# A Selective KDM5 Inhibitor, KDOAM-25, inhibits proliferation of MM1S myeloma cells *in vitro*.

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## Summary

The site- and methylation- state specific modification of lysine residues on histone proteins is a dynamic epigenetic modification that plays a key role in chromatin structure and gene regulation. Members of the KDM5 (also known as JARID1) subfamily are 2-oxoglutarate (2-OG) and $Fe^{2+}$ dependent oxygenases acting as histone 3 lysine 4 trimethyl (H3K4me3) demethylases, regulating proliferation, stem cell self-renewal and differentiation. Here we present the characterization of KDOAM-25, a chemical tool compound binding the co-substrate binding site in KDM5 enzymes. KDOAM-25 shows $IC_{50}$ values < 100 nM for KDM5A-D *in vitro* assays, with > 200-fold selectivity towards other subfamilies of 2-OG oxygenases as well as fifty-five representative off-target receptors and enzymes. The molecule is cell-active with an $EC_{50}$ of 60 µM. Selectivity towards other demethylases was confirmed in human cell assay systems. In support of the previously postulated role of H3K4 demethylases in cancer, we observe that overexpression of KDM5B in multiple myeloma is negatively correlated with overall survival, using data from 3 clinical trials. Application of KDOAM-25 to inhibit multiple myeloma MM1S cell proliferation *in vivo* is correlated with global increases of H3K4 methyl marks around transcriptional start sites.

## Introduction

The site- and methylation state specific modification of histone lysyl residues differentially regulates transcription by providing unique interaction sites that bind to methyl-lysine recognition (or “reader”) domains (Ng et al., 2009). Genome-wide analyses of tri- or di- methylated histone 3- lysine 4 (H3K4me3 or H3K4me2) sites demonstrate that these histone marks are associated with promoter regions and transcriptional start sites at the 5’ end of genes (Bernstein et al., 2012). Interaction of these di- or tri- methylated histone marks with the transcription initiation factor

TFIID (Lauberth et al., 2013) or with chromatin remodelling complexes leads to formation of a pre-initiation complex that can be rapidly activated by further recruitment of specific transcription factors (Chai et al., 2013,; Tanny, 2014).

The dynamic interplay between chromatin methylation and demethylation is catalysed by distinct classes of enzymes. The removal of methyl groups from histone lysine residues is catalysed by two classes of lysine (K) demethylases (KDMs) with different co-factor and substrate dependencies: the flavine adenine dinucleotide (FAD)-dependent monoamino oxidases (grouped as KDM1 subfamily) and the Fe(II) and 2-OG dependent oxygenases that contain a conserved catalytic Jumonji C-domain (JmjC, belonging to subfamilies KDM2- KDM7) (Johansson et al., 2014).

Members of the KDM5 subfamily of JmjC-KDMs are transcriptional co-repressors which specifically catalyse the removal of all possible methylation states from lysine 4 of histone H3 (H3K4me3/me2/me1), with highest activity found towards H3K4me3. KDM5 enzymes are often found as components of transcriptional complexes with repressors such as REST, histone deacetylases and histone methyl transferases (HMTs) (Pasini et al., 2008). In mammals the KDM5 subfamily encompasses four proteins, KDM5A (known as JARID1A or RBP2), KDM5B (known as JARID1B or PLU1), KDM5C (JARID1C or SMCX) and KDM5D (JARID1D or SMCY), the latter two encoded on the X and Y chromosomes, respectively.

The KDM5 family and domain architecture is conserved from yeast to humans, displaying a similar pattern with an N-terminal Jumonji domain (JmjN), a DNA binding ARID domain (AT-rich interactive domain), a catalytic JmjC-domain, a C5HC2 zinc finger motif located C-terminally to the JmjC-domain, a PLU-1 motif, as well as one to three methyl-lysine or methyl-arginine binding plant homeodomains (PHD) denoted PHD-1, PHD-2 and PHD-3 (Figure 2D). It is conceivable that these additional domains contribute critically to genomic KDM5 target gene occupation, since PHD domains are able to bind to modified lysine residues in a sequence-specific manner(Horton et al., 2016; Johansson et al., 2016).

