Structural basis for KDM5A histone lysine demethylase inhibition by diverse compounds

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SUMMARY

The KDM5/JARID1 family of Fe(II)- and α-ketoglutarate-dependent demethylases remove methyl groups from methylated lysine 4 of histone H3. Accumulating evidence supports a role for

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Author Contributions - J.R.H. performed crystallographic and ITC experiments, X.L. generated constructs, performed KDM5A/D kinetics and inhibition and ITC experiments; M.G. performed AlphaScreen, western blot analysis and colony formation assays; L.W. performed real-time RT-PCR and western blot analysis; J.R.S. performed routine protein purification; P.J.W. purified KDM5B full-length; X.Z. designed the KDM5A surface mutants and participated in discussion throughout; P.J.W. purified KDM5B full-length and KDM4A catalytic domain used in AlphaLISA experiment; J.S.K.B. analyzed ChIP-seq data; S.C.K performed AlphaLISA experiments; B.T.M., G.R., D.J.J. synthesized compounds; M.J.H., D.J.U., M.D.H., A.S. contributed to assay design and discussions throughout; D.J.M., A.J. identified compounds for synthesis & contributed to study designs; M.A.J and H.F. participated in organization and discussion of the study; J.R.H., P.M.V., Q.Y. and X.C. wrote the initial manuscript; and all were involved in analyzing data.

The atomic coordinates and structure factors of KDM5A in complex with αKG (code 5IVB), KDM5-C49 (code 5ISL), N3 (5IVC), N8 (code 5IVE), N10 (5IVF), N11 (5IVJ), N12 (5IVV), N16 (5IVY), and N19 (5IW0) have been deposited in the Protein Data Bank.

The authors declare that they have no conflicts of interest with the contents of this article.

SUPPLEMENTAL INFORMATION includes chemical synthesis, five figures and two tables.

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KDM5 family members as oncogenic drivers. We compare the in vitro inhibitory properties and binding affinity of ten diverse compounds with all four family members, and present the crystal structures of the KDM5A linked Jumonji domain in complex with eight of these inhibitors in the presence of Mn(II). All eight inhibitors structurally examined occupy the binding site of α-ketoglutarate, but differ in their specific binding interactions, including the number of ligands involved in metal coordination. We also observed inhibitor-induced conformational changes in KDM5A, particularly those residues involved in the binding of α-ketoglutarate, the anticipated peptide substrate, and intra-molecular interactions. We discuss how particular chemical moieties contribute to inhibitor potency and suggest strategies that might be utilized in the successful design of selective and potent epigenetic inhibitors.

**eTOC Blurb**

Horton et al. examine diverse compounds against all four members of KDM5 family. All inhibitors structurally examined occupy the binding site of α-ketoglutarate, but differ in the number of ligands involved in metal coordination. Inhibitor-induced conformational changes and inhibitor-specific interactions suggest strategies that might be used in the successful design of selective and potent epigenetic inhibitors.

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**INTRODUCTION**

Histone H3 lysine 4 (H3K4) methylation is a chromatin mark that on a genome-wide scale is broadly associated with gene activity. The mono-, di- and trimethylated forms of H3K4 are differentially enriched at promoters (predominantly H3K4me2/3), enhancers (H3K4me1) and other regulatory sequences (Deb et al., 2014; Shen et al., 2014). In mammals, six SET1/MLL1 methyltransferase complexes (Herz et al., 2013) and a tissue-specific PRDM9 (Mihola et al., 2009) are known to catalyze H3K4 methylation. Changes in gene state and the decommissioning of distal regulatory elements require the removal of H3K4 methylation, catalyzed by H3K4-specific demethylases, which include six enzymes...
belonging to two different families. The flavin adenine dinucleotide (FAD)-dependent demethylases LSD1/2 specifically remove methyl groups from low-degree (mono- or di-) methylated H3K4 (Shi et al., 2004; Zheng et al., 2015), whereas the Fe(II)- and \( \alpha \)-ketoglutarate (\( \alpha \)KG)-dependent demethylases KDM5A/B/C/D remove methyl groups from higher-degree (tri- or di-) methylated H3K4 forms (Cheng and Trievel, 2015; Christensen et al., 2007; Iwase et al., 2007; Klose et al., 2007; Lee et al., 2007; Xiang et al., 2007; Yamane et al., 2007). Mounting evidence from human tumors and model systems supports a role for the KDM5 family as oncogenic drivers (Rasmussen and Staller, 2014). KDM5A (also known as JARID1A or RBP2) was originally identified as a retinoblastoma (RB)-binding protein (Defeo-Jones et al., 1991; Klose et al., 2007), and indeed, the tumor-suppressive activity of RB is partially dependent upon its ability to sequester KDM5A (Benevolenskaya et al., 2005). Moreover, in estrogen receptor (ER) negative breast cancers, KDM5A mediates metastatic spread to the lung (Cao et al., 2014).

Extensive efforts have been devoted to develop inhibitors against the Jumonji family of histone lysine demethylases (Bavetsias et al., 2016; Heinemann et al., 2014; Kruidenier et al., 2012; Rotili et al., 2014; Wang et al., 2013; Westaway et al., 2016a; Westaway et al., 2016b). Some of these inhibitors, such as KDM5-C49 and its cell permeable ethyl ester derivative, KDM5-C70, are proposed to be potent and selective inhibitors of KDM5 demethylases in vitro and in cells (Patent WO2014053491). A number of additional compounds have been developed with various chemical moieties and a range of inhibitory activities (Chang et al., 2011; Rotili et al., 2014) (Supplementary Table S1).

The KDM5 family is unique among histone demethylases in that each member contains an atypical split catalytic Jumonji domain with insertion of a DNA-binding ARID and histone-interacting PHD1 domain separating it into two segments, JmjN and JmjC (Pilka et al., 2015) (Supplementary Figure S1A). We recently showed that the ARID and PHD1 domains are dispensable for in vitro enzymatic activity of KDM5 family members, whereas the Zn-binding domain immediately C-terminal to the JmjC is not (Horton et al., 2016). The linked JmjN-JmjC domain from KDM5A retains full structural integrity of the cofactor (metal ion and \( \alpha \)KG) binding characteristics of other structurally characterized Jumonji domain demethylases (Horton et al., 2016).

