Inhibitors

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Targeting Histone Lysine Demethylases by Truncating the Histone 3 Tail to Obtain Selective Substrate-Based Inhibitors**

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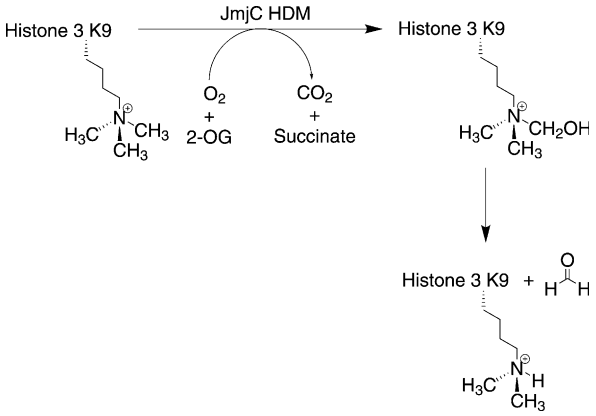
Histone tail modifications of specific amino acids in chromatin play a major role in gene regulation.[1] The patterns of these chemical modifications at a given area can code for either gene activation or for repression. These patterns constitute what is referred to as the epigenetic code.[2] These chemical modifications include phosphorylation of Ser and Thr residues, acetylation of Lys residues, and varying methylation states of Lys and Arg residues.[3]

A large number of enzymes involved in epigenetic regulation and reprogramming have been identified.[3,4] In the present study three jumonji-domain[5]-containing (JmjC) 2-oxoglutarate (2-OG)-dependent demethylases, JmjD2A (KDM4A), JmjD2C (KDM4C), and PHF8, were investigated. Trimethylation of lysine number 9 has been linked to oncogene-induced cellular senescence.[6–8] Overexpression of JmjD2 demethylases promotes tumor growth, which has been supported by several lines of evidence.[9–11] Mutations in the jumonji domain of human PHF8 has been reported to be involved in X-linked mental retardation and cleft lip palate.[12] All three enzymes have been reported to have demethylase activity towards Lys residue number 9 on the histone 3 tail (H3K9; see Scheme 1). JmjD2A has been reported to remove methyl groups from $H3K9Me_3/Me_2$,[13] JmjD2C from $H3K9Me_3/Me_2$,[9] and PHF8 from $H3K9Me_2/Me_1$.[14]



Scheme 1. Proposed mechanism of JmjC catalysis.[8] HDM = histone demethylase.

While the JmjD2 family of enzymes are candidate oncogenes, other demethylases are likely to function as tumor repressors. Consequently, selective inhibition of a specific member of the JmjC-domain-containing enzymes is a requirement for such inhibitors to be applied therapeutically in the treatment of cancer. In particular, selective inhibition of JmjD2C is desirable, since its involvement in diseases such as prostate and breast cancer[11] suggests that it could be an important target for anticancer therapy.[9] Unfortunately, the development of selective inhibitors is hampered by the high sequence and structural similarity of the enzymes, especially within the 2-OG cofactor pocket, which is the binding site of a number of previously reported small-molecule inhibitors.[15] In addition, these iron-chelating compounds also inhibit other histone-modifying enzymes, such as the histone deacetylases,[16] and several compound classes like the hydroxamic acids have been known for decades to interact with a number of different targets through iron metabolism inhibition.[17]

In the pursuit of selective inhibitors through a future peptidomimetic approach, a detailed investigation of the peptide-substrate kinetics of three different enzymes within the jumonji family, namely JmjD2A, JmjD2C, and PHF8, was initiated. The strategy was as follows. The shortest peptide fragment that could be recognized and processed by each enzyme was identified. Subsequently the coupling of a small molecule to the H3K9-lysine ε-amino group provided a substrate-based inhibitor that would be able to interact selectively with one of the enzymes within the same subfamily.