The KDM5 enzymes are suggested to play pivotal roles both during normal development and in pathological conditions such as cancer. KDM5A is ubiquitously expressed and involved in the control of cell proliferation and differentiation, and linked to several human cancers including acute myeloid leukemia (Wang et al.,

2009), hepatocellular carcinoma (Liang et al., 2013), gastric cancer (Li et al., 2014), (Zeng et al., 2010) and lung cancer (Teng et al., 2013; Wang et al., 2013). KDM5B plays an important role in stem cell biology by blocking differentiation in embryonic and hematopoietic stem cells (Cellot et al., 2013; Dey et al., 2008). Similar to KDM5A, elevated expression levels have been found in various primary cancers including melanoma (Roesch et al., 2010), breast (Barrett et al., 2002), testicular (Madsen et al., 2003) and ovarian cancer (Wang et al., 2015). KDM5C plays a role in neuronal development and shows highest expression in neuronal tissues. Inactivating mutations have been associated with X-linked mental retardation (Santos-Reboucas et al., 2011). The Y-chromosome encoded KDM5D, is expressed in all male tissues, and the protein has a suggested role in spermatogenesis (Akimoto et al., 2008).

Chemical tool compounds complement biological target identification strategies such as gene editing or knockdown, and play important roles in dissecting the function of specific domains in target biology. Although inhibitors have been reported for JmjC containing KDMs (Bavetsias et al., 2016; Gehling et al., 2016; Horton et al., 2016; Itoh et al., 2015; McAllister et al., 2016; Vinogradova et al., 2016; Westaway et al., 2016a; Westaway et al., 2016b; Wu et al., 2016), most of the published compounds have limitations such as lack of selectivity or cytotoxicity. Bavetsias *et. Al (Bavetsias et al., 2016)l.* reported a new class of cell active histone lysine demethylase inhibitors. The compounds are based on a cell permeable pyridopyrimidinone scaffold. However, these compounds do not show any selectivity between the KDM4 and KDM5 families. Another cell-active pan-KDM inhibitor is the prodrug of IOX1 (Schiller et al., 2014), yet this compound is affected by high cytotoxicity. All these limitation hamper the use of these molecules as tool compounds in a cellular context. Among the others, a recently reported KDM5A inhibitor (Liang et al., 2016), is the most remarkable tool compound/pre-clinical candidate for cancer therapy. This orally bioavailable pyrazolopyrimidinone shows both high inhibitions of KDM5 demethylases on a TR-FRET biochemical assay and good activity in cell-based assays making this compound as the best chemical tool reported in the literature to date.

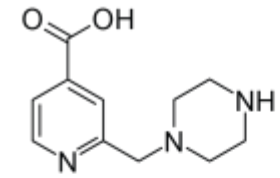
In this paper we report the biochemical and molecular genome-wide characterisation of KDOAM-25, a new, selective and cell-active pan-KDM5 inhibitor that can be used to study H3K4 methylation biology.

# Results and Discussion

## Discovery of KDOAM-25

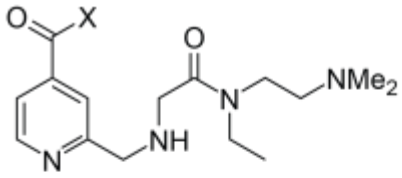
We previously described 4-carboxy-2-triazolopyridines as selective KDM2 inhibitors (England et al., 2014). In efforts to find alternative scaffolds based on the 4-carboxypyridine core, a range of compounds with a 2-aminomethyl group were synthesized and tested for KDM inhibition. Aminomethylpyridines such as KDOAM-1 showed preference for the KDM4 and KDM5 sub-families (Figure 1A) but we were unable to find analogues with potency in cellular immunofluorescence (IF) assays of H3K36 and H3K4 demethylation. Hence, we were interested when a patent application described very similar compounds and reported potency in an almost identical cellular IF assay (Labelle et al., 2014). In our hands two of the compounds described, named here KDOAM-20 and 21, showed low micromolar and sub-micromolar potency, respectively, in an H3K4me3 IF assay overexpressing KDM5B, but also inhibited H3K9me3 demethylation in accordance with its *in vitro* KDM4 activity (Figure S1A). We considered it possible that KDOAM-21 may be acting as an ester pro-drug akin to the KDM5/6 inhibitor GSK-J4 (Kruidenier et al., 2012). To our surprise the ester KDOAM-21 itself showed potent inhibition of KDM5B *in vitro* (Figure 1A). However, KDOAM-20 was also a sub-micromolar inhibitor of KDM4C and KDOAM-21 could potentially be hydrolyzed to KDOAM-20 *in situ*. Therefore, we sought to replace the ester of KDOAM-20 with a less labile group such as an amide to retain KDM5 potency and selectivity. There is some precedent for this as compound **16** in Bavetsias *et al.* is a weak KDM4/5 inhibitor with some selectivity for KDM5A/B over KDM4A/B (5 to 50-fold). The amides KDOAM-25, 28 and 29 were synthesized and KDOAM-25 was shown to be the most potent of the series with KDM5B $IC_{50}$ 19 nM and improved selectivity over KDM4C.