To gain insight into the structural and biochemical basis of inhibitory activity and how that may differ amongst members of the KDM5 family, we studied the binding modes of 10 chemically diverse, previously reported KDM5 demethylase inhibitors (Supplementary Table S1) in complex with the linked JmjN-JmjC domain of KDM5A at near atomic resolution by X-ray crystallography. In addition, we characterized the in vitro inhibitory activities and binding affinities of these with all four members of KDM5 family. We observed inhibitor-induced conformational changes in KDM5A, as well as inhibitor-specific binding interactions. We discuss how particular chemical moieties contribute to inhibition potency and how this may differ between families and amongst members of the KDM5 family. Overall, our results suggest strategies for future development of specific and potent KDM5 inhibitors.
RESULTS

Development of a KDM5A surface mutant with increased solubility and comparable demethylation activity

Previously we defined the minimal requirements for in vitro enzymatic activity of KDM5B and KDM5C to be the linked JmjN-JmjC domain coupled with the immediate C-terminal helical Zn-binding domain (Horton et al., 2016). We duplicated this approach to generate the corresponding constructs for the two other family members by deleting the ARID and PHD1 domains (ΔAP) from KDM5A and KDM5D truncated just beyond the Zn-binding domain, generating KDM5A(1-739)ΔAP and KDM5D(1-760)ΔAP (Supplementary Figure S1A). While KDM5D(1-760)ΔAP exhibited a similar expression level and solubility as that of KDM5B and C, KDM5A(1-739)ΔAP was totally insoluble, despite high expression in E. coli. We reasoned that some surface residues unique to KDM5A might affect protein solubility and/or aggregation. We substituted Cys626 and Cys636, both located in the Zn-binding domain and unique to KDM5A, to serine (C626S/C636S) (Supplementary Figure S1B). Though still less soluble than the other three family members, the KDM5A(1-739)ΔAP double mutant, KDM5A(1-739)ΔAP(2C-2S), generated a sufficient quantity of soluble, catalytically active protein for further study (Supplementary Figures S1C and S1D).

To investigate the effect of the Cys-to-Ser substitutions, we compared the kinetic parameters of KDM5A(1-739)ΔAP(2C-2S) and KDM5D(1-760)ΔAP to that of KDM5B(1-755)ΔAP and KDM5C(1-789)ΔAP, which we had previously characterized (Horton et al., 2016; Upadhyay et al., 2012). Under the optimal assay conditions established for KDM5B(1-755)ΔAP and KDM5C(1-789)ΔAP (Horton et al., 2016), the KDM5A(1-739)ΔAP(2C-2S) mutant showed kinetic parameters that were similar to that of the other three enzymes, with $K_m$ values for αKG in the 6–10 μM range, histone H3 peptide (residues 1-24, K4me3) in the 3–6 μM range, and $k_{cat}$ values of ~2–3 min⁻¹ (Figures 1A–1E). Thus we concluded that the substitutions of C626S/C636S have negligible effects on the in vitro enzymatic activity of KDM5A on peptide substrates.

Structure-function relationships among KDM5-directed agents

We compared the activities of 10 different small molecule inhibitors (Figure 2A) against all four members of the KDM5 family (Figure 2B and Supplementary Figure S2), in a formaldehyde dehydrogenase (FDH)-coupled demethylation assay (Horton et al., 2016). Among the 10 compounds, 8 contain an isonicotinic acid moiety with a carboxylic acid at the ring carbon-4 position and an extension either at the carbon-6 position (KDM5-C49, -C70, N19, N3 and N4) or carbon-5 position (N11, N12 and N16) (Figure 2A). Making use of the minimal catalytic domain constructs and assay conditions of 1 mM αKG, 0.5 μM enzyme, and 15 μM histone H3 peptide (1-24, K4me3) substrate, the compounds inhibited the demethylase activities of KDM5A and 5B to similar degrees (within ~2–3-fold, with compound N11 having the smallest and compound N19 having the largest difference between the two enzymes) and of KDM5C and 5D with nearly equal potency. This observation is in line with sequence conservation in that there is greater degree of shared sequence similarity within each pair (KDM5A and 5B vs. KDM5C and 5D) than there is
between them (Horton et al., 2016). Also, all of the compounds we examined inhibited the activities of KDM5A/B slightly better than that of KDM5C/D (approximately 3-fold). A similar observation was made recently with N-substituted 4-(pyridin-2-yl)thiazole-2-amine derivatives when comparing KDM5B and KDM5C, where a 2–5-fold lower IC₅₀ value is noted for KDM5B (Bavetsias et al., 2016). In previous work we found that JIB-04, a pan inhibitor of the Jumonji demethylase superfamily (Wang et al., 2013), inhibited the demethylase activity of KDM5B with ~8-fold increased potency compared to KDM5C (Horton et al., 2016), whereas GSK-J1, a semi-selective inhibitor of the KDM6/KDM5 subfamilies (Heinemann et al., 2014) had the opposite effect, and was ~8.5-fold more active against KDM5C than against KDM5B (Horton et al., 2016). Together, these observations suggest that selective inhibition within the KDM5 family members is attainable.

As previously observed with GSK-J1 and its ester derivative GSK-J4 (Kruidenier et al., 2012), the ethyl or methyl modification of the side group at the ring carbon-4 position (for example, between the pair of KDM5–C49 and KDM5–C70 or between N3 and N4) reduced in vitro inhibitory activity of the compounds by factors of 9- to 20-fold (compare IC₅₀ values between KDM5–C49 and –C70, and between N3 and N4; Figure 2B). This observation suggests that the maintenance of the carboxylic acid at carbon-4 position is critical to the inhibitory activity against the KDM5 family. In contrast, derivatization of the carbon-6 or the carbon-5 positions could accommodate vastly different chemical structures with little impact on inhibitory activity (for example, compare IC₅₀ values for KDM5–C49 to N19; and between N19 to N12). Interestingly, among the compounds examined, N8 and N11 exhibited the highest potency (IC₅₀ values of ~1 μM for KDM5A and 5B at 1 mM αKG and 0.5 μM enzyme concentrations) while sharing little structural similarity. N8 contains a pyrazolopyrimidine core with a nitrile functional group, whereas N11 is comprised of an isonicotinic acid with an extension containing an indazole ring and a difluoropiperidine ring (Figures 2A and 2B). The inhibitory activity of the different compounds correlated well with the relative binding affinities to KDM5A/5B linked Jumonji domain constructs as revealed by the dissociation constants (K_D) measured by isothermal titration calorimetry (Figure 2C and Supplementary Figure S3). Indeed, compounds N8 and N11 have the greatest binding affinities with K_D values of 25–50 nM.