Truncation of the $H3K9Me_3$ (JmjD2C and JmjD2A) and $H3K9Me_2$ (PHF8) substrates (starting with the entire his-

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tone 3 tail of 39 amino acids) was performed from both the C and N termini towards the K9 position, until no catalytic reaction could be detected (see Figure 1). In the beginning we

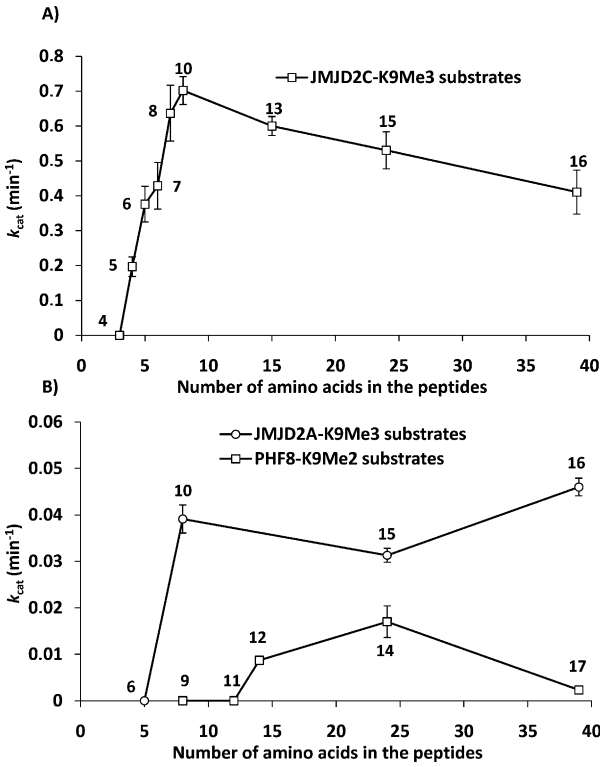


Figure 1. Correlation between A) JmjD2C, B) JmjD2A and PHF8, and their turnover number (k_{cat}) as a function of peptide-substrate chain length on the synthesized methylated lysine substrates; see Table 1 for the specific histone residues. k_{cat}, K_M, and k_{cat}/K_M can be found in Tables S1–S3, respectively, in the Supporting Information.

opted to remove larger portions of the substrate (going from 39 to 24 to 18 residues). As we began to see an effect on the catalytic activity we decided to proceed more carefully and truncate the peptide one amino acid at a time. The enzyme kinetics was determined using a formaldehyde dehydrogenase (FDH)-coupled enzyme assay,[18] and the demethylation of selected substrates was confirmed by MALDI-TOF mass spectrometry (MS). PHF8 can recognize and demethylate a peptide that consists of 14 amino acids ($H3_{(1-14)}K9Me_2$; **12**) with $k_{cat} = 0.01\ min^{-1}$, $K_M = 121\ \mu M$, and $V_{max} = 0.08\ \mu M^{-1} min^{-1}$ (k_{cat} = catalytic constant or turnover number, K_M = Michealis constant, and V_{max} = maximum rate of reaction). JmjD2A requires eight amino acids for catalytic activity: **10** ($H3_{(7-14)}K9Me_3$) with $k_{cat} = 0.04\ min^{-1}$, $K_M = 52\ \mu M$, and $V_{max} = 0.5\ \mu M^{-1} min^{-1}$, whereas JmjD2C was capable of recognizing and demethylating a substrate consisting of only four amino acids: **5** ($H3_{(7-10)}K9Me_3$) with $k_{cat} = 0.2\ min^{-1}$, $K_M = 35\ \mu M$, and $V_{max} = 1\ \mu M^{-1} min^{-1}$. Table 1 gives the complete list of substrates that were screened. The correlation between the turnover number (k_{cat}) as a function of peptide length is shown in Figure 1. k_{cat}, K_M, and k_{cat}/K_M for the various peptide substrates can be found in Tables S1–S3, respectively, in the Supporting Information.

Table 1: Peptides used as substrates as plotted in Figure 1. The table should be read as follows. Substrate **10**: the peptide is eight amino acids (AA) long, and consists of the sequence found in the histone tail H3 position 7–14: $H3_{(7-14)}$. $K_9(Me)_3$ indicates that the ε-amino group of lysine 9 (K_9) is trimethylated.