A



**KDOAM-1**

| AlphaScreen $IC_{50}$ (nM)* | |
|---|---|
| KDM5C | KDM4C |
| 980 | 1300 |



| | | AlphaScreen $IC_{50}$ (nM)* | |
|---|---|---|---|
| **KDOAM-** | **X** | **KDM5B** | **KDM4C** |
| **20** | OH | 7 | 170 |
| **21** | OEt | 49 | 2,400 |
| **28** | NHEt | 98 | *ND* ‡ |
| **29** | $NMe_2$ | 270 | *ND* ‡ |
| **25** | $NH_2$ | 19 | 4,800 |

B

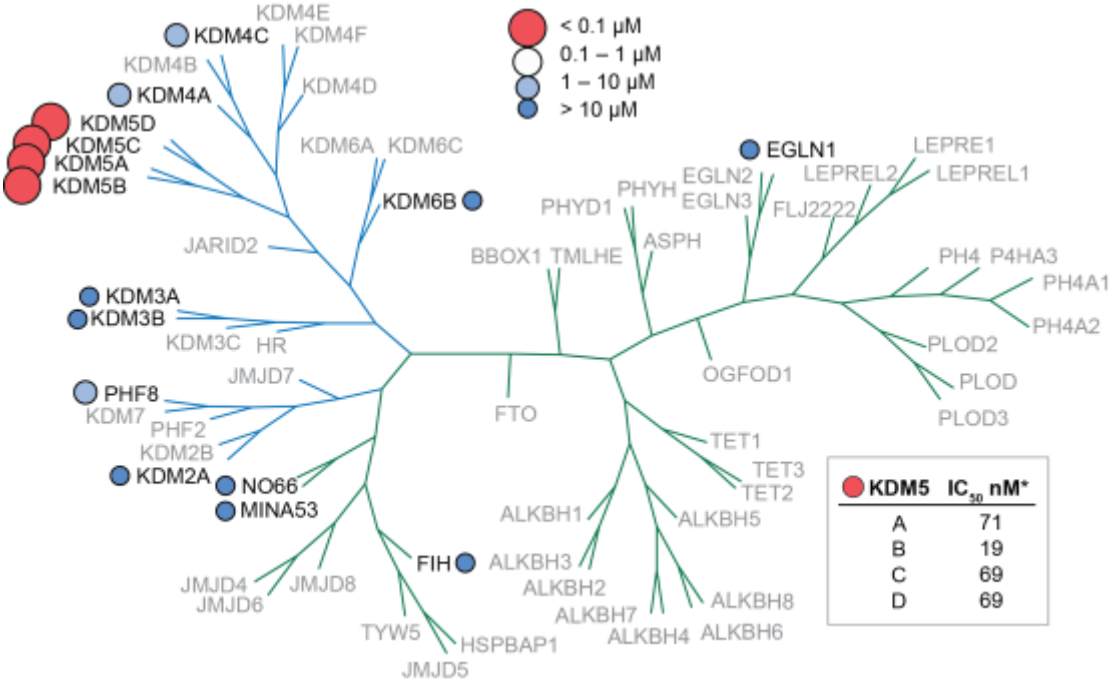



**Figure 1. Discovery of KDOAM-25** (A) KDOAM-21 is a weak KDM4/5 inhibitor. KDOAM-20 – 25 are potent and selective inhibitors of KDM5B over KDM4C. (B) KDOAM-25 was tested against a panel of 15 2-OG oxygenases (black text) including a representative from each of the lysine demethylases sub-families (blue branches). KDOAM-25 only inhibits the KDM5 sub-family members KDM5A-D with potencies <100 nM (box). *All $IC_{50}$ values are an average of at least 2 independent determinations (Table S1); ‡ Not Determined.

We also investigated the biochemical inhibitory effect of KDOAM-25 at different concentration of the cofactor 2-OG (Figure S1) on KDM5B. The experiment indicated

that the compound acts a partial competitor of the cosubstrate 2-OG, with $IC_{50}$ values increasing from 27 nM at 4 µM 2-OG, up to 558 nM at a 300 µM concentration of 2-OG.

With a potent KDM5B inhibitor available in the form of KDOAM-25, we profiled it extensively for *in vitro* potency in all our available JmjC KDM assays and assays for representative 2-OG dependent oxygenases (Figure 1B and Table S1) using AlphaScreen or MALDI-TOF enzymatic assays. KDOAM-25 was selective for the KDM5 subfamily with no inhibition below 4.8 µM for any of the other 2-OG oxygenases. KDOAM-25 was also profiled in the CEREP express panel of fifty-five common off-targets but showed no inhibition at 10 µM (Table S4).