**Structures of the KDM5A(1-588)ΔAP in complex with 8 inhibitors**

We determined the structure of the KDM5A linked Jumonji domain, KDM5A(1-588)ΔAP, in ternary complex with 8 of the 10 inhibitors and Mn(II) to the resolution range of 1.45–1.85 Å (the two least potent carbon-4 ester or methyl derivatives KDM5–C70 and N4 were excluded; Supplementary Table S2). We also determined the cofactor complex structure of KDM5A–αKG-Mn(II) to a higher resolution at 1.39 Å (Figure 3A). Like other structurally characterized αKG-dependent dioxygenases (Martinez and Hausinger, 2015), the metal ion was bound by six ligands in an octahedral coordination. The side chains of His–483, Glu–485, and His–571 (i.e. the HxE…H motif) provided coordination of the metal ion at three positions (1–3 in Figure 3B), while two oxygen atoms of αKG provide the fourth and fifth ligands and a water molecule provides the last. During the catalytic cycle, this sixth site would be occupied by a dioxygen O₂ molecule that initiates the demethylation reaction by
abstracting a hydrogen atom from the substrate. The αKG cofactor is involved extensively in both polar and hydrophobic interactions (Figures 3A and 3C).

The inhibitor bound forms of the KDM5A Jumonji domain are similar in overall structure to the αKG-bound form; for example, there was a root-mean-squared deviation of 0.3 Å across 293 pairs of Cα atoms between the αKG-bound and KDM5-C49-bound structures of KDM5A. Co-crystals of the KDM5A-αKG and KDM5A-KDM5-C49 complexes were obtained via traditional co-crystallization (see Experimental Procedures). The remaining seven compounds were soaked into pre-formed KDM5A-αKG crystals, thus allowing the inhibitor to compete away the bound αKG from the active site and replace it during the soaking process. In one instance (compound N19), we observed additional electron density near the active site that might represent the displaced αKG (see Discussion).

The isonicotinic acid moiety of KDM5-C49 occupies the αKG binding site, with its terminal carboxylic acid group (COO⁻) forming an extensive hydrogen bonding network with Lys-501, Tyr-409, and water-mediated interactions, similar to those normally engaged by αKG (compare Figures 3A and 3D). The carboxylic acids of all other compounds (N19, N3, N11, N12, and N16) exhibited nearly the same interaction (Figure 4). This finding underscores the importance of this local sub-structure to catalytic activity, and provides a molecular explanation for the observation that modification of the carboxylic acid (KDM5-C70 and N4) reduces inhibitory activity significantly.

**Inhibitor-induced conformational change at Asn-493**

Like αKG, KDM5-C49 provides two ligands for metal coordination, via the pyridine ring nitrogen and the aminomethyl nitrogen (Figure 3E), and has similar hydrophobic interactions with KDM5A (Figure 3F). Instead of occupying the ligand sites 4 and 5 as αKG does (Figure 3B), KDM5-C49 shifts to ligand site 5 and 6, with the position of a water molecule switching from the sixth to the fourth site (compare Figures 3B and 3E). The different binding contacts between αKG and KDM5-C49 in the bidentate interaction with the metal ion induce a side chain conformational change at Asn-493 (Figure 3G). In the αKG-bound form, Asn-493 bridges between αKG and Gln-557, resulting in a “dry” interface between αKG and Asn-493/Gln-557 (Figure 3A). In the inhibitor-bound form, Asn-493 forms a hydrogen bond to the water molecule occupying the fourth metal-ligand site, and a second water molecule is brought to the terminal carboxyl group of the isonicotinic acid moiety (Figure 3E). Superimposing the two structures reveals that Asn-493 moves from the αKG-interacting conformation to the inhibitor-bound conformation via a ~180° rotation of the side chain torsion angle χ1 (Figure 3G), resulting in a “wet” interface between the inhibitor and Asn-493/Gln-557 with at least three well-ordered water molecules in between (Figure 3D). Besides Asn-493, there is no significant change in the active site between the two structures, either in the αKG-bound or KDM5-C49-bound forms.

**Methyl-lysine substrate mimics**

Moving away from the metal binding center, the linear branch of KDM5-C49 extends into the proposed target methyl-lysine binding site (Figures 3H–3J). The amide nitrogen atom N11 and carbonyl oxygen atom O10 are joined via a peptide-like bond and the associated six
atoms are restrained in a planar conformation (Figure 3I) [inhibitor atoms in single letter format]. The closest distances between the branch atoms and KDM5 residues are C9:Glu-485 (3.1 Å), N11:Tyr-472 (3.7 Å) and the ethyl group off N11 and Asn-585 (3.2 Å). Finally, the terminal dimethyl amino group is flanked by Trp-470 and Asp-412 in an open sandwich conformation (Figure 3H).

The high structural similarity of KDM5A with other structurally characterized catalytic Jumonji domains (such as KDM6A) allowed us to model a methyl-lysine substrate into the active site of KDM5A (Horton et al., 2016). Superimposition of KDM6A in complex with histone H3 peptide (PDB 3AVR) (Sengoku and Yokoyama, 2011) places a trimethylated lysine residue in the active site of KDM5A, in a location occupied by the linear branch of KDM5-C49 (Figure 3I). We note that the methyl-lysine side chain and the linear branch of KDM5-C49 point in opposite directions. Interestingly, the ethyl group off the N11 nitrogen atom and one of the three methyl groups of trimethyl-lysine superimpose very well, and are surrounded by the main chain atoms of Val-584 and the side chain of Asn-585 (Figure 3J) – illustrating that methyl-lysine mimicry may be important for inhibitory activity of KDM5-C49. It is very unlikely that additional extension of the inhibitor could be accommodated in this location. Further, it appears that KDM5-C49 could also be competitive with the peptide substrate, but probably only after it takes the position of the αKG-binding site. However, this hypothesis will need to be validated by a peptide-bound co-crystal structure and detailed kinetic analyses.

Two metal ligands vs. one metal ligand

Like KDM5-C49, compounds with an extension out from the carbon-6 position of an isonicotinic ring (N19, N3) occupy two metal ligand positions (Figures 4A and 4B), whereas compounds with an extension out from the carbon-5 position (N11, N12 and N16), which point away from the metal binding center, occupy only one metal ligand position (Figures 4D–4F). Amongst those single-metal ligand compounds, water molecules occupy two out of six metal ligands (positions 4 and 6 in Figures 4D–4F). The number of metal ligands provided by each compound appears to be one of many factors determining inhibition potency, as demonstrated by similar IC50 values of N19 (possessing two metal ligands) and N12 (one metal ligand) (Figure 2A). Interestingly, all carboxylic-containing compounds have Asn-493 adopting an inhibitor-bound conformation, whereas N10 and N8 – the two compounds that do not contain a carboxylic moiety – have Asn-493 adopting the αKG-bound conformation (Figures 4C and 4G). Nevertheless, the interfaces between inhibitor and Asn-493/Gln-557 are all “damp”.

