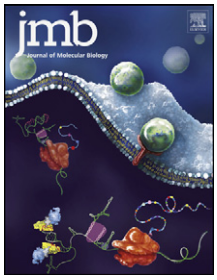

Studies on the Interaction of the Histone Demethylase KDM5B with Tricarboxylic Acid Cycle Intermediates

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Abstract

Methylation of lysine-4 of histone H3 (H3K4me$_n$) is an important regulatory factor in eukaryotic transcription. Removal of the transcriptionally activating H3K4 methylation is catalyzed by histone demethylases, including the Jumonji C (JmjC) KDM5 subfamily. The JmjC KDMs are Fe(II) and 2-oxoglutarate (2OG)-dependent oxygenases, some of which are associated with cancer. Altered levels of tricarboxylic acid (TCA) cycle intermediates and the associated metabolites D- and L-2-hydroxyglutarate (2HG) can cause changes in chromatin methylation status. We report comprehensive biochemical, structural and cellular studies on the interaction of TCA cycle intermediates with KDM5B, which is a current medicinal chemistry target for cancer. The tested TCA intermediates were poor or moderate KDM5B inhibitors, except for oxaloacetate and succinate, which were shown to compete for binding with 2OG. D- and L-2HG were moderate inhibitors at levels that might be relevant in cancer cells bearing isocitrate dehydrogenase mutations. Crystallographic analyses with succinate, fumarate, L-malate, oxaloacetate, pyruvate and D- and L-2HG support the kinetic studies showing competition with 2OG. An unexpected binding mode for oxaloacetate was observed in which it coordinates the active site metal via its C-4 carboxylate rather than the C-1 carboxylate/C-2 keto groups. Studies employing immunofluorescence antibody-based assays reveal no changes in H3K4me$_3$ levels in cells ectopically overexpressing KDM5B in response to dosing with TCA cycle metabolite pro-drug esters, suggesting that the high levels of cellular 2OG may preclude inhibition. The combined results reveal the potential for KDM5B inhibition by TCA cycle intermediates, but suggest that in cells, such inhibition will normally be effectively competed by 2OG.

Introduction

Dynamic changes in post-translational modifications to chromatin, in particular to the N-terminal tails of histone H3, contribute to the regulation of many cellular functions and modulate transcription in both healthy and diseased states [1–3]. Histone H3 lysine 4 (H3K4) methylation is an important modification that is normally activating with respect to transcription [4]. Removal of the H3K4 methylation regulates expression and is performed by histone demethylases from two different families [5]. The flavin adenine dinucleotide-dependent demethylases LSD1/2 (KDM1 family) specifically catalyze demethylation of di- and mono-methylated states of H3K4 (H3K4me$_1$ and H3K4me$_2$), while all methylated states of H3K4 (H3K4me$_1$, H3K4me$_2$ and H3K4me$_3$) are substrates for the KDM5 family of demethylases, also known as the JARID1s [1,6,7]. The KDM5 family contains the Jumonji C (JmjC) domain and belongs to the superfamily of Fe(II)/2-oxoglutarate (2OG)-dependent oxygenases [8,9]. The 2OG oxygenases use Fe(II) as a cofactor and 2OG as

a co-substrate, conversion of which to succinate and CO_2 is coupled to oxidation of methylated lysine residues in histone substrates to give hemiaminal intermediates, subsequent decomposition of which results in the formation of the corresponding demethylated lysine residue and formaldehyde [10].

The human KDM5 family comprises four isoforms (KDM5A-D), which in addition to the catalytic JmjC domain also contain an N-terminal Jumonji N-domain, a deoxyribonucleic acid (DNA)-binding ARID domain, a C5HC2 zinc finger, a PLU1-motif and two or three PHD domains [8,11,12]. KDM5 demethylation activity is involved in regulating cell differentiation and proliferation (KDM5A and KDM5B) and has been related to disease states, especially cancer [1,13]. The KDM5 enzymes have roles in tumor initiation, maintenance/survival and development of drug resistance [13,14]. KDM5A (JARID1A, RBP2) is a retinoblastoma RB-binding protein and mediates metastasis in estrogen receptor-negative breast cancers [15], while KDM5B (JARID1B or PLU-1) has roles in tumor maintenance [16], melanoma metastatic progression and resistance to cytotoxic agents and BRAF inhibitors [13]. KDM5C is involved in neuronal development [17], and KDM5D, which is encoded by a Y-chromosome-encoded (male specific) gene, has a role in spermatogenesis [18], Consequently, KDM5 members are current medicinal chemistry targets for cancer, with KDM5B being a particular focus due to its role in tumor maintenance and progression [13].

