRNA, suggest that they are strong candidates for the development of new anti-cancer drugs. However, because some of the family members have tumor suppressive functions, it is important that potential inhibitors are specific for their targets. So far we only have limited knowledge regarding the specific tumor subtypes that overexpress the JmjC demethylases, and a better definition of the patient group that would benefit from the use of JmjC inhibitors is required. Moreover, more functional evidence is required for the validation of the JmjC proteins as strong targets for preventing tumor maintenance.

7. Conclusion

Despite, the relatively recent discovery of HDMs several inhibitor scaffolds have already been disclosed. Most of the inhibitors are derived from inhibitors of other mechanistically related enzymes and most of the compounds are therefore not very selective. This is not necessarily a problem for therapeutic applications, but in order to achieve useful pharmacological tools for understanding the role of each HDM subtype more specific compounds are needed. A number of assays have been developed and several X-ray crystal structures have appeared, enabling discovery of new inhibitors using structure guided design. Therefore, it is expected that more potent and selective inhibitors of histone demethylases will be developed in the coming years. Many of the genes coding for HDMs are reported oncogenes and clearly methylation and demethylation is an important epigenetic event in both cancer development and progression, but also in other slowly progressing metabolic and central nervous system disorders epigenetic events may play a role, and these new inhibitors turn out to be clinically relevant as has been demonstrated with HDAC inhibitors.

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