## KDOAM-25 binds to KDM5B in the 2-Oxoglutarate and Substrate Sites

KDOAM-25 was crystallized in a JmjN-JmjC linked construct of KDM5B (Johansson et al., 2016) (Figure 2). The pose of the compound was identical to the reported structure of KDOAM-20 in the same construct (PDB ID 5A3T) with one key difference. In the carboxylate binding pocket, Y425 is shifted from a H-donor pose that interacts with the carboxylate of KDOAM-20 to allow an inverse H-bond interaction with the carboxamide of KDOAM-25. The shift of Y425 creates a cavity which is occupied by a water molecule. The propensity for Y425 to adopt this alternative pose in KDM5B may explain why KDOAM-25 is selective for KDM5 enzymes versus other KDMs that either form different H-bonds to the 4-carboxypyridines (KDM2/3/6/7) or have a less mobile homologous tyrosine (KDM4).

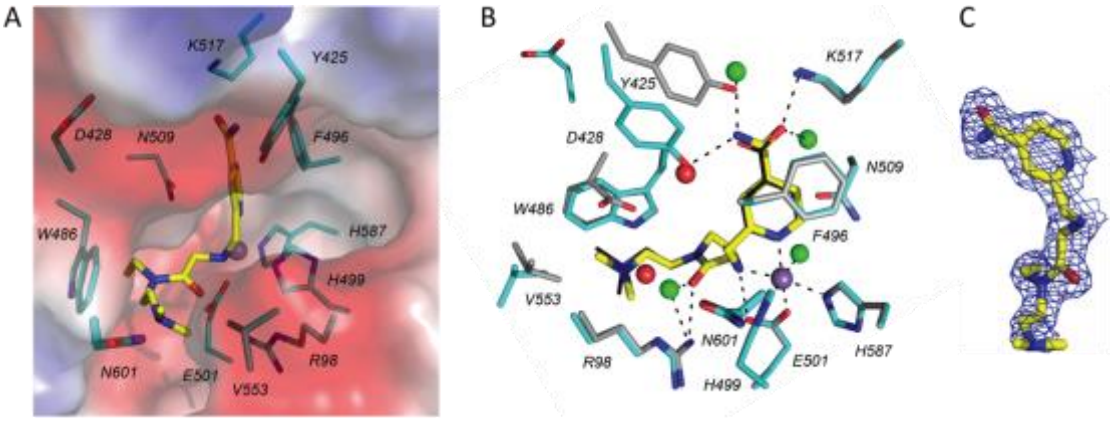


**Figure 2. Crystal Structure of KDOAM-25 bound to KDM5B** (A) Surface representation of KDOAM-25 buried in the 2-OG pocket of KDM5B (PDB 5A3N). (B) KDOAM-25 (yellow sticks) binds to KDM5B (cyan sticks) as a bidentate metal chelator. The Mn(II) ion (purple sphere), which serves as the crystallographic surrogate for the catalytic Fe(II), is held in the protein by one Asp and two His residues. Y425 is shifted to accommodate the carboxamide of KDOAM-25 compared to its pose (grey sticks) when binding KDOAM-20 (thin black sticks from PDB ID 5A3T), which allows binding of an additional water molecule within the pocket (KDOAM-25: green spheres, KDOAM-20: red spheres). (C) Electron density (blue mesh from 2F0-Fc map) of KDOAM-25 in the co-crystal structure with KDM5B.