Inhibitor-specific interactions with Gln-75 and Arg-73

Pairwise comparison between KDM5-C49 and other structured compounds indicates that none of the other seven compounds takes the route of KDM5-C49. For example, compound N19 contains a pyrazole ring connected to a benzene ring that points in a different direction (Figure 4A, panel 1). Nevertheless, N19 and KDM5-C49 exhibited nearly identical inhibitory activity as indicated by their similar IC50 and K_D values (Figures 2B and 2C). Interactions specific to N19 must occur to compensate for changes in the target methyl-lysine location, implying an adaptability of KDM5 enzymes to various inhibitors.
The benzyl moiety of compound **N19** contains a chlorine atom at ring position-4 and a methyl group at ring position-2, respectively. The chlorine atom is within the distance of 3.2 Å from the side chain amino group of Gln-75, which forms two hydrogen bonds with the main chain carbonyl oxygen and amide nitrogen atoms of Ser-479 (Figure 4A, panel 2). The hydrogen bonds between Gln-75 and Ser-479 are invariant among the KDM5A structures examined thus far; the side chain-main chain interactions might be important for the stability of the enzyme as the interactions connect two neighboring β strands. Besides Gln-75, the hydrophobic benzyl moiety is located in a hydrophilic environment consisting of Arg-73 (the closest distance of 3.7 Å to the ring carbon-3) and Asp-412 (the closest distance of 3.3 Å to the methyl group) (Figure 4A, panel 3).

The ability to interact with Gln-75 and Arg-73 is evident in compounds **N3, N10, N11** and **N12**. Compound **N3** contains a phenethylcarbamoyl moiety with its terminal phenyl ring being less rigid as indicated by broken electron density (Figure 4B, panel 3). Although the phenyl ring of **N3** superimposed well with the benzene ring of **N19** (Figure 4B, panel 1), the potency of **N3** is reduced by 3–5 fold relative to **N19** (Figure 2A), probably due to the lack of direct interaction with Gln-75. The **N10** compound contains a trifluorobutoxy moiety that interacts with Gln-75 and Arg-73 via C-F…H-N type of hydrogen bonds (Muller et al., 2007) (Figure 4C, panel 3). However, the electropositive environment of KDM5A is very different from the apolar pocket of heat shock protein 90, which can be occupied by a corresponding trifluorobutyl group of a HSP90 inhibitor (Zehnder et al., 2011). The fluorophilic setting of inhibitor binding in KDM5A might explain the apparent disparity between the ordered electron density observed in the KDM5A-**N10** structure and the relatively poor binding affinity (K_{D} >40 μM) and enzyme inhibition observed in solution (IC_{50} >90 μM) (Figures 2B and 2C). Another possibility for the disparity may arise from the labile nature of the trifluorobutoxy group and the potential for hydrolysis in solution. When the compound is bound in the active site, such hydrolysis would be inhibited, resulting in the selective retention of the non-hydrolyzed form upon soaking into the crystal (see Experimental Procedures). Additional data will be required to settle this point. The **N11** compound contains a 5-fluoro-1H-indazole ring with the fluorine atom within 3.2 Å from the side chain amino group of Gln-75, just like the chlorine atom of the **N19** compound (Figure 4D). The **N12** compound contains a 1-methyl-1H-pyrrolo[2,3-b]pyridine ring, which superimposes well with the fluoro-1H-indazole ring of **N11**, but without the Gln-75-interacting fluorine atom (Figure 4E). In addition, the **N11** compound contains a difluoropiperidine ring, which is disordered in the structure. Nevertheless, modeling suggests that the disordered difluoropiperidine ring could potentially form contacts with either Trp-470 or Gln-535 (Figure 4D, panel 4). Together, the Gln-75-interacting fluorine atom and the disordered difluoropiperidine ring may contribute to the ~3–8X enhanced potency of **N11** relative to that of **N12** (Figures 4E and 2B), whereas the strengths of inhibitor potency for **N12** and **N16** are approximately the same whether it is a 1-methyl-1H-pyrrolo[2,3-b]pyridine ring (**N12**) or a chlorophenyl ring (**N16**) occupying the space (Figure 4F).
**Inhibitor N8-induced conformational change of Tyr-409**

Among the compounds we examined, N8 and N11 demonstrated the strongest potency against the KDM5 family members (Figure 2B). However, the two compounds show little similarity in their chemical structures and limited overlap in the space they occupy in the active site, except that both have a single metal-interacting ligand position and a Lys501-interacting hydrogen bond (Figure 4G, panel 1). The nitrile group of the N8 compound makes a single interaction with the active-site metal ion, while the ring 1-position nitrogen atom forms a hydrogen bond with the side chain of Lys-501 and the carbonyl oxygen at the ring 7-position is within hydrogen bonding distance to the side chains of Asn-575, as well as Lys-501 (Figure 4G, panel 2).

In the preceding description of αKG or the isonicotinic acid based inhibitors, the side chain Asn-575 bridges between Lys-501 and Tyr-409, which forms a hydrogen bond with the carboxylic group of αKG or the isonicotinic acids of the inhibitors. By contrast, in the N8 complex, the side chain of Tyr-409 is pushed away by the bulky pyrazolopyrimidine ring, and is rotated nearly 90° from that of the αKG-bound form (Figure 4G, panel 3), resulting in a van der Waals contact with the 6-isopropyl substituent. In addition, the central pyrimidine ring sandwiches between the aromatic rings of Tyr-472 and Phe-480 (Figure 4H). It seems that the highly reactive nitrile functional group and the forced interaction with Tyr-409 contribute to potent inhibition with a similar strength to that of the N11 compound, which contains large and disordered additions in the current structure (Figure 4D).