Substrate	Length (AA)	Residues/modifications
4	3	$H3_{(7-9)}$: $ARK(Me)_3$
5	4	$H3_{(7-10)}$: $ARK(Me)_3$-S
6	5	$H3_{(7-11)}$: $ARK(Me)_3$-ST
7	6	$H3_{(7-12)}$: $ARK(Me)_3$-STG
8	7	$H3_{(7-13)}$: $ARK(Me)_3$-STGG
9	8	$H3_{(7-14)}$: $ARK(Me)_2$-STGGK
10	8	$H3_{(7-14)}$: $ARK(Me)_3$-STGGK
11	12	$H3_{(3-14)}$: $TKQTARK(Me)_2$-$STGGK_{(Ac)}$[a]
12	14	$H3_{(1-14)}$: $ARTKQTARK(Me)_2$-$STGGK_{(Ac)}$[a]
13	15	$H3_{(1-15)}$: $ARTKQTARK(Me)_3$-STGGKA
14	24	$H3_{(1-24)}$: $K_9(Me)_2$
15	24	$H3_{(1-24)}$: $K_9(Me)_3$
16	39	$H3_{(1-39)}$: full H3-histone tail with $K_9(Me)_3$
17	39	$H3_{(1-39)}$: full H3-histone tail with $K_9(Me)_2$

[a] $K_{(Ac)}$ indicates that the ε-amino group of lysine (K) is acetylated (Ac).

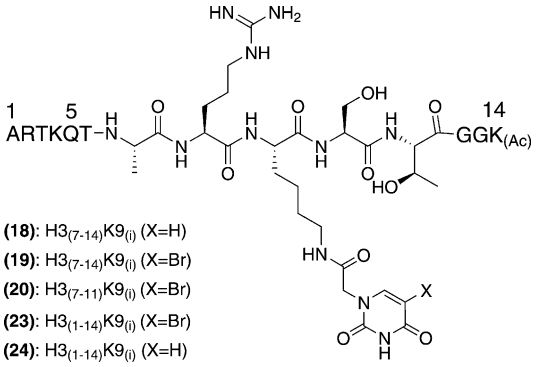
JmjD2C had the highest turnover number for the 8-mer substrate **10**: $k_{cat} = 0.7\ min^{-1}$, $K_M = 68\ \mu M$, $V_{max} = 4\ \mu M^{-1} min^{-1}$. This is consistent with previous results for JmjD2A, where the same substrate $H3_{7-14}K9Me_3$ was found to have the highest turnover number; however, no kinetic values were reported.[19] In this full kinetic characterization of JmjD2C, it is immediately apparent that there is a large difference in the turnover number between JmjD2C, JmjD2A, and PHF8, as seen in Figure 1. Interestingly, K_M for the three enzymes did not change significantly as a function of chain length, and plotting k_{cat}/K_M showed the same tendency (see Figure S1 in the Supporting Information). The turnover number k_{cat} for the same substrate **10** is 18 times higher for JmjD2C than JmjD2A, and for k_{cat}/K_M this value is 13 times higher. For PHF8 k_{cat} is 48 times lower than for JmjD2C, and k_{cat}/K_M is 74 times lower. These results demonstrate that JmjD2C has a significantly higher turnover number than PHF8 and JmjD2A. However, k_{cat} for JmjD2C is still sixfold lower than the turnover number for lysine-specific demethylase (LSD1) with $H3_{(1-21)}K_4(Me)_2$: $k_{cat} = 4.5\ min^{-1}$, $K_M = 21\ \mu M$[20] and JmjD2E at $H3_{(7-14)}K_9(Me)_3$: $k_{cat} = 4.56\ min^{-1}$, $K_M = 21.3\ \mu M$.[21] In general, the turnover number of the jumonji-class enzymes displays very low values compared to other enzyme classes.

In addition to the peptide length study, we also investigated the influence of the length of the lysine side chain by incorporating methylated ornithine instead of methylated lysines **1** and **2** (see Table S1 in the Supporting Information). No demethylation was observed, for any of the three enzymes, after removing one methylene group from the K9 side chain. Also, no enzymatic reaction could be observed for JmjD2C after removing alanine in the sequence $ARK(Me)_3$-ST to give substrate **3** $RK(Me)_3$-ST (see Table S1 in the Supporting Information), thereby underlining the importance of alanine in this position.