## KDOAM-25 increases H3K4me3 levels in HeLa cells

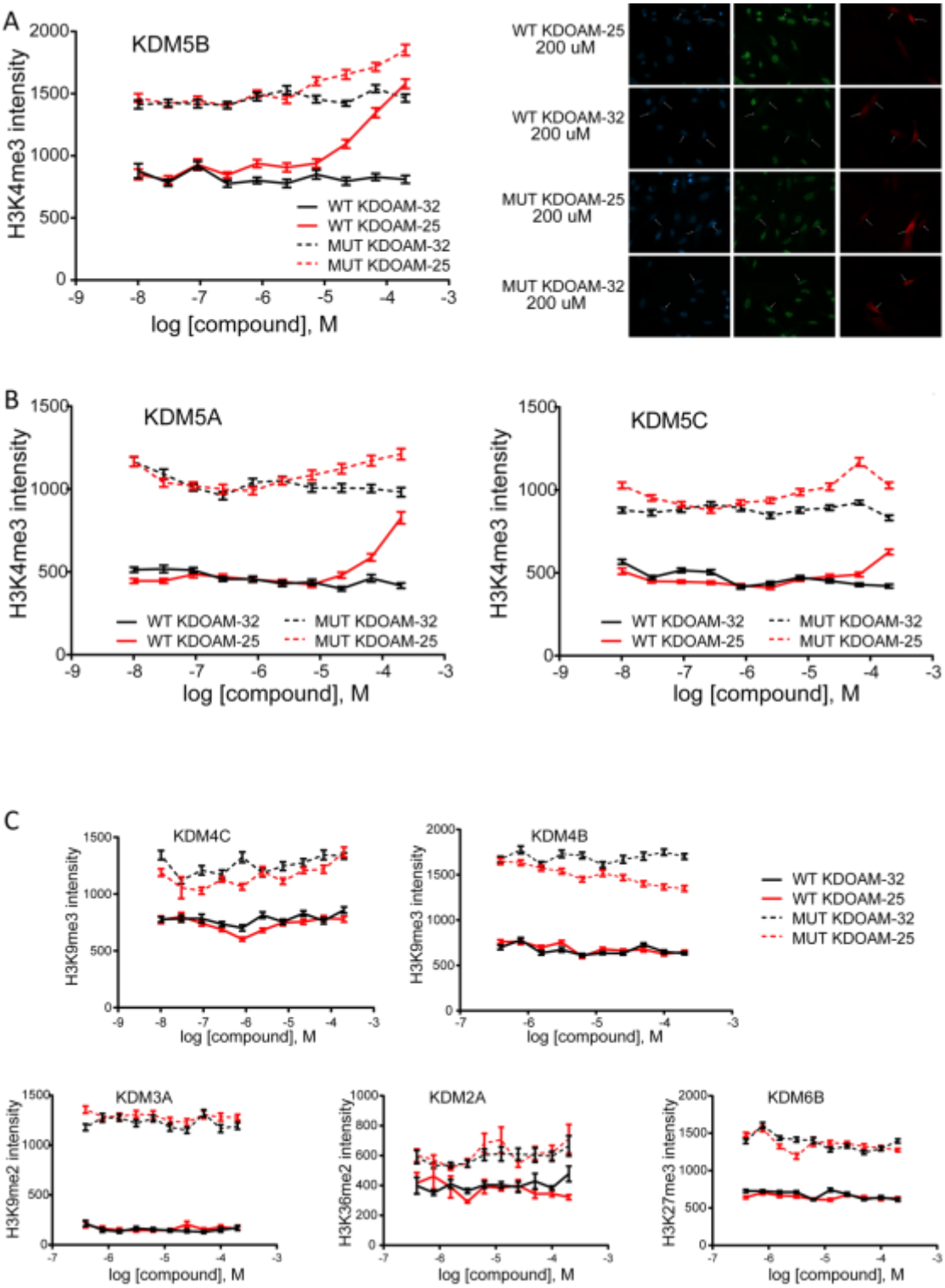
The cellular activity of KDOAM-25 was assessed in an IF assay by using an overexpression system, assessing the effect of KDOAM-25 and related compounds on the ectopically overexpressed wild-type (WT) demethylases compared to their catalytically dead mutants (MUT). As control we used a structurally related inactive compound KDOAM-32 (Figure S3) which is unable to coordinate the catalytic iron in the KDM5 active sites (Figure S1, Table S1). Consistent with its *in vitro* activity KDOAM-25 inhibited most potently KDM5B with an $IC_{50}$ of ~ 60 μM (Figure 3A) and the other KDM5 family members at concentrations above 100 μM (Figure 3B), but showed no cellular activity on any of the other tested JmjC family members (Figure 3C). At higher concentrations all compounds showed increased H3K4me3 levels for both WT and MUT overexpressing cells, indicating that the observed change in histone methylation is likely a combination of inhibition of endogenous KDM5 as well as the overexpressed enzyme, which made determination of an accurate $IC_{50}$ challenging.

KDOAM-25 also inhibited activity of endogenous KDM5 demethylases with potencies similar to those seen in the overexpression assay (Figure S1B) and was found to be active in increasing H3K4me3 levels using chromatin immunoprecipitation followed by parallel sequencing (ChIPseq) at similar concentrations in human multiple myeloma cells (see below).

The exquisite biochemical selectivity profile of KDOAM-25 indicates that the increase in the H3K4me3 mark is due to the inhibition of KDM5 enzymes. The other demethylase capable of removing the H3K4me3 mark is KDM2B. KDM2B is structurally and phylogenetically related to KDM2A (69% sequence similarity), for which KDOAM-25 is a weak inhibitor with $IC_{50}$ of 4.4 μM (Table S1).

The higher concentration (60 μM) required for cellular activity could be ascribed to different reasons: first to the partial competition with the cofactor 2-OG, which physiological intracellular concentration could be up to micromolar levels (Thirstrup et al., 2011); second to an estimated moderate-low cell permeability of KDOAM25. A Caco-2 cell permeability assay was used to evaluate the passive permeation through cellular membrane (Table S3) and KDOAM25 has shown values in the low permeability region. Combined altogether these effects could explain the partial loss of potency observed in the IF cell assay.