**Cellular effect of KDM5-C70**

As mentioned above, KDM5-C70 was designed as a cell permeable prodrug that is hydrolyzed by an esterase within the cell to generate KDM5-C49. We tested the activity of the KDM5-C49 compound against Flag-tagged full-length KDM5A, KDM5B and KDM5C purified from Sf21 insect cells (Sayegh et al., 2013). Under conditions of nanomolar enzyme concentration, KDM5-C49 inhibited KDM5A, KDM5B, and KDM5C with similar potency, exhibiting IC50 values in the nanomolar range by two independent assays (Figures 2D and 2E). KDM5-C49 showed greater inhibitory activity against the KDM5 family versus KDM6 and KDM4. The compound was unable to inhibit the activities of purified KDM6A or KDM6B catalytic domain (residues 1043-1682) by more than 50% even at 50 μM, the highest inhibitor concentration tested (Figure 2E), and had ~10-fold weaker potency against the KDM4A catalytic domain (residues 1-350) than against the corresponding KDM5B catalytic domain or full length KDM5B (Supplementary Figure S4B). These results further confirmed the inhibitory potency and specificity of KDM5-C49.

We examined the relationship between the in vitro findings for KDM5-C49 and cellular activity of KDM5-C70. We focused on breast cancer cells (MCF7, MDA-MB-231, BT474 and ZR-75-1) because KDM5A and KDM5B have been shown to be amplified/overexpressed in human breast cancers and to promote invasion and metastasis in human and mouse breast cancer models (Cao et al., 2014; Hou et al., 2012; Yamamoto et al., 2014). Moreover, previous studies have shown that knockdown of KDM5B inhibits the growth of luminal breast cancer cells, including MCF7, whereas more basal subtype breast cancer cells, such as MDA-MB231, are more resistant (Yamamoto et al., 2014; Yamane et al.,...
Similarly, knockdown of KDM5A suppressed the growth of ZR-75-1 breast cancer cells (Hou et al., 2012).

Consistent with an effect on the KDM5 family, treatment of MCF7 and MDA-MB-231 breast cancer cells with KDM5-C70 significantly increased global levels of H3K4me3, while having little impact on H3K4me2/me1, or modifications regulated by other histone lysine demethylases, such as H3K27me3 (substrate for the KDM6 family) and H3K9me3/H3K36me3 (substrates for the KDM4 family) (Figure 5A). To further examine the consequences of KDM5 inhibition in breast cancer cells, colony formation assays were performed. At 5 μM KDM5-C70, the growth of MCF7, BT474 and ZR-75-1 cells was inhibited by 85%, 97% and 70%, respectively (Figure 5B). For comparison, colony formation assays were also performed in cell lines shown to be relatively resistant to KDM5A or KDM5B knockdown, including MDA-MB-231 cells (Cao et al., 2014; Yamamoto et al., 2014), PC9 lung cancer cells (Sharma et al., 2010) and MCF10A immortalized, non-transformed mammary epithelial cells (Hou et al., 2012; Yamamoto et al., 2014). As expected, none were significantly affected by treatment with KDM5-C70 (Figure 5B). Interestingly, sensitivity to KDM5-C70 appears to be unrelated to a differential ability to hit the intracellular target, as KDM5-C70 led to a comparable accumulation of global H3K4me3 levels in sensitive (MCF-7) and more resistant (MDA-MB231) cell lines at equivalent doses (compare Figure 5A and 5B).

Several potential gene targets of KDM5-mediated repression have been identified in overexpression and/or knockdown and ChIP studies. We focused here on several genes (MT1F, MT1H, STK6, BRCA1, BUB3 and BUB1B) whose expression was altered upon overexpression and/or derepressed upon KDM5B knockdown (Scibetta et al., 2007; Yamane et al., 2007). Analysis of KDM5B ChIP-seq data from MCF7 cells (Yamamoto et al., 2014) indicated that KDM5B is enriched to varying degrees at the promoters of 5 of these genes, including MT1F, STK6, BRCA1, BUB3 and BUB1B (Supplementary Figure S5). Knockdown of KDM5B led to an increase in the ratio of H3K4me3 to H3K4me2 at most of these promoters, consistent with a local inhibition of H3K4me3 demethylase activity, albeit to varying degrees (Supplementary Figure S5). We therefore tested the impact of KDM5 inhibition on the expression of these genes. Treatment of MCF7 cells with KDM5-C70 under the same conditions used for the global histone analyses (5 μM for 3 days) led to an increase in the expression of two such targets, MT1F and MT1H, but not BRCA1, STK6, BUB3 and BUB1B (Figure 5C). The extent of the MT1F and MT1H upregulation caused by KDM5-C70 treatment was similar to that reported for KDM5B knockdown (Scibetta et al., 2007). These data suggest that whereas the demethylase activity of KDM5 enzyme(s) is important to maintain the repression of some genes (MT1F and MT1H), the KDM5 protein(s) (and hence the impact of KDM5 knockdown) may have additional functions in gene regulation beyond the catalytic activity (see Discussion).

**DISCUSSION**

KDM5A and KDM5B are amplified and overexpressed in breast cancer, and have oncogenic functions in cell proliferation, invasion and drug resistance (Rasmussen and Staller, 2014). We showed that KDM5A loss suppresses tumorigenesis and metastasis in multiple mouse...
cancer models (Cao et al., 2014; Lin et al., 2011), suggesting that KDM5A inhibition could be exploited for cancer treatment. Here we present eight structures of inhibitor-bound KDM5A Jumonji domain at high resolution. We note that the linked JmjN-JmjC domain of KDM5A is not active on its own but binds the cofactor αKG with a dissociation constant ($K_D$) of 80 μM (Horton et al., 2016). The lack of activity in the absence of the immediate C-terminal Zn-binding domain may stem from the inability to properly engage the H3 substrate. This result is similar to KDM6A, where the C-terminal helical Zn-binding domain is essential for enzymatic activity by binding a portion of the histone H3 peptide (Sengoku and Yokoyama, 2011). [We note that there was a 9-fold difference between the $K_D$ (80 μM without the C-terminal domain) and $K_m$ (9 μM with the C-terminal domain) for αKG. Whereas the exact reason for this discrepancy was not elucidated by the current study, one possible explanation is that the $K_m$ was measured in the presence of peptide substrate and the Zn-binding domain whereas the $K_D$ was determined in the absence of peptide substrate with the enzyme lacking the C-terminal domain. Thus additional H3 peptide interactions with the Zn-binding domain may have effect on enzyme activity.]