As seen from the k_{cat} profiles of the three enzymes, the overall catalytic rates of PHF8 and JmjD2A do not vary much with the substrate chain length, whereas JmjD2C is very

sensitive to the length of the substrate. The turnover number peaks at the eight amino acids with substrate **10** and drops gradually in k_{cat} for each removed amino acid. Only JmjD2C showed an enzymatic reaction towards the small peptides (AA < 8; see MALDI-TOF MS data in the Supporting Information), and we speculated that this could be exploited by coupling an iron-chelating group directly to the ε-amino group on one of the shorter substrate peptides. Although the enzymes are structurally very similar, there are subtle differences in the catalytic cavity and the enzyme surface, which is the contact area of the peptide backbone. We chose the 5-mer **6** ($H3_{(7–11)}K9Me_3$) as a scaffold, since this has 50% of the k_{cat} of the best substrate **10**; it is comparable to the histone 3 tail substrate and a good starting point for future peptidomimetic modifications.

A number of substrate-based inhibitors were synthesized with three different peptide lengths: 14, 8, and 5. The 14-mer was designed to target PHF8, the 8-mer to target JmjD2A and JmjD2C, while the 5-mer was anticipated to only target JmjD2C, based on the results from the substrate profiles of the enzymes in Figure 1. The synthesized substrate-based inhibitors are shown in Scheme 2.



Scheme 2. Synthesized substrate-based inhibitors with different peptide chain lengths.

Uracils are known as promiscuous iron chelators and we chose this class of compounds for several reasons: availability, ease of synthesis, and ability to coordinate iron. Initially they were tested in their free unconjugated state (along with other types of iron chelators), but none of them showed any inhibition in the low micromolar range. The identical molecules were then coupled to the peptides at the N^{ε}-amine of the lysine residue (see the Supporting Information for materials and methods). Attaching the small-molecule inhibitors to the shortest substrates recognizable for each of the three enzymes PHF8, JmjD2A, and JmjD2C had a dramatic effect on the enzymatic activity of these enzymes. Good correlation was found between the enzyme substrate activity and inhibition, as can be seen for the best substrate-based inhibitor for JmjD2C in our study, **20** ($H3_{(7–11)}K9_{(BrU)}$; see Table 2). Results for all tested inhibitors can be found in Table S4 in the Supporting Information, along with Michaelis–Menten kinetics for each substrate and inhibition curves.

Table 2: Enzyme activity of PHF8, JmjD2A, and JmjD2C correlated with inhibition as a function of peptide length. All substrates were $K9Me_3$, except for PHF8 where the substrate was $K9Me_2$. All inhibitors were measured against **10** ($H3_{(7–14)}K9Me_3$) except for PHF8 where **12** ($H3_{(1–14)}K9Me_2$) was used. (–) indicates that no substrate activity or inhibition could be detected by fluorescence and/or MALDI-TOF MS. Standard deviations for substrates: see Tables S1 and S2 in the Supporting Information.

Substrate activity k_{cat} [min^{-1}]:K_M [μM]			
Substrate	PHF8	JmjD2A	JmjD2C
$\mathbf{14}_{H3(1–14)}$	0.01:121	0.03:75	0.6:51
$\mathbf{8}_{H3(7–14)}$	–	0.04:52	0.8:68
$\mathbf{5}_{H3(7–11)}$	–	–	0.4:64

5-BrU inhibitor activity K_i [μM]			
Inhibitor	PHF8	JmjD2A	JmjD2C
23	20 ± 2	58 ± 5	66 ± 5
19	–	98 ± 6	52 ± 4
20	–	118 ± 2	27 ± 1

In the seminal work by Cole et al. a substrate-based inhibitor approach was used to target the related enzyme LSD1.[22] However, this substrate-based inhibitor was based on a peptide consisting of 21 residues ($H3_{(1–21)}K4_{(inhibitor)}$), thus making it a challenging lead structure for peptidomimetics and the development of non-peptide-based inhibitors.