Although KDOAM-25 is a potent KDM5 inhibitor in vitro, we could not rule out the possibility that some of the measured cellular affects may be due to amide hydrolysis to give KDOAM-20 in situ. For this reason we investigated the chemical stability of KDOAM-25 in phosphate buffer and the biological stability in Liver Microsomes (Table S4). Incubation of the compound at 37 ˚C in phosphate buffered saline did not show any hydrolysis of the amide after 24 hours; while the ester KDOAM-21 was partly hydrolyzed to the corresponding acid KDOAM-20 in 25% after the same time. Similarly, incubation of KDOAM25 with different liver microsomes (Human HLM, Mouse, MLM and Rat RLM) showed low intrinsic clearance in all the species (Table S4), indicating that the compound is likely to have good biological stability within a cellular environment.




250 **Figure 3. KDOAM-25 inhibits KDM5 activity in cells** (A) $EC_{50}$ curves for KDOAM-25 against cells overexpressing Flag-tagged KDM5B (left). Immunofluorescence assay showing inhibition of KDM5B (WT) mediated H3K4me3 demethylation by KDOAM-25, but no effect of the negative control compound KDOAM-32. Immunofluorescence images showing HeLa cells stained with DAPI nuclear stain

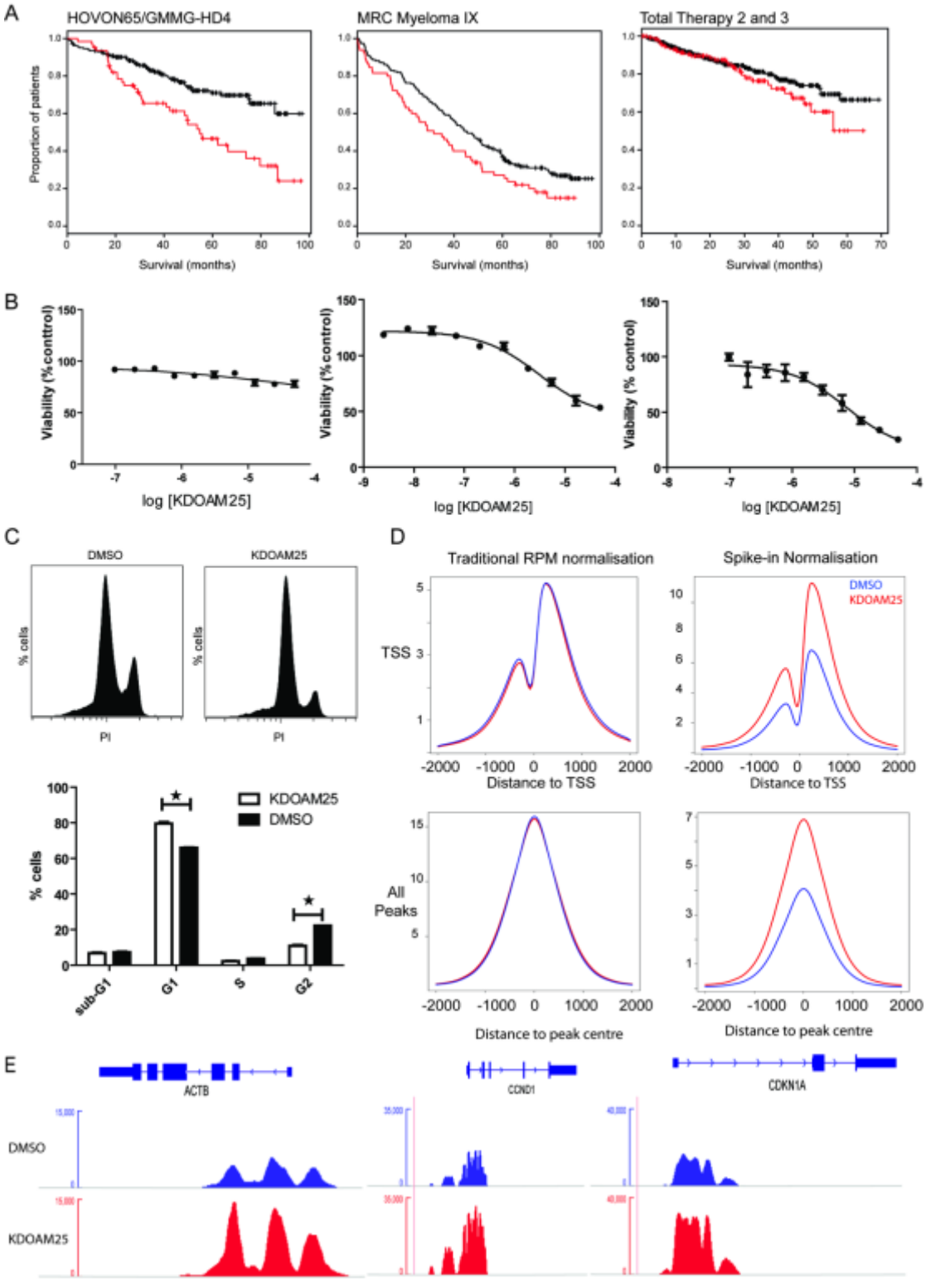
(blue), a specific antibody against H3K4me3 (green), and Flag-tag antibody (red) (right). Cells were transfected with KDM5B and treated with the indicated inhibitors. Arrowheads indicate cells transfected with KDM5B-Flag. (B) KDM5A and KDM5C are inhibited to a lesser extent by KDOAM-25 in cells overexpressing the respective demethylase. (C) KDOAM-25 has no effect on the histone substrates of the other KDM family members tested. No effect of any of the compounds is seen on any of the overexpressing catalytically inactive mutants, MUT, (dotted lines in A-C). IF was read after 24 h of compound treatment. Data represents the average and standard error of at least 100 cells.