Structural comparisons between the KDM5-C49-bound structure of KDM5A and that of KDM5B (PDB 5A3T), which includes the C-terminal domain and a similar ΔAP internal deletion, demonstrated that the compound KDM5-C49 binds the two family members in exactly the same manner even including the ordered water molecules (Figure 6A), and shows that the addition of the C-terminal helical Zn-domain does not interfere with either the folding of the linked JmjN-JmjC domain or its active site conformation. Furthermore, whether using the full-length KDM5B or the minimal catalytic domain of KDM5B(1-755)ΔAP, a similar inhibition was observed across the 10 inhibitors (Supplemental Figure S4B).

Our structural effort provides avenues for improving the potency and selectivity of KDM5 inhibitors. Firstly, as noted above, seven out of eight compounds were soaked into pre-formed crystals of KDM5A-αKG-Mn(II) complexes. During the structural refinement, we often observed residual electron densities near the compound binding sites, and sometimes we could model one or more glycerol molecules that were used for freezing crystals. In the compound N19-bound structure, we clearly observed additional density that could be fit by αKG, as suggested by the “Ligand Identification” module of PHENIX (Adams et al., 2010) (Figure 6B). The additional density is flanked between the aromatic benzyl ring and Cys-481 (bottom and top as shown in Figure 6B), a residue unique to the KDM5 family (Horton et al., 2016), and Gln-75 and His-483 (left and right), as well as a network of ordered water molecules (Figure 6B). The location of the “displaced αKG” and its proximity to Cys-481 provide a unique opportunity to explore a fragment-based approach; for example, by combining the existing compounds (such as N19) and the displaced αKG molecule, by using reversible covalent inhibitors that target noncatalytic Cys-481, or by extending further into the solvent channel (Figures 6C and 6D). While the active sites of many KDMs are similar (particularly among the KDM4, 5 and 6 families) (Cheng and Trievel, 2015), expansion of inhibitors into this water-filled channel (lined with many unique residues for the KDM5 family) opens an avenue for increasing the selectivity of an inhibitor for KDM5 over other similar KDM families or even among the family members themselves. For
instance, there is a water network that connects the “displaced αKG” to the side chain of Ser479 (Figure 6C), a residue conserved between KDM5A and B, but substituted with an alanine in KDM5C and D (Horton et al., 2016).

Secondly, compounds N19 and KDM5-C49 share the core structure of the isonicotinic acid moiety with the extension off the carbon-6 position, but pointing in different directions (Figure 5A, panel 1) and thus engaging a different set of interactions. Nevertheless, the two exhibited equipotent inhibitory activity (Figure 2B). Combining the two extensions into one molecule could potentially generate a more potent lead compound. Moreover, the apparent interactions between charged/polar residues Arg-73/Gln-75/Asp-412 and the compounds N11/N19 suggest that inhibitor potency may be further improved by replacing the ring fluorine atom (N11) or ring chlorine atom (N19) with group(s) that better maximize the placement of complementary polar/charged groups. It is noteworthy that, besides the aforementioned side chain conformational changes, the main chain Cα atom of Asp-412 undergoes an ~2Å shift comparing the N8-bound structure to that of other inhibitor-bound forms including αKG (Figure 6F). Lastly, replacement of the disordered difluoropiperidine ring of compound N11 with groups that maximize the hydrophobic interaction with Trp-470 or polar interaction with Gln-535 (Figure 5D, panel 4) might also increase the potency of this already potent inhibitor.

Using KDM5-C70, the cell permeable version of KDM5-C49, we showed that the demethylase activity of KDM5 enzymes is required for the growth of several breast cancer cell lines and for the repression of some genes. Of six genes known to be derepressed upon KDM5B knockdown in MCF7 cells (Scibetta et al., 2007; Yamane et al., 2007), only two were significantly upregulated upon KDM5-C70 treatment. Among these, MT1H, MT1F, and BRCA1 were occupied by KDM5B in directed ChIP assays (Scibetta et al., 2007; Yamane et al., 2007). ChIP-seq data from MCF7 cells (Yamamoto et al., 2014) further suggests that 5 of 6 genes studied are occupied by KDM5B, and exhibit some increase in the local ratio of H3K4me3/me2 upon KDM5B knockdown (Supplemental Figure S5). MT1H lacked KDM5B enrichment altogether, at least in this study (Yamamoto et al., 2014). The apparent discrepancy between knockdown studies and KDM5-C70 inhibitor studies could arise from differences in MCF7 strains, the sensitivity of the assay used (ChIP-qPCR versus ChIP-seq) or the influence of the compound on multiple KDM5 family members versus the knockdown of only one (KDM5B). Moreover, whereas the activity of the KDM5 demethylases may be important in maintaining the repression of some genes, the intact protein(s) may have additional, non-catalytic functions in gene repression (eg. a scaffolding function) that would be compromised upon knockdown, but perhaps unaffected by the inhibitor. Indeed, KDM5A is known to bind retinoblastoma (Defeo-Jones et al., 1991; Klose et al., 2007) and to interact with components of the Sin3 co-repressor complex (van Oevelen et al., 2008), and KDM5B has been suggested to interact with components of the NURD repressor complex (Klein et al., 2014), each of which could be disrupted upon KDM5A/B knockdown. Interestingly, differential sensitivity to KDM5-C70 appears unrelated to the ability to impact H3K4me3 levels and hence to hit the intracellular target, suggesting that there are additional factors that ultimately determine the cellular response. We note that the KDM5 enzymes contain a DNA binding ARID domain, two or three PHD (plant
homeodomain) domains and an uncharacterized PLU1 domain (Supplementary Figure S1A). The PHD domains have been shown to bind to the KDM5 substrate H3K4me2/3 (PHD3 in KDM5A (Wang et al., 2009), the demethylation product H3K4me0 (PHD1 in KDM5A (Torres et al., 2015)), and the repressive mark H3K9me3 (PHD1 in KDM5C (Iwase et al., 2007)). Thus, the future development of agents that target other regions of KDM5 demethylases might prove to be useful in combination with those directed at the catalytic domain to combat KDM5 oncogenic potential.

**Significance**

Our extensive efforts to characterize KDM5A interactions with diverse inhibitors provide important insights into structure activity relationships for KDM5 inhibitors and should aid in the successful design of selective and potent epigenetic inhibitors of KDM5 di-/trimethylated histone H3 Lys-4 demethylases. We present here the crystal structures of the KDM5A linked Jumonji domain in complex with eight of these inhibitors in the presence of Mn(II) at near atomic resolution of 1.45–1.85 Å. Inhibitor-induced conformational changes in the Asn-493 and Tyr-409 side chains were observed, as were inhibitor-specific interactions with Arg-73, Gln-85, Asp-412, Trp-470, Gln-535, Asn-575 or Asn-585. The following strategies might be utilized in the successful design of selective and potent epigenetic inhibitors: (1) use the additional space near Cys-481, a residue unique to the KDM5 family, (2) add chemical moieties onto existing inhibitors such that they extend into a nearby water-filled channel lined with many unique residues for the KDM5 family, (3) combine different branches onto one molecule and/or (4) modify the existing compounds to maximize the interactions with the involved amino acids.