The JmjC demethylases employ 2OG as a co-substrate and have potential to be inhibited by structurally related tricarboxylic acid (TCA) cycle metabolites [19]. Previous work has shown inhibition of certain 2OG oxygenases by succinate, fumarate [20–24] or D-/L-2-hydroxyglutarate (D-/L-2HG) [25–27], including the histone demethylase KDM4A [28,29]. These observations are of interest since mutations to genes encoding for succinate dehydrogenase or fumarate hydratase in cancer cells lead to the accumulation of succinate or fumarate, the metabolic changes that have been associated with tumorigenesis [30–32]. Elevated succinate and fumarate levels occur in paragangliomas, pheochromocytomas, gastric tumors (succinate) and hereditary leiomyomatosis (fumarate) [33,34]. "Gain of function" mutations to genes encoding for isocitrate dehydrogenase 1 or 2 (IDH1/2) in various tumors and leukemia lead to accumulation of high levels of D-2HG [35–37]. It is proposed that accumulation of some TCA cycle metabolites/2HG promotes tumorigenesis by altering modifications to chromatin, including histone and DNA methylation patterns [34]. Accumulation of D-2HG in cells has been reported to affect the H3K9 and H3K27 methylation status [38,39], and succinate dehydrogenase/fumarate hydratase mutant tumors have been linked to global increases in DNA methylation/hydroxymethylation levels [40,41]. Thus, the effects of TCA cycle metabolites on the levels of other histone methylation marks, including transcriptionally important H3K4 methylation, merit detailed investigation.

We report studies on the enzyme activity inhibition of isolated KDM5B, a current medicinal chemistry target, by TCA cycle metabolites and D- and L-2HG. Kinetic studies revealed a large difference in the extent of inhibition, with oxaloacetate and succinate being the most potent of the tested compounds. Crystallographic analyses support the findings suggesting competition of TCA cycle intermediates with 2OG and reveal an unexpected coordination mode for oxaloacetate. In contrast with some of the other 2OG oxygenases acting on chromatin, cellular studies on the effects of elevated TCA cycle metabolites on H3K4 methylation imply that the relatively high 2OG levels may "outcompete" the elevated TCA intermediate levels.

Results

In vitro biochemical studies show inhibition of KDM5B by succinate and oxaloacetate

We initially investigated inhibition of purified recombinant KDM5B (produced in Sf9 insect cells, M1-R822) by TCA cycle metabolites. KDM5B catalyzes demethylation of H3K4me$_3$, H3K4me$_2$ and (with lower activity) H3K4me$_1$; in line with the previous work, the H3K4me$_2$(1–21) peptide fragment was chosen for inhibition studies to avoid complications due to sequential demethylation that is observed when using the analogous H3K4me$_3$ peptide [8]. Previous kinetic studies on KDM5B and H3K4me$_2$ were conducted using a formaldehyde dehydrogenase (FDH)-coupled assay using 15-mer and 21-mer H3 peptide fragments, and recombinant truncated KDM5B constructs, without the PLU1, PHD2 and PHD3 domains (KDM5B(1–769) or KDM5B(1–822) [8,42]). Reported K_m^{app}(2OG) and K_m^{app}(H3K4me$_2$(1–15)) were 6 and 3.6 μM, respectively [42], whereas the K_m^{app} for H3K4me$_2$(1–21) was 0.85 μM [8]. The differences in the reported K_m^{app} values may be attributed, at least in part, to different lengths of the peptide substrates as precedented in work with other 2OG oxygenases [43–45].

We used matrix-assisted laser desorption/ionization–time of flight–mass spectrometry (MALDI-TOF-MS) for direct analysis of methylated and demethylated peptide ratios (by analyzing peak intensities), to avoid the possibility of FDH inhibition which could interfere with results when using the FDH-coupled enzyme assay. As a prelude to the inhibition studies, K_m^{app} values for 2OG and H3K4me$_2$(1–21) peptide were determined for KDM5B(1–822) using MALDI-TOF-MS to identify appropriate experimental