## Increased KDM5B expression is associated with shorter survival in myeloma patients and *ex vivo* inhibition with KDOAM-25 results in cell cycle arrest

After having identified a selective and cell-active KDM5 inhibitor, we then went on to employ this molecule in *ex vivo* experiments in MM1S multiple myeloma cells. In line with various reports on the oncogenic roles of the KDM5 enzymes (Kooistra and Helin, 2012) we found that the H3K4me3 demethylase JARID1B indeed is a predictive factor in multiple myeloma. We performed survival analysis using data from three separate, large clinical datasets of newly diagnosed myeloma patients for whom the level of (KDM5B) gene expression was measured at diagnosis. Univariate analysis of the data from each trial indicated that higher levels of expression of KDM5B were associated with worse overall survival, with significantly shorter survival seen in patients with expression in the upper quartile compared to those having lower expression levels. A further multivariate analysis of the data from the Myeloma IX trial, for which the most complete data set was available, indicates that the highest quartile of KDM5B expression at diagnosis remains associated with a statistically worse outcome compared to lower KDM5B expression ($p=0.039$). These data further highlight the importance of chromatin modification mechanisms and in particular the H3K4me3 demethylase KDM5B as an important factor in multiple myeloma (Figure 4A).

To investigate the role of the inhibition of H3K4 demethylation we screened the anti-proliferative effects KDOAM-25 in the MM1S multiple myeloma cell line. Using a

fluorescent cell viability assay, we found that after a delay of five to seven days, KDOAM-25 was able to reduce the viability of MM1S cells with an $IC_{50}$ of approximately 30 μM with little effect on cell viability after three days (Figure 4B) KDOAM-25 treatment did not show the same decrease in viability in a range of other MM cells or in a cell line derived from human mesenchymal stem cells (Figure S3). KDOAM-25 treatment resulted in a G1 cell-cycle arrest with an increased proportion of MM1S in G1 ($p=0.0286$) and a decrease of proportion of cells in G2 without increase the proportion of cells in the apoptotic sub-G1 phase (Figure 4C). ChIPseq was performed on MM1S cells treated with KDOAM-25 to investigate the change in the distribution of H3K4me3 marks across the genome. When distribution of H3K4me3 was measured following normalization to reads per million mapped reads (RPM) there was little difference seen in the coverage of H3K4me3 at either transcription start sites or across the totality of all peaks called. We then employed the ChIP-Rx strategy to enable quantification of the amount of pulled-down chromatin (Orlando et al., 2014). Use of this spike-in quantification revealed a global change in the level of H3K4me3 with approximately twice as much H3K4me3 found in cells treated with KDOAM-25 compared with the vehicle control (Figure 4D). The increase in H3K4me3 is global and is observed at the transcription start site of genes associated with endogenous “housekeeping” within the cell, such as beta actin (ACTB), pro-proliferative genes such as cyclin D1 (CCND1) and anti-proliferative genes such as cyclin-dependent kinase inhibitor 1a (CDKN1A) (Figure 4E).