**EXPERIMENTAL PROCEDURES**

Cloning, expression and purification

The N-terminal fragments of human KDM5A, B, C, and D and the internal deletion constructs - deleting ARID and PhD1 domains (ΔAP) - were prepared in a pET28 plasmid containing an N-terminal His-SUMO tag sequence, and purified as described (Horton et al., 2016). The surface mutant of KDM5A, C626S/C636S (Supplementary Figure S1B), was made by PCR, confirmed by sequencing, and expressed and purified similarly. The constructs used in this study exhibited variable expression levels, solubility, and activity (Supplementary Figure S1C). The enzymes were stored in the storage buffer at −80 °C [20 mM Hepes pH 8.0, 300 mM NaCl, 5% glycerol, 0.5 mM tris (2-carboxyethyl)phosphine (TCEP)].

Formaldehyde dehydrogenase (FDH)-coupled demethylase assay

FDH assays were performed in a total volume of 40 μL at room temperature (21 °C) as previously described (Horton et al., 2016). Briefly, the reaction buffer contains 50 μM (NH4)2Fe(SO4)2, 2 mM ascorbic acid, 0.6 mM APAD+ (3-acetylpyridine adenine dinucleotide, a more stable analogue of NAD+; Sigma-Aldrich A5251), 10 μg FDH (purified in-house), 0.5 μM [E] (and 7.5 mM NaCl carried over from the enzyme stock), and 50 mM MES (pH 6.8). Various αKG concentrations ranging from 0 to 50 μM with fixed 15 μM H3(1–24)K4me3 peptide [S] or various peptide [S] concentration ranging from 0 to 50 μM
with 1 mM αKG were incubated in the reaction buffer to determine the kinetic characteristics. The reaction was initialized by addition of αKG or peptide and APAD^+ into other assay components that had been pre-incubated at room temperature for 15 min. The fluorescence signal was recorded for 15 min in a BioTek Synergy 4 Hybrid Microplate Reader using 380/20 and 460/40 as the excitation and emission filters, respectively. Initial velocity was plotted against various αKG or peptide concentrations and fitted by the Michaelis–Menten equation using GraphPad Prism 5.0 software.

For inhibition assays, powders of inhibitor compounds were dissolved in 100% (v/v) DMSO to give a 20 mM or 50 mM concentration and stored in −20°C until needed. Various compounds with the concentrations ranging from 1.25 mM to 5 nM in a half-log serial dilution were pre-incubated with the reaction mixture for 15 min [0.5 μM enzyme [E], 15 μM peptide [S], 50 μM (NH₄)₂Fe(SO₄)₂, 1 mM αKG and variable inhibitor [I] in the KDM5 reaction buffer with 10% DMSO]. The addition of peptide and APAD^+ initiated the reaction for 15 min at room temperature. The dose response curves were fitted by a 4-parameter logistic equation using GraphPad Prism 5.0:

\[
\text{Inhibition} \% = B + \frac{M - B}{1 + 10^{(\log I_{50} - \log [I]) \times \text{HillSlope}}},
\]

where B is the baseline (usually 0 for no inhibition), M=maximum inhibition (usually 1), [I]=inhibitor concentration, and the Hill slope referring to the steepness of the curve (usually −1).

**Crystallography**

KDM5A(1–588)ΔAP (in the KDM5 storage buffer) was mixed with MnCl₂ and concentrated to ~50 mg/ml (~1.4 mM), and then diluted to ~10 mg/ml (280 μM) with αKG (~1.5 mM) or inhibitor (~1.5 mM) at a molar ratio of 1:5 at the time of co-crystallization. Experiments utilized the sitting drop technique and were conducted at 16 °C by mixing 0.2 μl of the complex with an equal volume of a well solution. Originally, clusters of crystals grew with αKG with a well solution containing 1.5 M ammonium sulfate, 12% glycerol, and 0.1 M Tris-HCl (pH 8.5), but these crystals were difficult to duplicate. However, some large, single crystals (amongst clusters) could be obtained by employing freshly crushed KDM5A(1–588)ΔAP micro-seeds and a 96-well screen containing 1.2–1.35 M (NH₄)₂SO₄, 0.1 M Tris-HCl (pH 8.6–9.2), 0–20% glycerol and 25 mM (Na/K) dibasic/monobasic phosphate. Crystals with αKG or KDM5-C49 were co-grown, whereas remaining inhibitors were soaked into preformed crystals of KDM5A-αKG-Mn(II) complexes by transferring a crystal into a drop not producing co-crystals but containing inhibitor, and then allowing the crystal to remain in this drop for 1–2 days before being mounted into nylon cryoloops (Hampton Research, Inc.). Crystals were frozen in liquid nitrogen after the addition of more glycerol (up to ~30% total) to the mother liquor as a cryoprotectant.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

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Highlights

- All four members of KDM5 demethylase family have similar kinetic parameters
- Structures of KDM5A with eight diverse inhibitors were solved at atomic resolution
- Inhibitor-induced and inhibitor-specific interactions are observed
- Inhibitors displace the bound α-ketoglutarate from the active site of KDM5A
Figure 1. Kinetic parameters of KDM5 family

(A–D), Michaelis-Menten kinetic plots of KDM5A(1–739)ΔAP(2C-2S) (A–B) and KDM5D(1–760)ΔAP (C–D) for the cofactors αKG (A and C) and substrate H3 peptide (B and D), measured by a FDH-coupled demethylase assay. Error bars, S.E. from two independent experiments. (E) Summary of kinetic parameters for the four members of the KDM5 family.