Since the jumonji-domain-containing enzymes are highly dependent on Fe^{2+} in the active site, we focused on small molecules that potentially could coordinate to iron as the starting point in our search for inhibitors. A large series of small-molecule iron-chelating inhibitors has been developed by Schofield's group towards cc-JmjD2E (KDM4E)[23,24] and many others have used this approach as well.[15,25] We speculated that selective hybrid inhibitors could be developed by conjugation of a metal-chelating group on the N^{ε}-amine of the lysine residue in combination with the peptide backbone, as these hybrids could also interact with the surface of the enzymes—rather than merely chelating to iron in the active site, which usually leaves the inhibitor unspecific.

The enzyme kinetics for substrate **6** ($H3_{(7–11)}K9Me_3$), MALDI-TOF mass spectrum, and inhibition curve for inhibitor **20** on our main target JmjD2C are shown in Figure 2. For the 14-mer inhibitor **23** with 5-bromouracil, only a little discrimination in inhibition could be observed for PHF8 over JmjD2C and JmjD2A. Through attachment of the small molecules to the histone-tail-based sequence, a significant increase in inhibition could be observed on going from milli- to micromolar levels. As is evident from Table 2, the 14-mer inhibitor **23** inhibited all three enzymes, whereas the 8-mer inhibitor **19** only inhibited JmjD2A and JmjD2C. The 5-mer inhibitor **20** had a fourfold difference in inhibitory effect between JmjD2A and JmjD2C and no inhibition of PHF8.

In conclusion, the substrate histone tail, a peptide consisting of 39 amino acids, can be significantly truncated to just four amino acids and still be recognized and demethylated by JmjD2C. This remarkable prevalence of JmjD2C after extensive truncation led to a JmjD2C-selective substrate, which subsequently was converted into a selective

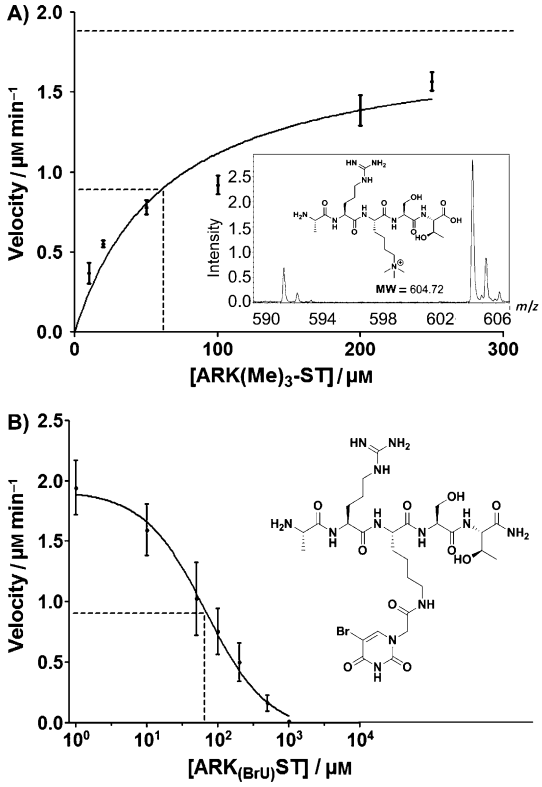


Figure 2. Profile of JmjD2C. A) Kinetics for **6** ($H3_{(7-11)}K9Me_3$); parameters: $V_{max} = 1.8\ \mu M\ min^{-1}$, $K_m = 64\ \mu M$, $k_{cat} = 0.4\ min^{-1}$. Inset: MALDI-TOF mass spectrum of the demethylation step from Me_3 to Me_2. B) Inhibition curve for **20** ($H3_{(7-11)}K9_{(BrU)}$) with substrate **10** ($H3_{(7-14)}K9Me_3$). K_i (inhibition constant) = 27 μM, $IC_{50} = 67\ \mu M$.

inhibitor by attaching a small molecule on the N^{ε}-amine of the lysine residue. These peptide-based inhibitors are proof-of-principle that our approach is viable and are excellent lead structures for the development of subtype-selective inhibitors of members of the jumonji-domain family of histone lysine demethylases. We believe that this truncation strategy is useful and applicable to all enzymes that depend on similar enzymatic reaction conditions involving long peptide substrates.

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