310 **Figure 4 KDM5B and KDOAM-25 in Multiple Myeloma Cells** (A) Increased histone H3K4me3 demethylase KDM5B expression is associated with shorter overall survival in multiple myeloma. Data from Affymetrix gene expression analysis with linked survival was available from 3 large datasets of myeloma patients at diagnosis

(Hovon65/GMMG-HD4 trial (n=246, GSE19784), MRC Myeloma IX trial (n=259), Total Therapy 2 and 3 trials (n=559, GSE2658)). Results for the probeset 201548_s_at (KDM5B) are shown as Kaplan-Meier survival curves for patients comparing those with the highest quartile of expression (red) of KDM5B to all others (black). (B) Viability of MM1S when treated with KDOAM-25 as measured after 3, 5 or 7 days (mean +/- standard error of three independent experiments, each in quadruplicate and normalized to DMSO control). (C) KDOAM-25 significantly increases the proportion of cells in G1 and reduces the proportion in G2 without increasing the sub-G1 population.. (D) Average coverage of H3K4me3 marks across transcription start sites (top) and across all called-peaks (bottom) when normalized by either reads per million (left) or spike-in quantification (right) (mean of three independent experiments). KDOAM-25 data shown in red and DMSO in blue. (E) Coverage tracks of H3K4me3 normalized by spike-in quantification for three genes, a “housekeeping” gene ACTB, a pro-proliferative gene cyclin D1 and an anti-proliferative gene CDKN1A.

## Significance

KDOAM-25 is a highly selective inhibitor of the KDM5 subfamily of histone lysine demethylases with strongest activity found against the catalytic domain of KDM5B. KDOAM-25 shows potent inhibition of the KDM5A-D enzymes *in vitro* (< 100 nM) and an expected corresponding increase in H3K4me3 levels using IF detection in an ectopic expression system in HeLa cells was seen with compound concentrations in the two-digit micromolar range. Structure-based design was used to generate KDOAM-25 without the need for the previously reported ester pro-drugs. KDOAM-25 is devoid of off-target activity on a Cerep express panel; it is well tolerated in several cell lines, even at high concentrations. Despite the fact that the compound cannot be considered as a chemical probe according to the SGC criteria (cellular $EC_{50}$ 1 uM), due to its good stability, high selectivity and low cytotoxicity KDOAM-25 may be a useful tool although results should be considered carefully due to the higher cellular $EC_{50}$ of 30 µM. While there is no inhibition of any other KDM sub-families at this concentration, other off-targets cannot be completely ruled out. Hence KDOAM-25 could help to investigate the biological roles of the KDM5 enzymes in relevant

cellular contexts. For example, here we reported that KDOAM-25 shows an inverse relationship between KDM5B expression and overall survival using data from 3 clinical multiple myeloma trials thus suggesting a potential application for KDM5B inhibitors in myeloma therapy. When human myeloma MM1S cells were treated with KDOAM-25 at concentrations consistent with inhibition of KDM5B mediated H3K4me3 demethylation in myeloma cells, anti-proliferative effects were observed with anticipated genome-wide increases in H3K4me3 levels as demonstrated by quantitative CHIP-seq experiments.

# Experimental Procedures

## Compound Synthesis

KDOAM-20 (KDM5-C49) and KDOAM-21 (KDM5-C70) were purchased from Xcessbio. KDOAM-25, 28 and 29 were prepared from KDOAM-21 via hydrolysis and amide coupling. KDOAM-32 was prepared from methyl 5-(bromomethyl)-nicotinate. KDOAM-1 was prepared from diethyl pyridine-2,4-dicarboxylate. See Supplemental Information (SI) for full synthetic protocols.

## Protein Production, Crystallography and X-Ray Data Collection

Recombinant KDM5B was expressed in Sf9 insect cells, purified and co-crystalized with KDOAM-25 using conditions analogous to those previously described (Johansson et al., 2016) (see SI for details).

## Determination of Cellular H3K4me3 Levels

The effect of KDM5 inhibition on H3K4me3 levels in HeLa cells was determined using a method similar to that previously published (Bavetsias et al., 2016) (see SI for details).

## Chromatin Immunoprecipitation and Sequencing

MM1S cells were treated with either 50 μM KDOAM-25 or DMSO for 7 days. Immediately prior to fixation in 1% formaldehyde, 3 million Sf9 cells were added to each sample. Cells were fixed, lysed, sonicated and the chromatin immunoprecipitated with anti-H3K4me3 antibody (Millipore Cat 07-473 Lot 2207281). See SI for additional experimental details including data processing.

## Survival Curves from Clinical Trials

Gene expression profiling was performed on newly diagnosed multiple myeloma patients' CD138 selected plasma cells (see SI for details).

## Author Contributions

Conceptualization, methodology, writing, visualization: PB, UO, SM; investigation, formal analysis: AK, AN, AS, AT, CB, CJ, CP, CS, CS, CY, EW, FD, GM, GR, KE, NA, NB, NL, NW, OF, PB, PS, RN, SH, SM, SM, SV, UO; supervision: CS, NL, PB, UO, SM; funding acquisition: CB, NA, PB, UO, SM.

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