<table>
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<tr>
<th></th>
<th>αKG</th>
<th>H3(1-24)K4me3</th>
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<tr>
<td>KDM5</td>
<td>K_m (μM)</td>
<td>k_cat (min⁻¹)</td>
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<tr>
<td>5A(1-739)ΔAP(2C-2S)</td>
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<td>2.1</td>
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<tr>
<td>5B(1-755)ΔAP</td>
<td>9</td>
<td>1.9</td>
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<tr>
<td>5C(1-789)ΔAP</td>
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<td>1.9</td>
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<tr>
<td>5D(1-760)ΔAP</td>
<td>10</td>
<td>2.6</td>
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Figure 2. Inhibition of KDM5 family by diverse inhibitors

(A) Chemical structures of the 10 compounds examined. (B) Summary of the IC$_{50}$ values of inhibition of demethylation of four KDM5 members by various inhibitors on H3(1–24)K4me3 substrate under the indicated experimental conditions (see Supplementary Figure S2 for original inhibition data). The labels (*) indicated the upper limit of IC$_{50}$ values of N8 and N11 compounds (being close to or lower than the probe concentration of [E]). Error Bars, S.E. Average IC$_{50}$ and S.E. values determined from 3 independent experiments. (C) Summary of ITC measurement of dissociation constants (K$_D$) of various compounds to KDM5A and KDM5B (see Supplementary Figure S3 for original binding data). (D) Summary of the IC$_{50}$ values of inhibition of demethylation for KDM5A (residues 1-1090) and full-length of KDM5B and KDM5C under the indicated experimental conditions by AlphaLISA (see Supplementary Figure S4A for original inhibition data). Average IC$_{50}$ and S.E. values determined from 3 independent experiments. (E) Full-length (FL) KDM5 enzymes, full-length KDM6A, and KDM6B (residues 1043-1682) enzyme activities in the presence of compound KDM5-C49 as determined by AlphaScreen, under the indicated experimental conditions. Graphs show representative experiments. Average IC$_{50}$ and S.E. values determined from n independent experiments: n=4 for KDM5A, n=5 for KDM5B, n=3 for KDM5C, n=2 for KDM6A and n=4 for KDM6B.
Figure 3. Structures of αKG and KDM5-C49 bound KDM5A Jumonji domain

(A–C) Structure of KDM5A-αKG-Mn(II) complex showing the binding of αKG in the active site involving (A) polar interactions, (B) six metal ligands, and (C) hydrophobic interactions. The omit electron densities, contoured at 5σ and 3σ above the mean, are shown for Mn(II) (magenta mesh) and αKG (gray mesh), respectively. (D–F) Structure of KDM5A-C49-Mn(II) complex showing the binding of KDM5-C49 compound in the active site involving (D) polar and water-mediated interactions, (E) altered metal ligands, and (F) hydrophobic interactions. (G) Superimposition of αKG and KDM5-C49 bound KDM5A structures, illustrating the conformational change of Asn-493. (H) The terminal dimethyl amino group of KDM5-C49 is flanked by Trp-470 and Asp-412. The amide nitrogen atom N11 and carbonyl oxygen atom O10 are joined via a peptide-like bond and the associated six atoms are restrained in a planar conformation. (I) Chemical structure of KDM5-C49 showing that the ethyl group off of the N11 nitrogen atom and one of the three-methyl groups of proposed trimethyl-lysine superimpose well. (J) The ethyl group off of the N11 nitrogen atom is packed against the wall of Val-584 and Asn-585.
Figure 4. Structures of seven inhibitors soaked into KDM5A active site
(A) Compound N19 superimposed with KDM5-C49 (panel 1), conserved interactions involving the isonicotinic acid moiety (panel 2), and specific interactions with the 4-chloro-2-methylbenzyl moiety. (B) Compound N3 superimposed with N19 (panel 1), conserved interactions involving the isonicotinic acid moiety (panel 2), and specific interactions with the phenthylcarbamoyl moiety. (C) Compound N10 superimposed with N19 (panel 1), conserved interactions involving side chains of Lys-501, Tyr-409, and Asn493 (panel 2), and specific interactions with the trifluorobutoxy moiety. (D) Compound N11 superimposed with N19 (panel 1), conserved interactions involving the isonicotinic acid moiety (panel 2), specific interactions with the 5-fluoro-indazole ring (panel 3), and the disordered difluoropiperidine ring (panel 4). (E) Compound N12 superimposed with N11 (panel 1), conserved interactions involving the isonicotinic acid moiety (panel 2), and specific interactions with the 1-methyl-pyridine ring (panel 3). (F) Compound N16 superimposed with N11 (panel 1), conserved interactions involving the isonicotinic acid moiety (panel 2), and specific interactions with the 4-chlorophenyl ring (panel 3). (G) Compound N8 superimposed with N11 (panel 1), specific interaction with Asn-575 (panel 2), and forced conformational change of Tyr-409 (panel 3). (H) Aromatic stacking interaction with the pyrazolopyrimidine core of N8.
Figure 5. Cellular activities of KDM5-C70

(A) Western blot analysis of MDA-MB-231 and MCF7 cells treated with DMSO or 5 μM KDM5-C70 for 3 days. Fold represents the relative ratio of band intensity for H3K4me3 divided by total H3, normalized to DMSO control. Light and dark indicate different exposures for the same blot. Representative data from biological duplicate experiments performed in duplicate are shown. (B) Colony formation assays of cancer cell lines treated with DMSO or 5 μM KDM5-C70. Representative images are shown on the left, with quantification on the right. Ratio of % intensity indicates the measured percent intensity normalized to the average percent intensity for DMSO-treated cells. Error bars represent SEM. **, p=0.0011; ***, p<0.001; ns, not significant. Representative data from biological duplicate experiments performed in triplicate are shown. (C) Real time RT-PCR analysis of the indicated mRNAs in MCF7 cells treated with DMSO or 5 μM KDM5-C70 for 3 days. Error bars represent SEM. ***, p<0.001; ns, not significant. Data from biological triplicate experiments are shown.

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Figure 6. A water-filled channel with inhibitor bound in the active site of KDM5A
(A) Superimposition of KDM5A (colored) and KDM5B (grey) showing identical interactions with **KDM5-C49** compound in the absence (KDM5A) and presence of immediate C-terminal helical Zn domain (KDM5B; PDB 5A3T). (B–C) An example of “displaced αKG” from the active site by a αKG-competitive inhibitor (N19). (D) Conformational changes of Asp-412 comparing all nine structures. The compound N8-bound structure (in yellow) exhibited the largest difference in main chain as well as side chain conformations of Asp-412. (E) A surface model of KDM5A Jumonji domain showing the surface charge distribution with red for negative, blue for positive, and white for neutral. (F–G) An enlarged water-filled channel with an inhibitor (KDM5-C49) bound in the active site of KDM5A.